

Heterologous Expression of Human $\alpha 6\beta 4\beta 3\alpha 5$ Nicotinic Acetylcholine Receptors: Binding Properties Consistent with Their Natural Expression Require Quaternary Subunit Assembly Including the $\alpha 5$ Subunit

Vladimir P. Grinevich, Sharon R. Letchworth, Kari A. Lindenberger, Jean Menager, Veronique Mary, Khalima A. Sadieva, Lori M. Buhlman, Georg Andrees Bohme, Laurent Pradier, Jesus Benavides, Ronald J. Lukas, and Merouane Bencherif

Targacept, Inc., Winston-Salem, North Carolina (V.P.G., S.R.L., K.A.S., M.B.); Division of Neurobiology, Barrow Neurological Institute, Phoenix, Arizona (K.A.L., L.M.B., R.J.L.); and Aventis Pharma SA, Vitry Sur Seine, France (J.M., V.M., G.A.B., L.P., J.B.)

Received July 28, 2004; accepted September 8, 2004

ABSTRACT

Heterologous expression and lesioning studies were conducted to identify possible subunit assembly partners in nicotinic acetylcholine receptors (nAChR) containing $\alpha 6$ subunits ($\alpha 6^*$ nAChR). SH-EP1 human epithelial cells were transfected with the requisite subunits to achieve stable expression of human $\alpha 6\beta 2$, $\alpha 6\beta 4$, $\alpha 6\beta 2\beta 3$, $\alpha 6\beta 4\beta 3$, or $\alpha 6\beta 4\beta 3\alpha 5$ nAChR. Cells expressing subunits needed to form $\alpha 6\beta 4\beta 3\alpha 5$ nAChR exhibited saturable [³H]epibatidine binding ($K_d = 95.9 \pm 8.3$ pM and $B_{max} = 84.5 \pm 1.6$ 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 μ M), similar to that for nAChR in dopamine

neurons displaying a distinctive pharmacology. 6-Hydroxydopamine lesioning studies indicated that $\beta 3$ and $\alpha 5$ subunits are likely partners of the $\alpha 6$ 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 $\alpha 6$ 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 $\alpha 5$ subunit makes a critical contribution to $\alpha 6\beta 4\beta 3\alpha 5$ nAChR assembly into a ligand-binding form with native $\alpha 6^*$ -nAChR-like pharmacology and of potential physiological and pathophysiological relevance.

Distinct nicotinic acetylcholine receptor (nAChR) subtypes

Support for these studies was provided by Targacept, Inc. Work in Phoenix, part of which was conducted in the Charlotte and Harold Simensky Neurochemistry of Alzheimer's Disease Laboratory, also was funded by the Roberta and Gloria Wallace Foundation and by endowment and/or capitalization funds from the Men's and Women's Boards of the Barrow Neurological Foundation.

Portions of this work have been presented in abstract form: Buhlman LM, Lindenberger KA, Xu L, Fuh LP-T, Kuo Y-P, and Lukas RJ (2003) Function of human nicotinic acetylcholine receptors containing the $\alpha 6$ subunit ($\alpha 6^*$ nAChR) heterologously expressed in human SH-EP1 epithelial cells, Program 45.8; and Grinevich VP, Sadieva KA, Hauser TA, Buhlman LM, Lindenberger KA, Lukas RJ, Bencherif M, and Letchworth SR (2003) Pharmacology of novel $\alpha 6$ -containing nicotinic receptors containing subunit stably expressed in SH-EP1 cells, in the *Society for Neuroscience 33rd Annual Meeting*, 2003 November 8-12; New Orleans, LA. Program 410.19, Society for Neuroscience, Washington, DC.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.104.075069.

expressed in mesostriatal dopaminergic neurons are involved in modulation of striatal dopamine (DA) release (Wonnacott, 1997; Champiaux et al., 2002; Luetje, 2004); nAChR containing $\alpha 4$ and $\beta 2$ subunits ($\alpha 4\beta 2^*$ nAChR) or containing $\alpha 6$ subunits ($\alpha 6^*$ nAChR) participate directly, and $\alpha 7^*$ nAChR participate indirectly. Whereas the biochemistry of $\alpha 4\beta 2^*$ and $\alpha 7^*$ nAChR is well characterized, the subunit composition and functional properties of $\alpha 6^*$ nAChR remain unclear. Suggested roles for $\alpha 6^*$ 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 $\alpha 6$ subunit is restricted

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor(s); DA, dopamine; α CtxMII, 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; α Btx, α -bungarotoxin; CYT, cytosine; CAR, carbachol; DH β E, dihydro- β -erythroidine; LOB, lobeline; MEC, mecamlamine; 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, $\alpha 6$ is the predominant form of nAChR subunit mRNA that extensively colocalizes with nAChR $\beta 3$ subunit mRNA (Le Novère et al., 1996). Immunoprecipitation studies in rats and mutant mice, however, have suggested that $\alpha 4\beta 2^*$, $\alpha 6\beta 2^*$, and $\alpha 6\alpha 4\beta 2^*$ are the primary nAChR subtypes in rodent DA terminals, with a possible contribution of $\alpha 6(\alpha 4)\beta 2\beta 3$, $\alpha 6\beta 3\beta 2$, and $\alpha 4\alpha 5\beta 2$ subunit combinations (Zoli et al., 2002; Champtiaux et al., 2003; Salminen et al., 2004). The subunit composition of $\alpha 6^*$ nAChR expressed by human or monkey DA neurons remains unknown, although there is greater relative expression of $\beta 4$ subunit in primates (Quik et al., 2000, 2004). To date, $\alpha 6^*$ nAChR have been identified primarily by sensitivity to α -conotoxin MII (α CtxMII), an $\alpha 6$ -selective antagonist (Champtiaux et al., 2002). In humans with Parkinson's disease and in monkeys treated with the DA neurotoxin MPTP, α CtxMII 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 $\alpha 6^*$ nAChR.

Heterologous expression studies suggest that nAChR $\alpha 6$ subunits may coassemble with $\beta 2$ or $\beta 4$ subunits. Chick or rat nAChR $\alpha 6$ subunits form functional channels when coexpressed with human $\beta 4$ subunit in *Xenopus* oocytes, but the $\alpha 6\beta 2$ combination failed to form functional channels (Gerzanich et al., 1997). Chick $\alpha 6$ subunits also formed functional receptors when coexpressed with chick $\beta 2$ or $\beta 4$ subunits in BOSC 23 cells (Fucile et al., 1998). More recently, Kuryatov et al. (2000) reported functional expression of human $\alpha 6\beta 4$ nAChR in *Xenopus* oocytes, although suggesting $\alpha 6\beta 2$ nAChR form ligand-binding (nonfunctional) aggregates and that $\beta 3$ subunits may facilitate $\alpha 6^*$ nAChR trafficking to the cell membrane. Coexpression of a human $\alpha 6/\alpha 4$ chimeric subunit with human $\beta 4$ subunit in *Xenopus* oocytes and HEK-293 human embryonic kidney cells results in functional receptors with a pharmacological profile representative of wild-type $\alpha 6^*$ nAChR, although expression of wild-type $\alpha 6$ subunits with $\beta 4$ subunits did not produce ligand-binding and functional $\alpha 6^*$ nAChR (Evans et al., 2003).

The objectives of this study were to heterologously express $\alpha 6^*$ nAChR of different but defined subunit composition to identify possible $\alpha 6$ 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 $\alpha 6^*$ 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'Arbresle, 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 α CtxMII 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, α Btx, cytosine (CYT), carbachol (CAR), dihydro- β -erythroidine (DH β E), EPI, leupeptin trifluoroacetate, lobeline

(LOB), mecamlamine (MEC), methylcarbamylocholone (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. (-)-Alitricline (SIB-1508Y) and 3-(2,4)-dimethoxybenzylidene anabaseine (GTS-21) were synthesized at Aventis Pharma SA. 2-(3-Pyridinyl-1-azabicyclo[2,2,2]octane) 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 $\alpha 6$ 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 $\beta 3$ subunit cDNA was amplified with hB3N (CTAGAAGCTTAACCCCTTTTC-CAGTG) and hB3C (GTACGAATTCGCGATTCGGGGTTCGTA) primers containing HindIII and EcoRI restriction sites, respectively. The desired 1.6-kB band was gel-extracted and cloned into pcDNAneo. 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: $\alpha 6$, in pcDNA3.1-hygro, cut with FspI; $\beta 4$, in pcDNA3.1-zeo, cut with PvuI; $\beta 3$, in pcDNA3.1-neo, cut with PvuI; and $\alpha 5$, in pEF6 (conferring blasticidin 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 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B (all obtained from Invitrogen, Carlsbad, CA) plus 5% fetal bovine serum (HyClone, Logan, UT) in a humidified atmosphere containing 5% CO₂ 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 μ g of DNA dissolved in TE (10 mM Tris-HCl containing 1 mM EDTA) buffer, pH 7.4 (minimum DNA concentration of 0.1 μ g/ μ l), was diluted in supplement-free DMEM to a total volume of 300 μ l, then mixed with 60 μ l 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 μ F; 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 μ l of buffer containing 20 mM HEPES, 87 mM NaCl, 5 mM KCl, 0.7 mM NaHPO₄, and 6 mM dextrose, pH 7.05. After the addition of 100 μ g of DNA (in TE buffer), the sample was

trituated 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- $\alpha 6\beta 2$, SH-EP1- $\alpha 6\beta 4$, SH-EP1- $\alpha 6\beta 2\beta 3$, SH-EP1- $\alpha 6\beta 4\beta 3$, and SH-EP1- $\alpha 6\beta 4\beta 3\alpha 5$ were created by sequential transfection with pcDNA3.1-hygro- $\alpha 6$, and pcDNA3.1-zeo-h $\beta 2$ or pcDNA3.1-zeo-h $\beta 4$, and pcDNA3.1-neo-h $\beta 3$, and pEF6-h $\alpha 5$.

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 μ g of mRNA was incubated with 4 units of DNase I in a 50- μ l reaction at 37°C for 30 min. DNase I was then inactivated by the addition of 5 μ l of 25 mM EDTA and incubation at 65°C for 10 min. RT was carried out using 2 μ g of DNA-free total RNA, oligo(dT) 12-18 primer, and a Superscript II preamplification system (Invitrogen) in a 20- μ l 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 μ l of cDNA preparation, 1 μ l of 10 μ M each of 5' and 3' gene-specific primers, 1 μ l of 10 mM dNTP, and 2.5 units of RedTaq (Sigma-Aldrich) in 50- μ l 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 40,000g 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 $\alpha 6$ 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 CaCl₂, 2.67 mM KCl, 1.47 mM KH₂PO₄, 0.49 mM MgCl₂, 137.93 mM NaCl, and 4.29 mM Na₂HPO₄, pH 7.4 in either 48-well or 96-well plates. Each sample (performed in triplicate at minimum) contained 50 μ l of test compound in solution at the desired concentration, 50 μ l of 4 \times [3 H]EPI stock solution, and 100 μ l of membrane suspension. Incubation was conducted for 2 h at room temperature. Total and non-specific bindings were measured in the presence of assay buffer or 100 μ M 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 femtomoles 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 μ l 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 μ l 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 μ M NIC or 10 μ M MLA, respectively.

Competition Binding Assays Using α CtxMII. Test samples (triplicates in 48-well plate) containing 100 μ l of α CtxMII solution at the desired concentration, 750 μ l 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 μ l 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 μ M nicotine, respectively. Next, 50 μ l 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 μ g 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 CO₂ 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 \times 2 min at 20 Hz. Chloroform (200 μ l) was added to the homogenate, and samples were sonicated for 20 s and centrifuged at 12,000g for 15 min. The aqueous phase (600 μ l) 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 μ l 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 (Prologis France SAS, Paris, France) following the Applied Biosystems RT reaction procedure. The final RT reaction mix included template, 1 \times cDNA first strand synthesis buffer, 5.5 mM MgCl₂, 0.5 mM each dNTP, 0.4 U/ μ l of RNase inhibitor, 2.5 μ M oligo(dT), and 1.25 U/ μ l of Multiscribe reverse transcriptase in a total volume brought to 100 μ l 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 TaqDNA polymerase, dNTP, MgCl₂, and SYBR Green I dye; Roche Diagnostics, Mannheim, Germany) was mixed with 1 μ l of cDNA and primers at 0.4 μ M in a final volume of 20 μ l 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 \pm S.E.M.) and the maximum number of binding sites B_{max} (mean \pm 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^{(logIC₅₀ - X)/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).

Results

Expression of Human $\alpha 6^*$ nAChR by SH-EP1 Human Epithelial Cells. Based on the current understanding of the possible composition(s) of nAChR containing $\alpha 6$ subunits as well as findings from the lesioning studies presented below, we sought to determine whether and how efficiently nAChR could be formed containing: $\alpha 6$ subunits with $\beta 2$ or $\beta 4$ subunits as simple, binary complexes; $\alpha 6$ and $\beta 2/\beta 4$ subunits with $\beta 3$ subunits thought to be required for formation of α CtxMII-sensitive $\alpha 6^*$ nAChR; and $\alpha 6$ and both $\beta 3$ and $\alpha 5$ 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: $\alpha 6\beta 2$, $\alpha 6\beta 4$, $\alpha 6\beta 2\beta 3$, $\alpha 6\beta 4\beta 3$, and $\alpha 6\beta 4\beta 3\alpha 5$.

Little-to-No Radioligand Binding to Human $\alpha 6\beta 2$ -, $\alpha 6\beta 4$ -, $\alpha 6\beta 2\beta 3$ -, and $\alpha 6\beta 4\beta 3$ nAChR Stably Expressed in SH-EP1 Human Epithelial Cells. The $\alpha 6\beta 2$ and $\alpha 6\beta 4$ subunit combinations stably expressed in SH-EP1 human epithelial cells did not produce any meaningful high affinity binding capacity for [³H]EPI or [³H]MLA. Also, only barely detectable high affinity binding levels were observed for [³H]MLA or [³H]NIC using membranes made from cells transfected with $\alpha 6\beta 2\beta 3$ or $\alpha 6\beta 4\beta 3$ combinations. Although the density of [³H]EPI binding sites on membranes from SH-EP1- $\alpha 6\beta 2\beta 3$ cells (data not shown) or SH-EP1- $\alpha 6\beta 4\beta 3$ 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 μ M α CtxMII (a selective antagonist for $\alpha 6$ -containing nAChR), at the concentration that exceeded 800-fold its K_i determined for another $\alpha 6^*$ nAChR (see Table 1).

Pharmacological Characterization of Human $\alpha 6\beta 4\beta 3\alpha 5$ nAChR Stably Expressed in SH-EP1 Human Epithelial Cells. Specific, α CtxMII-sensitive [³H]EPI binding was observed with membrane preparations derived from SH-EP1- $\alpha 6\beta 4\beta 3\alpha 5$ cells (Fig. 1). Saturation binding analysis indicated that the binding of [³H]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

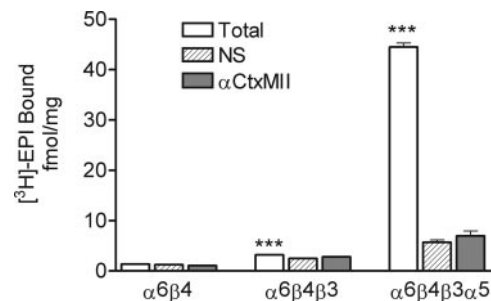


Fig. 1. Density of specific [³H]EPI binding on membranes of SH-EP1- $\alpha 6\beta 4$, - $\alpha 6\beta 4\beta 3$, and - $\alpha 6\beta 4\beta 3\alpha 5$ cells. Data expressed as femtomoles of bound [³H]EPI per milligram of membrane protein are means \pm S.E.M. Each data set was generated from three independent experiments using 0.5 nM [³H]EPI alone (total binding; open bars) or in the presence of 100 μ M NIC (NS, nonspecific binding; cross-hatched bars) or 5 μ M α CtxMII (shaded bars). Data on SH-EP1- $\alpha 6\beta 4\beta 3\alpha 5$ 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 α CtxMII samples. ***, $p < 0.001$.

model ($R^2 = 0.97$; $F_{2,6} = 0.539$; $p = 0.61$). Competition binding assays using α -CtxMII were conducted to confirm the $\alpha 6$ -specificity of the expressed binding sites. α CtxMII 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 α CtxMII in competition toward [³H]EPI binding, with $K_i = 1.75$ (1.42–2.14) nM. Compared with A-85380, MLA, and NIC, α CtxMII did not displace completely [³H]EPI (with an estimated $11 \pm 2\%$ 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- $\alpha 6\beta 4\beta 3\alpha 5$ 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 [³H]EPI was TC-2429 ($K_i = 1.75$ nM) > α CtxMII ($K_i = 6.15$ nM) \geq LOB ($K_i = 6.39$ nM) > A-85380 ($K_i = 8.71$ nM) > CYT \approx NIC \approx MLA ($K_i = 156$ –200 nM) > ABT-418 \approx MCC \approx SIB-1508Y \approx GTS-21 ($K_i = 0.97$ –1.29 μ M) > CAR ($K_i = 3.25$ μ M) > dihydro- β -erythroidine, DH β E \approx α Btx ($K_i > 10$ μ M). All compounds examined had a slope value near unity (range: -0.80 to -1.15).

The pharmacological profile of $\alpha 6\beta 3\beta 4\alpha 5$ nAChR was distinctly different from that of $\alpha 4\beta 2^*$ nAChR as probed with [³H]NIC on rat cortex membranes, or of $\alpha 7^*$ nAChR as probed with [³H]MLA on membranes of the rat hippocampus (Fig. 3b and Table 1). Indeed, whereas α CtxMII was more than 500 times more potent at $\alpha 6\beta 4\beta 3\alpha 5$ nAChR than at $\alpha 4\beta 2^*$ nAChR (Table 1 and Fig. 3a), typical $\alpha 4\beta 2^*$ nAChR agonists such as A-85380, NIC, CYT, ABT-418, MCC, and SIB-1508Y were at least 100 times less potent at $\alpha 6\beta 4\beta 3\alpha 5$ nAChR than at $\alpha 4\beta 2^*$ nAChR. Moreover, the $\alpha 4\beta 2^*$ nAChR antagonist DH β E did not display any affinity for $\alpha 6\beta 4\beta 3\alpha 5$ nAChR. Likewise, α CtxMII was 100 times more potent at $\alpha 6\beta 4\beta 3\alpha 5$ nAChR than at $\alpha 7^*$ nAChR (Table 1 and Fig. 3a), and the $\alpha 7^*$ nAChR antagonist α -Bgt did not displace [³H]EPI binding to SH-EP1- $\alpha 6\beta 4\beta 3\alpha 5$ cell-derived membranes. MLA, another potent $\alpha 7^*$ nAChR antagonist, displayed moderate affinity for $\alpha 6\beta 4\beta 3\alpha 5$ nAChR ($K_i = 200$ nM).

$\alpha 6$ 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 $\alpha 6\beta 3\beta 4\alpha 5$ nAChR stably expressed in SH-EP1 cells, at native rat $\alpha 4\beta 2^*$ nAChR (cortex) or at $\alpha 7^*$ nAChR (hippocampus)

Compound	$\alpha 6\beta 3\beta 4\alpha 5$	$\alpha 4\beta 2^*$	$\alpha 7^*$
	$[^3\text{H}]\text{EPI}$ Binding	$[^3\text{H}]\text{NIC}$ Binding	$[^3\text{H}]\text{MLA}$ Binding
		<i>nM</i>	
EPI	0.096 \pm 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)
α CtxMII	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)
DH β E	>10,000 ^c	24.6 (16.9–35.8)	7700 (4510–13100)
α Btx	>10,000	>10,000	2.16 (1.56–3.01)

^a K_d , mean \pm S.E.M.

^b Reported in Bencherif et al., 1998.

^c Data represent two independent experiments.

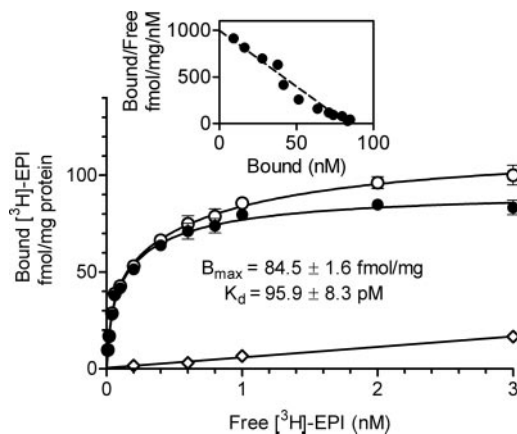


Fig. 2. Saturation $[^3\text{H}]\text{EPI}$ binding to membranes of SH-EP1 cells stably expressing human $\alpha 6\beta 4\beta 3\alpha 5$ nAChR. Saturation analysis was conducted using a concentration range of 0.01 to 3.0 nM $[^3\text{H}]\text{EPI}$. Data expressed as fmol/mg protein are means \pm S.E.M. of three independent experiments using cells of passages 34–35. Curves for total (\circ) and specific binding (\bullet) were generated using nonlinear regression (a one-site model). Linear regression was used to plot nonspecific binding (\diamond). The inset illustrates Scatchard transformation of the specific binding data.

composition of $\alpha 6^*$ 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 $\alpha 6$ subunits and its possible assembly partners ($\alpha 4$, $\alpha 5$, $\beta 2$, $\beta 3$, $\beta 4$) was quantified eight months later for 6-OHDA-lesioned animals and normalized to levels of message in saline-injected controls. The level of $\alpha 6$ expression was decreased in lesioned animals to 19% of controls (Fig. 4a). Whereas mRNA levels for $\alpha 5$ subunits were decreased to 44% of controls, $\alpha 4$ subunit expression remained high after lesion at 84% of controls. Furthermore, destruction of the nigro-striatal pathway resulted in loss of $\beta 3$ subunit expression identical to that for the loss of $\alpha 6$ subunit message levels (19% of controls, Fig. 4a), whereas expression of $\beta 2$ and $\beta 4$ subunits remained unaffected (92 and 107%, respectively) by the lesion. These data suggest that $\beta 3$ and $\alpha 5$ subunits are likely partners of the $\alpha 6$ subunits in nicotinic receptors expressed in dopaminergic cell bodies. Quantitative real-time RT-PCR indicated

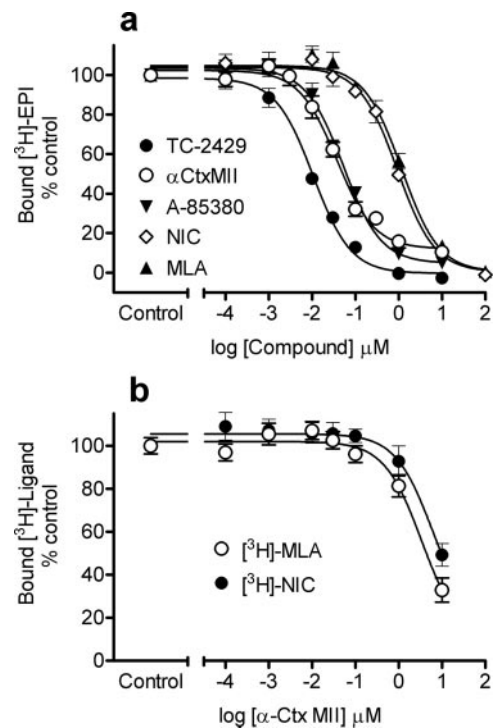


Fig. 3. A, competition of α CtxMII and other nicotinic ligands for $[^3\text{H}]\text{EPI}$ binding to human $\alpha 6\beta 4\beta 3\alpha 5$ nAChR. Data expressed as percent of control are means \pm S.E.M. of three to five independent experiments. Curves were generated using nonlinear regression (individual data were best fitted to a one-site model) for TC-2429 (\bullet), α CtxMII (\circ), A-85380 (\blacktriangledown), nicotine (\diamond), and methyllycaconitine (\blacktriangle) competing for specific $[^3\text{H}]\text{EPI}$ binding. B, α CtxMII binding to rat $\alpha 4\beta 2^*$ or $\alpha 7^*$ nAChR. Data expressed as a percent of control are mean \pm S.E.M. of three to four independent experiments for α CtxMII competition toward $[^3\text{H}]\text{MLA}$ binding to $\alpha 7^*$ nAChR (\circ) or toward $[^3\text{H}]\text{NIC}$ binding to $\alpha 4\beta 2^*$ nAChR (\bullet). Curves were generated using nonlinear regression (individual data were best fitted to a one-site model).

that $\alpha 6$ 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 $\alpha 6$ subunit are specifically enriched in this brain region most affected in Parkinson's disease.

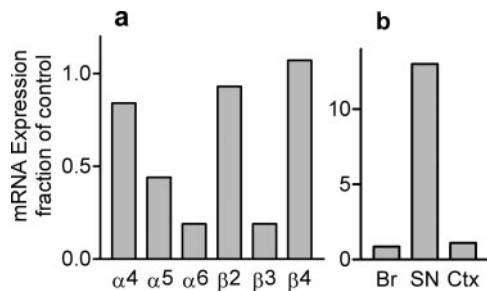


Fig. 4. A, lesion of the nigro-striatal dopaminergic pathway coeffects $\alpha 6$, $\alpha 5$, and $\beta 3$ nAChR subunit gene expression. Rats ($n = 10$) that received microinjections of $8 \mu\text{g}$ 6-OHDA in the medial forebrain bundle were tested for nAChR subunit gene expression 8 months later. 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 [^3H]EPI and αCtxMII , a selective antagonist for $\alpha 6^*$ nAChR (Champtiaux et al., 2002). Using standard nicotinic ligands to displace [^3H]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 non-functional 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 $\beta 4$. About half of the $\alpha 6$ -containing nAChR in retina also contains $\beta 3$ and/or $\alpha 3$, whereas less than 10% contain $\beta 2$ and none contain $\alpha 4$ or $\alpha 5$ subunits (Vailati et al., 1999). The presence of the $\alpha 6\beta 4\beta 3$ subunit combination in chick retina was confirmed in a later study (Vailati et al., 2000). Chick $\alpha 6\beta 4$ 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 $\alpha 6$ and $\beta 3$ subunits in combination with $\beta 4$ instead of $\beta 2$ subunit would most probably generate physiologically-relevant $\alpha 6^*$ nAChR, although a requirement for $\alpha 5$ subunits as we demonstrate here was not carefully addressed in previous studies.

The chick $\alpha 6\beta 4$ nAChR heterologously expressed in BOSC 23 cells (Barabino et al., 2001) exhibits a pharmacological profile similar to that of human $\alpha 6\beta 3\beta 4\alpha 5$ nAChR as reported here for some of the ligands, such as α CtxMII, NIC, MLA, CAR, and DH β E. There are notable variations, however, such as CYT and EPI, which exhibited 25- to 30-fold lower affinities for human $\alpha 6\beta 4\beta 3\alpha 5$ nAChR than for chick $\alpha 6\beta 4$ nAChR (Barabino et al., 2001). Interestingly, α CtxMII was found to have lower affinity for human $\alpha 6\beta 3\beta 4\alpha 5$ 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 ($EC_{50} = 2 \pm 1$ nM; $E_{max} = 40\%$ of NIC- or EPI-evoked responses) although it lacks activity as an agonist at $\alpha 4\beta 2$ 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 $\alpha 6^*$ nAChR. Moreover, the observed rank potency for a broad spectrum of nicotinic ligands in competition with [3 H]EPI at $\alpha 6\beta 4\beta 3\alpha 5$ nAChR revealed a profile distinctly different from that of $\alpha 4\beta 2^*$ or $\alpha 7^*$ nAChR and clearly characteristic of $\alpha 6^*$ nAChR.

Lesioning studies presented in this report support coexpression of $\alpha 6$, $\beta 3$, and $\alpha 5$ subunits in rat DA neurons (Charpantier et al., 1998; Elliott et al., 1998; Zoli et al., 2002; Champiaux et al., 2003). Diverse lines of investigation suggest that rodent DA neurons in the substantia nigra and ventral tegmentum express a mixture of $\alpha 6\beta 2(\pm \alpha 4)(\pm \beta 3)$ nAChR and $\alpha 4\beta 2$ nAChR on terminals and a predominance of $\alpha 4\beta 2^*$ nAChR over $\alpha 6^*$ nAChR somatodendritically (Ruben et al., 2000; Zoli et al., 2002; Champiaux et al., 2003; Salminen et al., 2004). Although immunoprecipitation studies suggested that a small fraction of $\alpha 6^*$ nAChR contained $\alpha 5$ (12%) and/or $\beta 3$ (8%) subunits, there were very large reductions in the expression of these subunits in radioligand binding complexes upon DA neuronal lesioning or in $\alpha 6^-/-$ mice (Le Novère et al., 1996; Champiaux et al., 2003). However, $\beta 4$ mRNA is not abundant in rodent dopaminergic cells (Le Novère et al., 1996), and studies on $\beta 2$ -null mutant mice have demonstrated that functional heteromeric nAChR (containing $\alpha 4$ and/or $\alpha 6$ subunits) expressed by dopaminergic neurons contain predominantly the $\beta 2$ subunit (Grady et al., 2001). Thus, the bulk of natural expression evidence points to involvement of $\beta 2$ subunits in formation of rodent $\alpha 6^*$ nAChR.

However, results from our studies and others indicate that heterologously expressed $\alpha 6\beta 2$ or $\alpha 6\beta 2\beta 3$ nAChR fail to properly assemble (Gerzanich et al., 1997; Kuryatov et al.,

2000; Evans et al., 2003), making uncertain roles of $\beta 2$ subunit as a mandatory assembly partner for human $\alpha 6$ subunits. Moreover, the greater relative expression of $\beta 4$ nAChR subunits in primate DA neurons (Quik et al., 2000) suggests that $\alpha 6^*$ nAChR in primates may have a different subunit composition from that in rodents. Perhaps some human $\alpha 6$ nAChR, as heterologously expressed in SH-EP1 cells, contain $\beta 4$, $\beta 3$, and $\alpha 5$ subunits, whereas their rodent "counterpart" utilizes the $\beta 2$ instead of the $\beta 4$ subunit and do not require the $\alpha 5$ subunit for binding and function.

The results from our studies suggest possible differences in the phenotype of $\alpha 6^*$ nAChR expressed across species. The relative high abundance of $\alpha 6$ mRNA in the substantia nigra from human compared with other brain regions is consistent with a role for $\alpha 6^*$ nAChR in motoric function observed in animal studies. Whereas it remains to be established what roles in general the $\alpha 5$ subunit plays in human nAChR, the current studies represent initial steps in exploring $\alpha 6^*$ nAChR in human health and disease.

Acknowledgments

We are grateful to Melanie Kiser and Sara Woodson at Targacept, Inc. for expert technical assistance.

References

- Azam L, Winzer-Serhan UH, Chen Y, and Leslie FM (2002) Expression of neuronal nicotinic acetylcholine receptor subunit mRNAs within midbrain dopamine neurons. *J Comp Neurol* **444**:260–274.
- Barabino B, Vailati S, Moretti M, McIntosh JM, Longhi R, Clementi F, and Gotti C (2001) An $\alpha 4\beta 4$ nicotinic receptor subtype is present in chick retina: identification, characterization and pharmacological comparison with the transfected $\alpha 4\beta 4$ and $\alpha 6\beta 4$ subtypes. *Mol Pharmacol* **59**:1410–1417.
- Bencherif M and Schmitt JD (2002) Targeting neuronal nicotinic receptors: a path to new therapies. *Curr Drug Targets CNS Neurol Disord* **1**:349–357.
- Bencherif M, Schmitt JD, Bhatti BS, Crooks P, Caldwell WS, Lovette ME, Fowler K, Reeves L, and Lippello PM (1998) The heterocyclic substituted pyridine derivative (\pm)-2-(3-pyridinyl)-1-azabicyclo[2.2.2]octane (RJR-2429): a selective ligand at nicotinic acetylcholine receptors. *J Pharmacol Exp Ther* **284**:886–894.
- Champiaux N, Gotti C, Cordero-Erausquin M, David DJ, Przybylski C, Lena C, Clementi F, Moretti M, Rossi FM, Le Novère N, et al. (2003) Subunit composition of functional nicotinic receptors in dopaminergic neurons investigated with knock-out mice. *J Neurosci* **23**:7820–7829.
- Champiaux N, Han ZY, Bessis A, Rossi FM, Zoli M, Marubio L, McIntosh JM, and Changeux JP (2002) Distribution and pharmacology of $\alpha 6$ -containing nicotinic acetylcholine receptors analyzed with mutant mice. *J Neurosci* **22**:1208–1217.
- Charpantier E, Barneoud P, Moser P, Besnard F, and Sgard F (1998) Nicotinic acetylcholine subunit mRNA expression in dopaminergic neurons of the rat substantia nigra and ventral tegmental area. *Neuroreport* **9**:3097–3101.
- Cui C, Booker TK, Allen RS, Grady SR, Whiteaker P, Marks MJ, Salminen O, Tritto T, Butt CM, Allen WR, et al. (2003) The $\beta 3$ nicotinic receptor subunit: a component of α -conotoxin MII-binding nicotinic acetylcholine receptors that modulate dopamine release and related behaviors. *J Neurosci* **23**:11045–11053.
- Dani JA (2001) Overview of nicotinic receptors and their roles in the central nervous system. *Biol Psychiatry* **49**:166–174.
- Eaton JB, Peng JH, Schroeder KM, George AA, Fryer JD, Krishnan C, Buhlman L, Kuo YB, Steinlein O, and Lukas RJ (2003) Characterization of human $\alpha 4\beta 2$ -nicotinic acetylcholine receptors stably and heterologously expressed in native nicotinic receptor-null SH-EP1 human epithelial cells. *Mol Pharmacol* **64**:1283–1294.
- Elliott KJ, Jones JM, Sacaan AI, Lloyd GK, and Corey-Naeve J (1998) 6-Hydroxydopamine lesion of rat nigrostriatal dopaminergic neurons differentially affect nicotinic acetylcholine receptor subunit mRNA expression. *J Mol Neurosci* **10**:251–260.
- Evans NM, Bose S, Benedetti G, Zwart R, Pearson KH, McPhie GI, Craig PJ, Benton JP, Volsen SG, Sher E, et al. (2003) Expression and functional characterization of a human chimeric nicotinic receptor with $\alpha 6\beta 4$ properties. *Eur J Pharmacol* **466**:31–39.
- Fucile S, Matter JM, Erkman L, Ragozzino D, Barabino B, Grassi F, Alema S, Ballivet M, and Eusebi F (1998) The neuronal $\alpha 6$ subunit forms functional heteromeric acetylcholine receptors in human transfected cells. *Eur J Neurosci* **10**:172–178.
- Gerzanich V, Kuryatov A, Anand R, and Lindstrom J (1997) "Orphan" $\alpha 6$ nicotinic AChR subunit can form a functional heteromeric acetylcholine receptor. *Mol Pharmacol* **51**:320–327.
- Gerzanich V, Wang F, Kuryatov A, and Lindstrom J (1998) $\alpha 5$ Subunit alters desensitization, pharmacology, Ca^{2+} permeability and Ca^{2+} modulation of human neuronal $\alpha 3$ nicotinic receptors. *J Pharmacol Exp Ther* **266**:311–320.
- Grady SR, Meinerz NM, Cao J, Reynolds AM, Picciotto MR, Changeux JP, McIntosh JM, Marks MJ, and Collins AC (2001) Nicotine agonists stimulate acetylcholine

- release from mouse interpeduncular nucleus: a function mediated by a different nAChR than dopamine release from striatum. *J Neurochem* **76**:258–268.
- Groot-Kormelink PJ, Boorman JP, and Sivilotti LG (2001) Formation of functional $\alpha 3\beta 4\alpha 5$ human neuronal nicotinic receptors in *Xenopus* oocytes: a reporter mutation approach. *Br J Pharmacol* **134**:789–796.
- Hernandez M-C, Erkman L, Matter-Sadzinski L, Roztocil T, Ballivet M, and Matter J-M (1995) Characterization of the nicotinic acetylcholine receptor $\beta 3$ gene. *J Biol Chem* **270**:3224–3233.
- Klink R, de Kerchove d'Exaerde A, Zoli M and Changeux JP (2001) Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic neurons. *J Neurosci* **21**:1452–1463.
- Kulak JM, Sum J, Musachio JL, McIntosh JM, and Quik M (2002) 5-Iodo-A-85380 binds to α -conotoxin MII-sensitive nicotinic acetylcholine receptors (nAChR) as well as $\alpha 4\beta 2^*$ subtypes. *J Neurochem* **81**:403–406.
- Kuryatov A, Olale F, Cooper J, Choi C, and Lindstrom J (2000) Human $\alpha 6$ AChR subtypes: subunit composition, assembly and pharmacological responses. *Neuropharmacology* **39**:2570–2590.
- Le Novère N, Zoli M, and Changeux JP (1996) Neuronal nicotinic receptor $\alpha 6$ subunit mRNA is selectively concentrated in catecholaminergic nuclei of the rat brain. *Eur J Neurosci* **8**:2428–2439.
- le Novère N, Zoli M, Lena C, Ferrari R, Picciotto MR, Merlo-Pich E, and Changeux JP (1999) Involvement of $\alpha 6$ nicotinic receptor subunit in nicotine-elicited locomotion, demonstrated by in vivo antisense oligonucleotide infusion. *Neuroreport* **10**:2497–2501.
- Luetje CW (2004) Getting past the asterisk: the subunit composition of presynaptic nicotinic receptors that modulate striatal dopamine release. *Mol Pharmacol* **65**:1333–1335.
- Lukas RJ (1986) Immunochemical and pharmacological distinctions between curaremimetic neurotoxin binding sites of central, autonomic and peripheral origin. *Proc Natl Acad Sci USA* **83**:5741–5745.
- Lukas RJ, Fryer JD, Eaton JB, and Gentry CL (2001) Some methods for studies of nicotinic acetylcholine receptor pharmacology, in *Nicotinic receptors in the nervous system* (Levin ED ed) pp 3–27, CRC Press LLC, Boca Raton, FL.
- Lukas RJ, Norman SA, and Lucero L (1993) Characterization of nicotinic acetylcholine receptors expressed by cells of the SH-SY5Y human neuroblastoma clonal line. *Mol Cell Neurosci* **4**:1–12.
- Pacheco MA, Pastoor TE, Lukas RJ, and Wecker L (2001) Characterization of human $\alpha 4\beta 2$ neuronal nicotinic receptors stably expressed in SH-EP1 cells. *Neurochem Res* **26**:683–693.
- Quik M, Bordia T, Forno L, and McIntosh JM (2004) Loss of α -conotoxinMII- and A85380-sensitive nicotinic receptors in Parkinson's disease striatum. *J Neurochem* **88**:668–679.
- Quik M and Kulak JM (2002) Nicotine and nicotinic receptors; relevance to Parkinson's disease. *Neurotoxicology* **23**:581–594.
- Quik M, Polonskaya Y, Gillespie A, Lloyd GK, and Langston JW (2000) Differential alterations in nicotinic receptor $\alpha 6$ and $\beta 3$ subunit messenger RNAs in monkey substantia nigra after nigrostriatal degeneration. *Neuroscience* **100**:63–72.
- Ramirez-Latorre J, Yu CR, Qu X, Perin F, Karlin A, and Role L (1996) Functional contributions of $\alpha 5$ subunit to neuronal acetylcholine receptor channels. *Nature (Lond)* **380**:347–351.
- Reuben M, Boye S, and Clarke PB (2000) Nicotinic receptors modulating somatodendritic and terminal dopamine release differ pharmacologically. *Eur J Pharmacol* **393**:39–49.
- Salminen O, Murphy KL, McIntosh JM, Drago J, Marks MJ, Collins AC, and Grady SR (2004) Subunit composition and pharmacology of two classes of striatal presynaptic nicotinic acetylcholine receptors mediating dopamine release in mice. *Mol Pharmacol* **65**:1526–1535.
- Vailati S, Hanke W, Bejan A, Barabino B, Longhi R, Balestra B, Moretti M, Clementi F, and Gotti C (1999) Functional $\alpha 6$ -containing nicotinic receptors are present in chick retina. *Mol Pharmacol* **56**:11–19.
- Vailati S, Moretti M, Balestra B, McIntosh M, Clementi F, and Gotti C (2000) $\beta 3$ subunit is present in different nicotinic receptor subtypes in chick retina. *Eur J Pharmacol* **393**:23–30.
- Wang F, Gerzanich V, Wells GB, Anand R, Peng X, Keyser K, and Lindstrom J (1996) Assembly of human neuronal nicotinic receptor $\alpha 5$ subunits with $\alpha 3$, $\beta 2$, and $\beta 4$ subunits. *J Biol Chem* **271**:17656–17665.
- Wonnacott S (1997) Presynaptic nicotinic Ach receptors. *Trends Neurosci* **20**:92–98.
- Zoli M, Moretti M, Zanardi A, McIntosh JM, Clementi F, and Gotti C (2002) Identification of the nicotinic receptor subtypes expressed on dopaminergic terminals in the rat striatum. *J Neurosci* **22**:8785–8789.

Address correspondence to: Merouane Bencherif, Targacept, Inc., 200 East First Street, Suite 300, Winston-Salem, NC 27101. E-mail: merouane.bencherif@targacept.com
