0026-895X/07/7106-1563–1571$20.00
MOLECULAR PHARMACOLOGY
Vol. 71, No. 6
31492/3207013
Mol Pharmacol 71:1563–1571, 2007
Printed in U.S.A.

Pharmacology of α-Conotoxin MII-Sensitive Subtypes of Nicotinic Acetylcholine Receptors Isolated by Breeding of Null Mutant Mice

Outi Salminen,[1] Jennifer A. Drapeau, J. Michael McIntosh, Allan C. Collins, Michael J. Marks, and Sharon R. Grady

Institute for Behavioral Genetics, University of Colorado, Boulder, Colorado (O.S., J.A.D., E.L., M.C., A.C.C., M.J.M., S.R.G.); and Departments of Biology and Psychiatry, University of Utah, Salt Lake City, Utah (J.M.M.)

Received October 4, 2006; accepted March 6, 2007

ABSTRACT

Subtypes of nicotinic acetylcholine receptors (nAChR) containing α6 subunits comprise 25 to 30% of the presynaptic nAChRs expressed in striatal dopaminergic terminals in rodents and 70% in monkeys. This class of receptors, potentially important in nicotine addiction, binds α-conotoxin MII (α-CtxMII) with high affinity and is heterogeneous, consisting of several subtypes in mice, possibly an important consideration for the design of compounds that selectively activate or antagonize the α6 subclass of nAChRs. Selected-null mutant mice were bred to generate isolated subtypes of α6β2* nAChRs expressed in vivo for assessing pharmacology of α6β2* nAChRs. Binding to striatal membranes and function in synaptosomes from (α4−/−)(β3+/+) and (α4−/−)(β3−/−) mice were measured and compared with wild-type (α4+/+)(β3+/+) mice. Gene deletions (α4 and β3) decreased binding of ^{125}I-α-CtxMII without affecting affinity for α-CtxMII or inhibition of α-CtxMII binding by epibatidine or nicotine. Deletion of the α4 subunit substantially increased EC_{50} values for both nicotine- and cytisine-stimulated α-CtxMII-sensitive dopamine release from striatal synaptosomes. A further increase in EC_{50} values was seen upon the additional deletion of the β3 subunit. The data indicate that one α-CtxMII-sensitive nAChR subtype, prevalent on wild-type dopaminergic terminals, has the lowest EC_{50} for a nicotine-mediated function so far measured in mice. In conclusion, the gene deletion strategy enabled isolation of α6* subtypes, and these nAChR subtypes exhibited differential activation by nicotine and cytisine.

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels assembled as pentamers of various subunits (α2–α7 and β2–β4). Most subtypes are heteromeric, containing α and β subunits, although α7 forms homomeric receptors (Lindstrom 2003). Many brain nAChRs are expressed presynaptically, where they facilitate neurotransmitter release (Wonnacott 1997) by promoting an influx of calcium directly through nAChRs or via voltage-sensitive calcium channels (Soliakov and Wonnacott, 1996; Kulak et al., 2001).

Identifying the nAChR subtypes that modulate dopamine release is of interest because dopamine plays a vital role in reinforcing effects of nicotine (Dani and Heinemann, 1996). Early pharmacological studies established that nicotinic agonists elicit robust increases in [^{3}H]dopamine release from rodent striatal synaptosomes (Rapier et al., 1988, 1990; Grady et al., 1992). The discovery that α-conotoxin MII (α-CtxMII) is a partial inhibitor established that at least two nAChR subtypes modulate [^{3}H]dopamine release (Grady et al., 1997; Kulak et al., 1997). Approximately 25 to 30% of agonist-evoked [^{3}H]dopamine release from striatal synaptosomes is mediated by α-CtxMII-sensitive nAChRs in rodent (Kulak et al., 1997; Kaiser et al., 1998; Salminen et al., 2004). A larger percentage (~70%) is α-CtxMII-sensitive in monkey (McCallum et al., 2005).

Expression of multiple nAChR subtypes in dopaminergic

This work was supported by National Institute on Drug Abuse grants DA12242 (to M.J.M.) and DA03194 (to A.C.C), National Institute of Mental Health grant MH53631 (to J.M.M.), Colorado Tobacco Research Grant 3F-034 (to O.S.), and an Emil Aaltonen Foundation grant (to O.S.). Production of the null mutant mice was supported by animal resources grant DA015663 (to A.C.C.).

A portion of this work has been presented as an abstract: Salminen OS, Marks MJ, McIntosh JM, Collins AC, and Grady SR. (2005) Characterization of a novel presynaptic nicotinic acetylcholine receptor subtype (62) on mouse brain dopaminergic terminals. *Soc Neurosci Abstr* **31:**954.7

[1] Current affiliation: Division of Pharmacology and Toxicology, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.106.031492.

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; α-CtxMII, α-conotoxin MII; ACh, acetylcholine iodide; A-85380, 3-((2*S*)-azetidinylmethoxy)pyridine dihydrochloride; BSA, bovine serum albumin; DHβE, dihydro-β-erythroidine; MLA, methyllycaconitine; ST, striatum; OT, olfactory tubercle; NAcc, nucleus accumbens; SC, superior colliculus.

neurons is suggested by detection of mRNA for eight nAChR subunits (α3–α7, β2–β4) in dopamine cell bodies (Klink et al., 2001; Azam et al., 2002). Studies with nAChR subunit-null mutant mice demonstrate that certain subunits play vital roles in those nAChRs that modulate dopamine release. Agonist-induced dopamine release is totally lost from striatal synaptosomes (Grady et al., 2001) and slices (Zhou et al., 2001) obtained from β2-null mutant mice, establishing that the β2 subunit plays an essential role in all nAChR subtypes that modulate dopamine release. Although α-CtxMII binds with high affinity to both α6*- and α3β2*-nAChRs (Cartier et al., 1996; McIntosh et al., 2004) (* indicates the possibility of additional subunits; Lukas et al., 1999), this binding, as well as α-CtxMII-sensitive agonist-stimulated dopamine release, is totally absent in dopaminergic neurons of α6-null mutant mice (Champtiaux et al., 2002, 2003), establishing that α6* rather than α3* nAChRs modulate dopamine release. The β3 gene deletion substantially decreases the α-CtxMII-sensitive component of dopamine release, indicating that the β3 subunit is important in nAChRs that bind α-CtxMII with high affinity (Cui et al., 2003). We have analyzed the effects of α4, α5, α7, β2, β3, and β4 gene deletion on α-CtxMII-sensitive and -resistant dopamine release (Salminen et al., 2004). Results with β2 and β3 deletions replicated those in our previous reports (Grady et al., 2001; Cui et al., 2003). Deleting the α4 gene totally eliminated the α-CtxMII-resistant component of dopamine release as well as 50 to 60% of the α-CtxMII-sensitive component, whereas deleting the α5 subunit decreased maximal α-CtxMII-resistant dopamine release. We concluded that α4β2 and α4α5β2 nAChRs modulate the α-CtxMII-resistant component of dopamine release and that α6α4β3β2, α6β3β2, and perhaps α6β2 nAChRs modulate the α-CtxMII-sensitive component of dopamine release. Using an immunochemical approach to assess expression of α6-containing nAChRs, Gotti et al., (2005) also concluded that the α-CtxMII-sensitive nAChRs expressed in dopaminergic neurons are comprised of α6α4β3β2, α6β3b2, and α6β2 nAChRs.

Studying the pharmacology of α6*-nAChRs has been hampered by the difficulty of expressing α6β2-nAChRs in *Xenopus laevis* oocytes (Kuryatov et al., 2000). However, under conditions in which some expression was achieved, addition of α6 to α4 and β2 subunits did not change EC_{50} values for activation by ACh or nicotine, and chimeric α6/α3 expression with β2 increased EC_{50} values compared with α4β2-nAChRs (Kuryatov et al., 2000). These results contrast with our measurements for agonist-stimulated dopamine release, where the α-CtxMII-sensitive α6β2*-nAChRs exhibited significantly lower EC_{50} values than the α-CtxMII-resistant α4β2*-nAChRs (Salminen et al., 2004). Recombinant receptors may not have the same pharmacological selectivity as native receptors (Nicke et al., 2003). On the other hand, the more complex α6α4β3β2 nAChR, not yet studied in oocytes, may be responsible for the increased sensitivity to agonists observed for dopamine release.

The studies described here used wild-type, α4-null mutant and α4/β3 double-null mutant mice to characterize the pharmacology of α6β2* nAChRs. The sequential gene deletion strategy is designed to simplify α6* expression from that of wild-type (α6α4β3β2, α6β3β2, and α6β2) to that of α4-null mutant mice (α6β3β2 and α6β2), to that of the α4/β3 double mutation (α6β2), thereby allowing the characterization of the pharmacological properties of functional receptors assembled with normal processing and expressed in appropriate dopaminergic cells.

Materials and Methods

Materials. 7,8-[^{3}H]Dopamine was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA) (specific activity, 40–60 Ci/mmol). HEPES and sucrose were products of Roche Applied Science (Indianapolis, IN). Sigma-Aldrich (St. Louis, MO) was the source for the following compounds: acetylcholine iodide (ACh), ascorbic acid, aprotinin, atropine sulfate, A-85380, bovine serum albumin (BSA), cytisine, dihydro-β-erythroidine (DHβE), diisopropyl fluorophosphate, (−)-epibatidine hydrochloride, EDTA, EGTA, leupeptin trifluoroacetate, methyllycaconitine (MLA), (−)-nicotine tartrate, nomifensine, pargyline, polyethylenimine, pepstatin A, and phenylmethylsulfonyl fluoride. α-CtxMII was synthesized as described previously (Cartier et al., 1996), as was ^{125}I-α-CtxMII (Whiteaker et al., 2000) (specific activity, 2200 Ci/mmol). All other chemicals were reagent grade. OptiPhase SuperMix scintillation fluid was purchased from PerkinElmer Life Sciences and Analytical Sciences–Wallac Oy (Turku, Finland).

Animals. Animal care and experimental procedures were in accordance with the guidelines and approval of the Animal Care and utilization Committee of the University of Colorado, Boulder. Mice used in this study were bred and maintained at the Institute for Behavioral Genetics, University of Colorado (Boulder, CO). Mice were weaned at 25 days of age and housed with same-sex littermates. A 12-h light/dark cycle (lights on from 7 AM to 7 PM) at 22°C was used. Mice had free access to food (Teklad Rodent Diet; Harlan, Madison, WI) and water. DNA was extracted from tail clippings, taken at 40 days of age, using the DNeasy kit from QIAGEN (Valencia, CA) and analyzed by polymerase chain reaction for assignment of genotype (Salminen et al., 2004).

The α4-null mutant mice, originally obtained from the laboratory of John Drago (Ross et al., 2000), were bred onto C57BL/6 background for 1 generation, and the β3-null mutants, from the laboratory of Stephen Heinemann (Cui et al., 2003), were bred onto C57BL/6 background for nine generations. Wild-type mice [the (α4+/+)(β3+/+) genotype] were littermates of the α4-null mutant mice [the (α4−/−)(β3+/+) genotype] and both were generated by breeding mice heterozygous for the α4 subunit [the (α4+/−)(β3+/+) genotype] and were of mixed genetic background. Mice with the double α4- and β3-null mutations [the (α4−/−)(β3−/−) genotype] were generated by first breeding mice of the (α4−/−)(β3+/+) genotype with mice of the (α4+/+)(β3−/−) genotype to generate mice of the (α4+/−)(β3+/−) genotype. These mice were subsequently bred together and pups were genotyped for both null mutations. This procedure generated both (α4−/−)(β3+/+) and (α4−/−)(β3−/−) mice that were littermates and on a mixed background with a greater contribution of C57BL/6 than the wild-type and α4-null mutants described above. No differences in binding or functional measures were found between the mixed background wild-type mice and C57BL/6 mice or between the two mixed backgrounds of the (α4−/−)(β3+/+) mice. The double knockout mice generated were subsequently bred for one generation to produce sufficient numbers of defined double-knockout mice.

Synaptosome Preparation for Release Experiments. After a mouse was sacrificed by cervical dislocation, its brain was removed and placed immediately on an ice-cold platform. The striatum (ST), olfactory tubercle (OT), and/or nucleus accumbens (NAcc) were dissected. Tissues from each mouse were homogenized in 0.5 ml of ice-cold 0.32 M sucrose buffered with 5 mM HEPES, pH 7.5. A crude synaptosomal pellet was prepared by centrifugation at 12,000*g* for 20 min. The pellets were resuspended in "uptake buffer": 128 mM NaCl, 2.4 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES, pH 7.5, 10 mM glucose, 1 mM ascorbic acid, and 0.01 mM pargyline using 1.6 ml for ST or OT from one mouse and 0.8 ml for NAcc.

Uptake of [^{3}H]Dopamine. Synaptosomes were incubated at 37°C in uptake buffer for 10 min before addition of 100 nM [^{3}H]dopamine (1 μCi for every 0.2 ml of synaptosomes), and the suspension was incubated for an additional 5 min.

Perfusion and Release. All experiments were conducted at room temperature using methods described previously (Grady et al., 1997, 2001) with modifications for collection into 96-well plates. In brief, aliquots of synaptosomes (80 μl) were distributed onto filters and perfused with the perfusion buffer (uptake buffer containing 0.1% BSA and 1 μM nomifensine) at 0.7 ml/min for 10 min before fractions were collected. For experiments using ACh as agonist, the synaptosomes were treated with diisopropyl fluorophosphate (10 μM) during the last 5 min of the uptake procedure, and atropine (1 μM) was added to the perfusion buffer. Fractions (~0.1 ml) were collected into 96-well plates every 10 s using a Gilson FC204 fraction collector with a multicolumn adapter (Gilson, Inc., Middleton, WI). Radioactivity was determined by scintillation counting using a 1450 MicroBeta Trilux scintillation counter (Perkin Elmer Life and Analytical Sciences–Wallac Oy) after addition of 0.15 ml of OptiPhase SuperMix scintillation cocktail. Instrument efficiency was 40%.

Membrane Preparation for ^{125}I-α-CtxMII Binding. Each mouse was sacrificed by cervical dislocation, and the brain was removed and placed on an ice-cold platform. Olfactory tubercles, striatum, and superior colliculus were dissected. Samples were homogenized in ice-cold 2× physiological buffer (288 mM NaCl, 3 mM KCl, 4 mM $CaCl_2$, 2 mM $MgSO_4$, and 40 mM HEPES, pH 7.5) using a glass-Teflon tissue grinder. The homogenate was then incubated with 1 mM phenylmethylsulfonyl fluoride at 22°C for 15 min to inactivate endogenous serine proteases and centrifuged at 20,000*g* (15 min, 4°C, RC-2B centrifuge; Sorvall, Newton, CT). The pellet was resuspended in distilled water and centrifuged as before. The pellet was washed once more by resuspension and centrifugation and then resuspended in a final volume of distilled water (0.250 ml for individual regions). Protein concentrations in the membrane preparations were measured using the method of Lowry et al. (1951), with BSA as the standard.

^{125}I-α-CtxMII Binding to Membranes. Membrane binding of ^{125}I-α-CtxMII was performed as described in Salminen et al. (2005). Samples were incubated in 1.2 ml of siliconized polypropylene tubes arranged in a 96-well format, using 40 to 50 μg of membrane protein per tube. The binding buffer contained 144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM HEPES, pH 7.5, and was supplemented with 0.1% (w/v) BSA, 5 mM EDTA, 5 mM EGTA, and 10 mg/ml each of aprotinin, leupeptin trifluoroacetate, and pepstatin A to protect the ligand from endogenous proteases. The small reaction volume (30 μl) minimized the amount of tissue and radioisotope required. All incubations progressed for 2 h at 22°C. Six replicates were used to determine the total and nonspecific (in the presence of 1 μM epibatidine) binding. When inhibition of ^{125}I-α-CtxMII binding was measured, various concentrations of competing ligands were included in triplicate wells. After the 2-h incubation, each sample was diluted with 1 ml of binding buffer containing 0.1% (w/v) BSA and incubated for 4 min at 22°C to reduce nonspecific binding to the membrane preparation. Binding reactions were terminated by filtration of samples onto a Unifilter^R GF/B filter (Whatman Inc., Clifton, NJ) treated with 5% (w/v) nonfat dry milk for 30 min, using a modified Inotech Cell Harvester (Inotech Biosystems, Rockville, MD). Samples were subsequently washed with four changes of ice-cold binding buffer containing 0.1% (w/v) BSA. Washes were performed at 30-s intervals, each lasting approximately 5 s. All filtration steps were performed at 4°C. Bound ligand was quantified by gamma counting at 60% efficiency, using a 1450 MicroBeta Trilux scintillation counter (Perkin Elmer Life and Analytical Sciences–Wallac Oy) after addition of 25 μl of Optiphase SuperMix scintillation cocktail to each well.

Data Analysis. Data were analyzed using the SigmaPlot 5.0 and SPSS for DOS (SPSS Inc., Chicago, IL). Perfusion data were plotted as counts per minute versus fraction number. Fractions collected before and after the peak were used to calculate baseline as a single exponential decay. The calculated baseline was subtracted from the experimental data. Fractions that exceeded baseline by 10% or more were summed to give total released counts per minute. Counts per minute released above baseline were normalized to baseline to give units of release [(counts per minute − baseline)/baseline] (Grady et al., 1997, 2001). (Note that because of the modifications required to use 96-well plates, the data are normalized to a 10-s baseline rather than an 18-s baseline that was used in previous studies from our laboratory. This results in higher R_{max} values for normalized data.)

Agonist dose response data were fit to the Hill equation (Grady et al., 2001). IC_{50} values for inhibition of release were calculated by fitting the data to a single site, partial-inhibition equation [release = $R/((1 + [An]/IC_{50}) + C)$] where R is the maximum amount of release that was inhibited by the antagonist, C is the amount of release that could not be inhibited by the antagonist and [An] is the antagonist concentration) (Grady et al., 2001). For competitive antagonists, K_i values were estimated from IC_{50} values using the equation: $K_i = IC_{50}/(1 + EC_{50}/[Ag])$ where [Ag] is concentration of agonist.

Results for inhibition of binding were calculated using a one-site fit: $B = B_0/[1+(I/IC_{50})]$ where B is ligand bound at inhibitor concentration I, B_0 is the binding in the absence of inhibitor, and IC_{50} is the concentration of inhibitor required to reduce binding to 50% of B_0.

Statistical significance was assessed from the R_{max}, EC_{50}, and IC_{50} values by one-way (genotype) or two-way (genotype and brain region) analysis of variance with Tukey's post hoc test. The EC_{50} and IC_{50} data were calculated using log transformation of the data.

Results

ST, OT, and superior colliculus (SC) contain significant ^{125}I-α-CtxMII binding (Whiteaker et al., 2000; Salminen et al., 2005); therefore, these three brain areas were assayed for α-CtxMII binding in wild-type mice (α4+/+)(β3+/+), mice with the α4-null mutation (α4−/−)(β3+/+), and mice with the double-null mutation (α4−/−)(β3−/−). ^{125}I-α-CtxMII binding was detectable in all three regions of all three genotypes (Fig. 1), but binding was reduced considerably after α4 gene deletion (α4−/−)(β3+/+) and double α4 and β3 gene deletions, (α4−/−)(β3−/−). Binding in ST of (α4−/−)(β3+/+) mice and of (α4−/−)(β3−/−) mice was reduced to 47% and 20%, respectively, of that of wild-type mice. In OT, binding in (α4−/−)(β3+/+) mice and in (α4−/−)(β3−/−) mice was 38% and 34%, respectively, of that of wild-type mice. In SC, re-

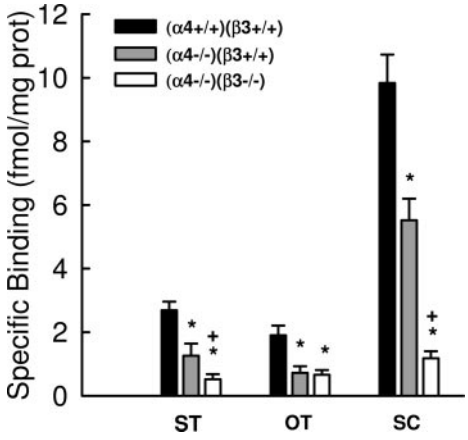

Fig. 1. α-CtxMII binding in ST, OT, and SC for three genotypes. ^{125}I-α-CtxMII membrane binding was measured using a single ligand concentration of 0.5 nM in ST, OT, and SC membranes prepared from (α4+/+)(β3+/+), (α4−/−)(β3+/+), and (α4−/−)(β3−/−) mice. *, significant difference from wild type, $P < 0.05$; +, significant difference from α4 subunit deletion, $P < 0.05$.

sidual ^{125}I-α-CtxMII binding in (α4−/−)(β3+/+) mice and (α4−/−)(β3−/−) mice was 56% and 12%, respectively, of that of wild-type mice.

The inhibition of ^{125}I-α-CtxMII (0.5 nM) binding by nicotine (Fig. 2a) and epibatidine (Fig. 2b) were measured for each of the three genotypes, using membrane preparations from the three regions combined. Data are presented as percentage of uninhibited controls. Controls were in agreement with the binding data of Fig. 1, with binding to membranes of the (α4−/−)(β3+/+) mixed regions at 49 ± 6% of wild-type and to (α4−/−)(β3−/−) at 18 ± 3% of wild-type binding. IC_{50} values for inhibition of ^{125}I-α-CtxMII binding by nicotine and epibatidine were not affected by deletion of either the α4 subunit or both α4 and β3 subunits (Fig. 2c).

Striatal synaptosomes were prepared from wild-type, α4-null mutant, and α4β3 double-null mutant genotypes to assess amounts of α-CtxMII-sensitive ACh-stimulated [^{3}H]dopamine release. Deletion of the α4 subunit eliminates α-CtxMII-resistant nAChR-mediated dopamine release (Champtiaux et al., 2003; Salminen et al., 2004); therefore, in both (α4−/−)(β3+/+) and (α4−/−)(β3−/−) mice, all agonist-stimulated dopamine release should be α-CtxMII-sensitive. In wild-type (α4+/+)(β3+/+) mice only a portion (29%) of the ACh-stimulated dopamine release was inhibited by α-CtxMII. As expected, in the (α4−/−)(β3+/+) and (α4−/−)(β3−/−) genotypes, virtually all dopamine release stimulated by 10 μM ACh was inhibited by α-CtxMII (30 nM). This result confirms previous reports that α4β2* nAChRs mediate dopamine release resistant to inhibition by α-CtxMII. Furthermore, the amount of dopamine release stimulated by ACh (10 μM) that was sensitive to inhibition by α-CtxMII was significantly lowered, but not eliminated, by deletion of the α4 subunit and further decreased by deletion of the α4 plus the β3 subunits (Fig. 3b). Note that the α-CtxMII-resistant activity seen in wild-type mice (71% of the total wild-type activity or 17.80 ± 2.32 units) is not included in Fig. 3b but is represented in Fig. 3a. Deletion of the α4 subunit decreased α-CtxMII-sensitive dopamine release by 62% (38% of wild-type remaining), and deletion of both the α4 and the β3 subunits decreased release by 84% from the wild-type (16% remaining) (Fig. 3b), similar percentages to the binding data for ST above. When the effect of gene deletion on total

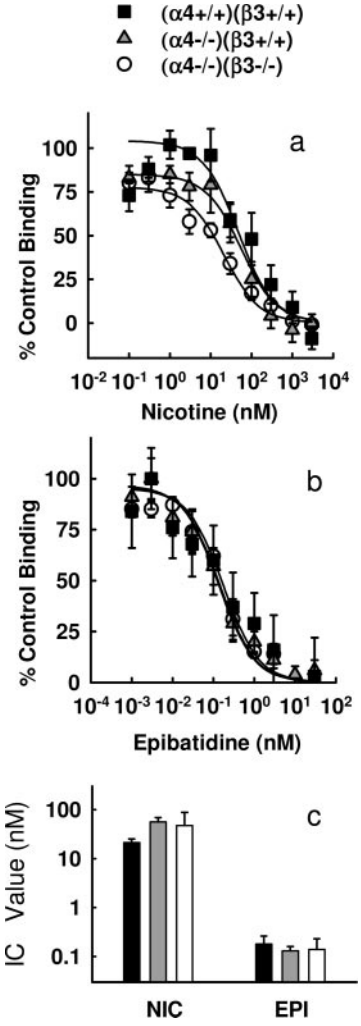

Fig. 2. Inhibition of ^{125}I-α-CtxMII binding by nicotine and epibatidine in three genotypes of mice. Mouse brain particulate fractions from combined ST, OT, and SC were incubated at 22°C with 0.5 nM ^{125}I-α-CtxMII. In all cases nonspecific binding was determined by including 1 μM unlabeled epibatidine. Each point represents the mean ± S.E.M. of 5–6 separate determinations. All data are presented as percentage of control binding to membranes of the same genotype. Inhibition by nicotine (a) and epibatidine (b) are shown. No significant differences in IC_{50} values with genotype were detected (c).

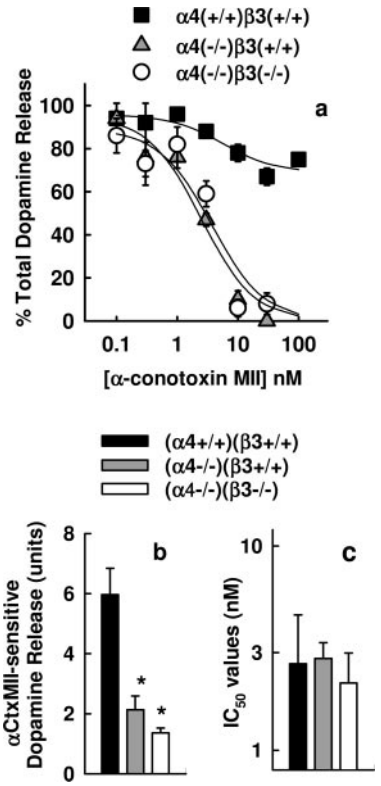

Fig. 3. Inhibition of agonist-induced DA release by α-CtxMII in three genotypes. Aliquots of synaptosomes were exposed to varying concentrations of α-CtxMII, as indicated, for 5 min immediately preceding exposure to 10 μM ACh for 10s. For a, data are expressed as percentage of control release (no α-CtxMII). Curves shown are fits to single-site partial inhibition for (α4+/+)(β3+/+) [release = 29/((1+[An]/IC_{50})+71)] and, for (α4−/−)(β3+/+) and (α4−/−)(β3−/−), to single-site full inhibition [release = 100/(1+[An]/IC_{50})]. For b, DA release via α-CtxMII-sensitive nAChRs for the three genotypes are compared. Only wild-type mice showed α-CtxMII-resistant activity, which, for these experiments, was 17.80 ± 2.32 units of dopamine release (data not shown). c presents a comparison of IC_{50} values for α-CtxMII for the three genotypes. *, significantly different from wild type, $P < 0.05$; +, significantly different from α4-null mutant, $P < 0.05$.

activity is considered, the residual activity in the (α4−/−)(β3+/+) and in (α4−/−)(β3−/−) mice is 11% of total (89% decrease) and 5% of total (95% decrease), respectively. The IC_{50} value for the inhibition by α-CtxMII did not differ significantly among genotypes and ranged from 2.13 to 2.81 nM (Fig. 3c).

Because ACh-evoked dopamine release was measurable in all three genotypes, the pharmacology of this nAChR-mediated function could be investigated. Concentration-effect curves for nicotine-stimulated α-CtxMII-sensitive dopamine release were determined in synaptosomes prepared from ST (Fig. 4a), NAcc (Fig. 4b), and OT (Fig. 4c) of the wild-type, α4-null mutant and α4β3 double-null mutant genotypes. Gene deletions altered both maximal responses and EC_{50} values for nicotine-stimulated α-CtxMII-sensitive dopamine release similarly in each brain region (two-way analysis of variance revealed no significant genotype by brain region interactions). As was the case for the ACh-stimulated release (Fig. 3), maximal nicotine-stimulated α-CtxMII-sensitive dopamine release for both the α4-null- and the α4/β3 double-null-mutants was significantly lower than that of wild-type mice in each brain region (Fig. 4d). In addition, a significant and substantial increase in EC_{50} values was observed after α4, and α4 plus β3, gene deletions (Fig. 4e). EC_{50} values for nicotine were 4- to 7-fold higher in (α4−/−)(β3+/+) than in wild-type mice. EC_{50} values for (α4−/−)(β3−/−) mice were an additional 4- to 7-fold higher than for mice with only the α4 subunit gene deletion. Consequently, EC_{50} values for the (α4−/−)(β3−/−) mice were 25- to 34-fold higher for nicotine-stimulated dopamine release than those for α-CtxMII-sensitive nicotine-stimulated dopamine release in wild-type mice.

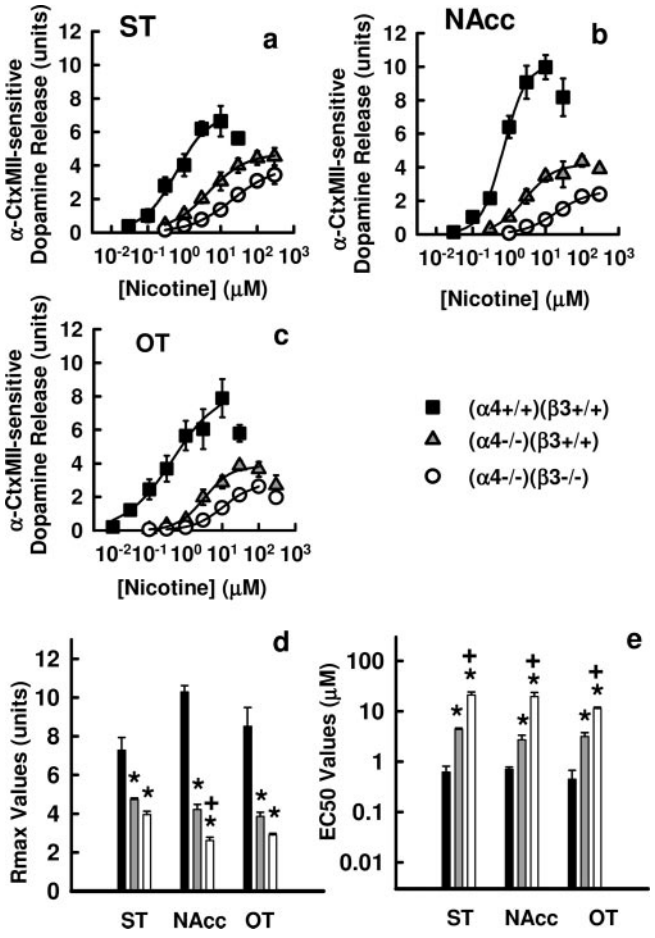

Fig. 4. Nicotine dose-response curves for α-CtxMII-sensitive DA release determined from synaptosomes prepared from ST, NAcc and OT of three genotypes. Dopamine release was stimulated by an 18s exposure to the indicated concentrations of nicotine. For the (α4+/+)(β3+/+) genotype, release was determined with and without a 5 min exposure to α-CtxMII (50 nM) immediately preceding the nicotine exposure. Data shown is determined by difference (total release - α-CtxMII-resistant release). Curves are from fits of the data to the Hill equation. Comparison of maximum release and the EC_{50} values for these agonists are shown in d and e, respectively. *, significantly different from wild type, $P < 0.05$; +, significantly different from α4-null mutant, $P < 0.05$.

Dose response curves for stimulation of α-CtxMII-sensitive dopamine release by four other nicotinic agonists were determined in wild-type, α4-null mutant, and α4/β3 double-null mutant genotypes. These assays were confined to ST because there seemed to be no significant regional differences when nicotine was used as test compound. Data for A-85380, acetylcholine, epibatidine, and cytisine are presented in Fig. 5a, b, c, and d, respectively. The effects of deletion of the α4 and α4 plus β3 subunits on maximal nAChR-stimulated dopamine release were similar for each agonist (Figs. 4d and 5f). The largest effect for each agonist was elicited by deletion of the α4 subunit. This deletion eliminated all α-CtxMII resistant release and significantly reduced the α-CtxMII sensitive release. R_{max} values in α4−/− mice for epibatidine (48%), ACh (49%) and A85380 (44%) were less than half those of WT mice, whereas R_{max} values for both cytisine (63%) and nicotine (65%) were slightly less affected by α4 gene deletion. Agonist-stimulated α-CtxMII-sensitive dopamine release was further reduced by deletion of the β3 subunit using nicotine with NAcc synaptosomes and using ACh with ST synaptosomes. With other agonists and regions, this further decrease was not statistically significant. In mice with both α4 and β3 gene deletions, R_{max} values were approximately 40% of those measured for WT mice for epibatidine, ACh, and A85380 and 50% for cytisine and nicotine.

EC_{50} values are compared in Fig. 5e. Large and significant increases in EC_{50} values for cytisine-evoked dopamine release were seen after deletion of the α4 subunit (~30-fold) and with the double deletion (α4−/−)(β3−/−) (~100-fold). For ACh and epibatidine, increases were 5-fold or less for the (α4−/−)(β3+/+) mice compared with the wild type and between 3- and 13-fold for the (α4−/−)(β3−/−) genotype. Subunit deletions did not produce significant changes in EC_{50} values for A85380.

For nicotine and cytisine, the two agonists for which gene deletions elicited large changes in EC_{50}, the dose response data were subjected to further analysis to estimate EC_{50} values for individual nAChR subtypes. The assumption here is that a change in EC_{50} value by gene deletion indicates that the EC_{50} value for the subtype removed differs significantly from the remaining subtypes. Minimal effect of gene deletion on EC_{50} value means the subtypes have similar EC_{50} values. Subtraction of curves allows an estimate of the EC_{50} value of the removed subtype, assuming no large changes in expression in response to the gene deletion (see *Discussion*). By subtracting the α4-null mutant data from the wild-type data, EC_{50} values for dopamine release mediated by the α-CtxMII-sensitive subtype unique to the wild-type mouse could be estimated. Likewise, subtracting the data for the double-null mutation (α4−/−)(β3−/−) from the data for the α4-null mutant should isolate the contribution of the α-CtxMII-sensitive

subtype without the $\alpha4$ subunit but with the $\beta3$ subunit ($\alpha6\beta3\beta2$-containing subtype). Table 1 presents EC_{50} values for curves generated by subtraction as well as those from the curves from Figs. 4 and 5, and values for α-CtxMII-resistant ($\alpha4\beta2$* nAChR) (Salminen et al., 2004) for comparison. This analysis indicated that the EC_{50} values for the subtype unique to the wild-type mice ($\alpha6\alpha4\beta3\beta2$-nAChR) were significantly lower than those for the sum of all α-CtxMII-sensitive subtypes, and the EC_{50} values for $\alpha6\beta3\beta2$-nAChR were lower than the values measured for the sum of subtypes in the $\alpha4$-null mutant, but higher than that measured for the sum of subtypes in the wild-type.

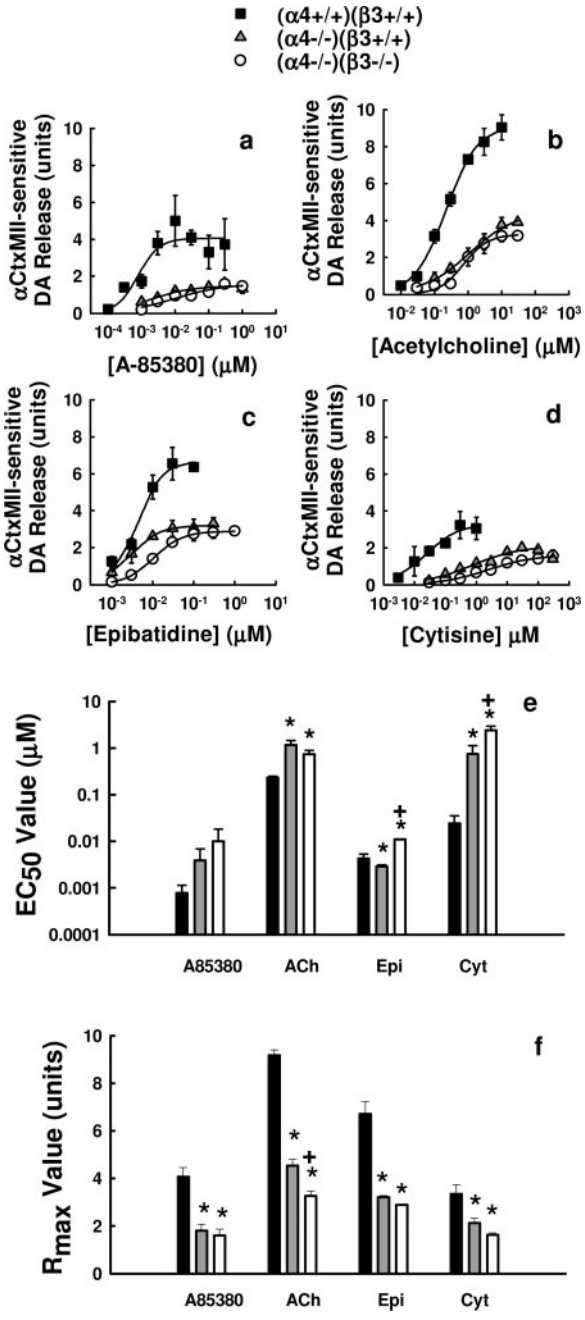

Fig. 5. Dose-response curves for agonist-stimulated α-CtxMII-sensitive dopamine release determined from synaptosomes prepared from ST of three genotypes. Dopamine release was stimulated by an 18-s exposure to the indicated concentrations of agonist. For the $(\alpha4+/+)(\beta3+/+)$ genotype, release was determined with and without a 5-min exposure to α-CtxMII (50 nM) immediately preceding the agonist exposure. Data shown is determined by difference (total release − α-CtxMII-resistant release). Curves are from fits of the data to the Hill equation. A comparison of the EC_{50} and R_{max} values for the four agonists by genotype is shown in e and f, respectively. *, significantly different from wild type, $P < 0.05$; +, significantly different from $\alpha4$-null mutant, $P < 0.05$.

Unlike the rather large differences seen for some of the agonists, effects of antagonists did not differ much for wild-type, $\alpha4$-null mutant, and $\alpha4\beta3$ double-null mutant genotypes. Inhibition of α-CtxMII-sensitive ACh-stimulated dopamine release from striatal synaptosomes by DHβE and MLA are shown in Fig. 6, a and b. IC_{50} values are compared in Fig. 6c. IC_{50} values for DHβE did not differ significantly among genotypes and ranged from 2.83 to 3.89 μM, equivalent to a K_i value of 0.21 ± 0.08 μM. IC_{50} values for MLA ranged from 0.83 to 1.92 μM. The small decrease in IC_{50} value for MLA in mice with the double subunit deletion $(\alpha-/-)(\beta-/-)$ was significant compared with both wild-type mice and mice with the $(\alpha4-/-)(\beta+/+)$ genotype; however this difference was not evident when calculated as K_i values (0.04, 0.20, and 0.06 μM for the three genotypes, respectively).

Discussion

Nicotinic agonist-stimulated dopamine release can be separated into two subclasses based on differential sensitivity to inhibition by α-CtxMII (Kulak et al., 1997; Cui et al., 2003). The α-CtxMII-sensitive subclass is more sensitive to ACh stimulation of dopamine release than the α-CtxMII-resistant component (Salminen et al., 2004). Further subdivision within the α-CtxMII-sensitive subclass is supported by immunoprecipitation experiments using subtype specific antibodies, indicating that three different α-CtxMII-sensitive nAChRs ($\alpha6\alpha4\beta3\beta2$, $\alpha6\beta3\beta2$, and $\alpha6\beta2$) are expressed in mouse striatum (Champtiaux et al., 2003; Gotti et al., 2005). The ^{125}I-α-CtxMII binding experiments reported here clearly support the assertion that there are three different α-CtxMII-sensitive nAChRs expressed in mouse striatum. Deletion of the $\alpha4$ gene (which should result in loss of $\alpha6\alpha4\beta3\beta2$ nAChRs) markedly decreased (>50%) ^{125}I-α-CtxMII binding. Deletion of both the $\alpha4$ and the $\beta3$ genes (which should eliminate the $\alpha6\beta3\beta2$ nAChRs but not the $\alpha6\beta2$-type nAChRs), further decreased but did not eliminate ^{125}I-α-CtxMII binding, consistent with the assertion that $\alpha6\beta3\beta2$ and $\alpha6\beta2$ nAChRs are expressed. Further evidence, supporting the postulate that three α-CtxMII-sensitive nAChRs are expressed in wild-type brain, is provided by the observation that maximal agonist-stimulated [^{3}H]dopamine release progressively decreased when measured from synaptosomes prepared from wild-type, $\alpha4$-null mutants, and $\alpha4\beta3$

TABLE 1
EC_{50} values for dopamine release from striatal synaptosomes

Genotype/Subtype	EC_{50}	
	Nicotine	Cytisine
	μM	
Wild-type $(\alpha4+/+)(\beta3+/+)$ α-CtxMII-sensitive	0.62 ± 0.19	0.024 ± 0.011
$\alpha4$-null/$\beta3$-null $(\alpha4-/-)(\beta3-/-)$	21.24 ± 3.21	2.38 ± 0.56
$\alpha6\alpha4\beta3\beta2$ by subtraction	0.23 ± 0.08	0.0080 ± 0.0033
$\alpha6\beta3\beta2$ by subtraction	1.52 ± 0.19	0.082 ± 0.032
C57BL/6 α-CtxMII-resistant	1.61 ± 0.19[a]	0.47 ± 0.09[a]

[a] Data from Salminen et al. (2004).

double-null mutants. The finding that the dose-response curves for both nicotine- and cytisine-stimulated [^{3}H]dopamine release showed progressive shifts to the right (increased EC_{50} values) as the α4, and then both the α4 andβ3 genes were deleted, reinforces the idea that at least three distinct nAChRs modulate the α-CtxMII-sensitive component of dopamine release. These experiments indicate that $\alpha6\alpha4\beta3\beta2$ nAChRs make up 50 to 60% of the α6* nAChRs expressed in dopaminergic nerve terminals of wild-type mice and that this subtype is more sensitive to activation by nicotine than are any of the other nAChR subtypes expressed in dopaminergic nerve terminals. Thus, the $\alpha6\alpha4\beta3\beta2$ subtype may play a very important role in regulating dopamine-related behaviors such as locomotor activity and the reinforcing effects of nicotine.

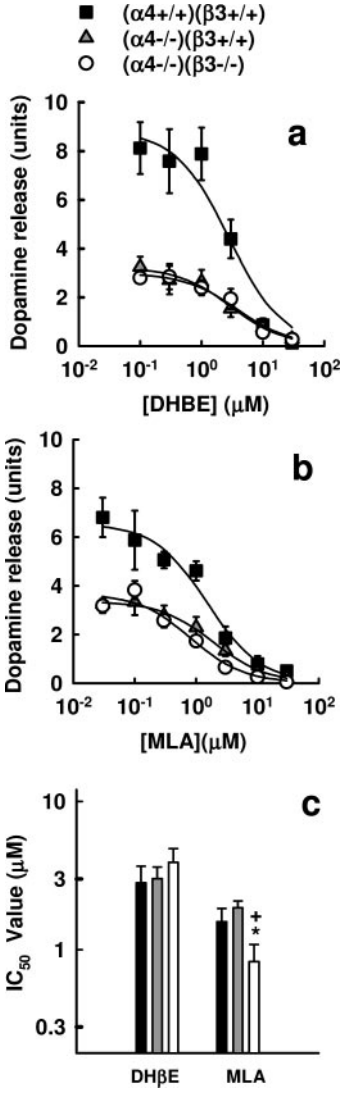

Fig. 6. Inhibition curves for α-CtxMII-sensitive dopamine release determined from synaptosomes prepared from ST of three genotypes of mice. Dopamine release was stimulated by an 18-s exposure to 10 μM ACh simultaneously with the indicated concentrations of DHβE or MLA. For the (α4+/+)(β3+/+) genotype, release was determined with and without a 5-min exposure to α-CtxMII (50 nM) immediately preceding the agonist ($\pm$ antagonist) exposure. Data shown is determined by difference (total release $-$ α-CtxMII-resistant release). Single-site inhibition curve-fits to the data are shown. c shows a comparison of IC_{50} values. *, significantly different from wild-type, $P < 0.05$; +, significantly different from α4-null mutant, $P < 0.05$.

The studies reported here comprise a first attempt to compare the pharmacology of the three native $\alpha6\beta2$* nAChRs expressed in mouse striatum. In the past, pharmacological comparisons of nAChR subtypes have used transfected cell lines or *X. laevis* oocytes (see Papke and Heinemann, 1994; Parker et al., 2001; Wu et al., 2006, for a few of many examples). Unfortunately, for reasons that remain unknown, α6*-nAChRs have been difficult to express in *X. laevis* oocytes (Gerzanich et al., 1997). A chimera between the extracellular domain of α6 and the transmembrane domain of α3 has been successfully expressed (Kuryatov et al., 2000); however, its pharmacology may not reflect that of native subtypes (Nicke et al., 2003). Recently human α6 subunits were expressed in human embryonic kidney cells (Tumkosit et al., 2006), indicating that comparisons of α6* nAChRs may be possible in the future. We chose to study the pharmacological properties of native α6* nAChRs by using breeding strategies to limit the nAChR subtypes expressed in dopaminergic nerve terminals. This approach has several advantages: 1) functional receptors are formed in neurons where they are normally expressed, 2) receptors are composed of native subunits, and 3) receptors are assembled with normal processing. However, analysis does require differentiation among the native subtypes, and gene deletion may elicit compensatory changes in the remaining subtypes.

Compensatory changes in nAChR subunits expressed in dopaminergic nerve terminals, if they occur, would serve to complicate results obtained with the breeding strategy. However, there is little evidence for major compensatory changes in nAChR subunits. None of the known nAChR subunit mRNA levels are changed by either α4 (Ross et al., 2000) or β3 (Cui et al., 2003) gene deletion. Deletion of the β2 subunits eliminates all agonist-stimulated dopamine release; β4 subunits cannot replace β2 (Picciotto et al., 1998; Grady et al., 2001). Deletion of the α6 subunit eliminates all α-CtxMII-sensitive agonist-stimulated dopamine release, as well as α-CtxMII-sensitive ^{125}I-epibatidine binding, indicating that α3 subunits cannot substitute for α6 (Champtiaux et al., 2003). However, immunoprecipitation and binding experiments indicate that there is some increase in α4 subunits after α6 deletion (Champtiaux et al., 2003). With the α4 gene deletion, virtually all the agonist-stimulated α-CtxMII-resistant response is eliminated, indicating that no subunit is capable of substituting for α4 in dopaminergic terminals (Champtiaux et al., 2003; Salminen et al., 2004). In addition, the α4 gene deletion eliminates approximately half of the α-CtxMII-sensitive dopamine release, showing that additional α6 subunits do not take the place of α4 (Salminen et al., 2004). Some deletions of minor subunits do increase function of alternate subtypes of nAChR. For example, in β3 subunit-null mutant mice, an increase is seen in the agonist-stimulated dopamine release supported by the α-CtxMII-resistant subtypes (Cui et al., 2003; Salminen et al., 2004), but immunoprecipitation experiments establish that levels of α4, α5, or β2 proteins do not accompany these functional changes (Gotti et al., 2005).

Results reported here are consistent with a lack of compensatory changes in other nAChR subtypes. The decreased α-CtxMII binding that we found in ST (53%) is consistent with loss of the wild-type population of $\alpha6\alpha4\beta3\beta2$-nAChR subtype estimated by immunoprecipitation (Gotti et al., 2005). Likewise, the decrease in ^{125}I-α-CtxMII binding of an

additional 27% in ST in the double mutants is consistent with the immunoprecipitation estimate of the $\alpha6\beta3\beta2$-nAChR population (Gotti et al., 2005). The agreement between binding and immunological results indicates that additional nAChR subtypes, or greater numbers of remaining subtypes, are not formed in these null mutant mice; i.e., expression of new or novel nAChRs does not easily occur in nAChR-null mutant mice.

Deletion of $\alpha4$ and $\alpha4$-plus-$\beta3$ subunits profoundly affected maximal agonist-induced dopamine release. The amounts of decrease in dopamine release stimulated by ACh (Fig. 3) were similar to the decreases in ^{125}I-α-CtxMII binding. Qualitatively similar results were measured for R_{max} values for all five agonists, although decreases were somewhat less by this measurement. Although minor compensatory increases in $\alpha6\beta2$* nAChR expression cannot be ruled out, these results could also indicate some differences in kinetic or other functional properties of the different subtypes.

Gene deletion of $\alpha4$ had little effect on EC_{50} value for A-85380, some effect for ACh and epibatidine, but elicited marked changes in EC_{50} values for both cytisine- and nicotine-stimulated dopamine release. Subsequent deletion of the $\beta3$ gene elicited further increases for these agonists. These results build on the observation that the EC_{50} value for ACh-induced α-CtxMII-sensitive dopamine release is not changed significantly after deletion of the $\alpha4$ gene (Salminen et al., 2004), whereas a significant increase (8-fold) in the EC_{50} value for nicotine-stimulated dopamine release has been reported (Champtiaux et al., 2003). We measured a 31-fold increase in EC_{50} value for cytisine activation of the α-CtxMII-sensitive nAChR in $(\alpha4-/-)(\beta3+/+)$ mice over the wild-type nAChR and a 99-fold increase was seen with the $(\alpha4-/-)(\beta3-/-)$ mice over the wild type. Nicotine was also able to distinguish all three subtypes with 7- and 31-fold increases, respectively. Other compounds, including the agonists ACh and epibatidine and the antagonist MLA, did not differentiate as well among variations of $\alpha6$*-nAChR subtypes. Affinity for A-85380, DHβE, and α-CtxMII did not differ with genotype. For those agonists showing large differences, subtraction of data allowed estimation of EC_{50} values for individual subtypes (Table 1). The $\alpha6\alpha4\beta3\beta2$ nAChR has the highest sensitivity for nicotine-stimulated activity measured to date in the mouse, with an EC_{50} value for dopamine release of 0.2 μM, well within the range achieved by long-term treatment (Marks et al., 2004).

In summary, experiments reported here confirm the postulate that three $\alpha6$* nAChR subtypes play important roles in modulating the α-CtxMII-sensitive component of dopamine release (Salminen et al., 2004; Gotti et al., 2005). The $\alpha6\alpha4\beta3\beta2$ subtype is unique in that it has an EC_{50} value for nicotine-stimulated release that are 7-fold lower than the EC_{50} value for nicotine-induced stimulation of the α-CtxMII-resistant component of dopamine release (Salminen et al., 2004). Likewise, the EC_{50} value for activating this receptor is 7-fold lower than the EC_{50} value for nicotine-stimulated GABA release (Lu et al., 1998) and more than 200-fold lower than the EC_{50} value for nicotine-stimulated-ACh release (Grady et al., 2001) from synaptosomes. Thus, the $\alpha6\alpha4\beta3\beta2$ receptors may play a very important role in modulating behavioral effects of nicotine, particularly those associated with tobacco addiction. The results of the experiments reported here also indicate that interbreeding nAChR-null mutant mice may be used successfully to characterize pharmacological properties of native nAChRs normally expressed along with other, more abundant, nAChR subtypes. This strategy of sequential gene deletion revealed that large, and agonist selective, differences in sensitivity exist among $\alpha6$*-nAChR subtypes. This approach may facilitate the identification of new compounds capable of selectively modulating nAChR subtypes in vivo that may be of therapeutic benefit and also may lead to a greater understanding of the natural role of these receptors.

Acknowledgments

We thank Natalie M. Meinerz, Theresa delVecchio, and Tianna Purrington for technical assistance in genotyping. We also thank Megan Canon and Eric Laudenslager for assistance in dopamine release assays.

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Address correspondence to: Sharon R. Grady, Institute for Behavioral Genetics, University of Colorado, 447UCB, Boulder, CO 80309. E-mail: sharon.grady@colorado.edu