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Reporter Mutation Studies Show That Nicotinic Acetylcholine Receptor (Nachr) Î±5 Subunits And/Or Variants Modulate Function Of Î±6*-Nachr

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Reporter Mutation Studies Show That Nicotinic Acetylcholine Receptor (nAChR) α5 Subunits and/or Variants Modulate Function of α6*-nAChR*

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Background: We asked how partner subunits influence α6*-nicotinic receptor (nAChR) function.
Results: We found several, novel ways to manipulate effects of α5 subunits on α6*-nAChR function.
Conclusion: Extracellular domains in α6 and cytoplasmic/transmembrane domains in α6/α3/α5/β3 subunits have unexpected influences on α6*-nAChR function.
Significance: We found factors that influence assembly and function of α6*-nAChRs, which play important roles in mood, reward, and nicotine dependence.

To further the understanding of functional α6α5*-nicotinic acetylcholine receptors (nAChR; the asterisk (*) indicates known or possible presence of other subunits), we have heterologously expressed in oocytes different, mouse or human, nAChR subunit combinations. Coexpression with wild-type α5 subunits or chimeric α5/β3 subunits (in which the human α5 subunit N-terminal, extracellular domain is linked to the remaining domains of the human β3 subunit) almost completely abolishes the very small amount of function seen for α6β4*-nAChR and does not induce function of α6β2*-nAChR. Coexpression with human α5$^{V9'S}$ subunits bearing a valine 290 to serine mutation in the 9′ position of the second transmembrane domain does not rescue the function of α6β4*-nAChR or induce function of α6β2*-nAChR. However, coexpression with mutant chimeric α5/β3$^{V9'S}$ subunits has a gain-of-function effect (higher functional expression and agonist sensitivity and spontaneous opening inhibited by mecamylamine) on α6β4*-nAChR. Moreover, N143D + M145V mutations in the α6 subunit N-terminal domain enable α5/β3$^{V9'S}$ subunits to have a gain-of-function effect on α6β2*-nAChR. nAChR containing chimeric α6/α3 subunits plus either β2 or β4 subunits have some function that is modulated in the presence of α5 or α5/β3 subunits. Coexpression with α5/β3$^{V9'S}$ subunits has a gain-of-function effect more pronounced than that in the presence of α5$^{V9'S}$ subunits. Gain-of-function effects are dependent, sometimes subtly, on the nature and apparently the extracellular, cytoplasmic, and/or transmembrane domain topology of partner subunits. These studies yield insight into assembly of functional α6α5*-nAChR and provide tools for development of α6*-nAChR-selective ligands that could be important in the treatment of nicotine dependence, and perhaps other neurological diseases.

Nicotinic acetylcholine receptors (nAChR)[3] exist as a variety of subtypes composed from different combinations of genetically distinct subunits, with α2-α7 and β2-β4 subunits being expressed in the nervous system (1). Some of these subunits form homopentameric receptors when expressed in heterologous expression systems (α7, α8, and α9), whereas other subunits assemble into heteropentameric structures with various combinations of α and β subunits. Since its discovery in rat (2) and chicken (3) brain as part of a gene cluster with nAChR α3 and β4 subunits, work on the α5 subunit has defined distribution of its mRNA, some of the effects of its incorporation on properties of heterologously expressed nAChR, and how its genetic elimination affects some behaviors (4–8). nAChR α5 subunits do not form functional receptors when expressed alone nor in combination with any other single types of subunit, but they are capable of integrating as accessory subunits into complexes containing at least one other α and one other β subunit (9–12).

nAChR α6 and α5 subunit messages share very similar expression patterns, and some studies suggest that the α5 subunit may participate in formation of functional α6*-nAChR (where the asterisk (*) indicates the known or possible presence of additional subunits in the complex), perhaps promoting assembly and stability of mature α6*-nAChR (10, 13). Interest in α6α5*-nAChR is increasing because α6α5*-nAChR, likely expressed predominantly in dopaminergic midbrain regions where they might modulate dopamine release, may be implicated in pleasure, reward, and drug (including nicotine) dependence, and could be involved in schizophrenia and Parkinson disease (6, 7, 14–16). α6*-nAChR that may exist *in vivo* are not easily recreated in artificial expression systems (10). It

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[3] The abbreviations used are: nAChR, nicotinic acetylcholine receptor(s); ACh, acetylcholine; I_{max}, peak current response; M2, second transmembrane domain; E1, first, large, extracellular domain; h, human; m, mouse.

has been difficult to demonstrate the function of heterologously expressed nAChR containing α6 and α5 subunits.

Recently Broadbent *et al.* (17) and we (18) observed that incorporation of the human (h) nAChR β3 (hβ3) subunit has a dominant-negative effect on the function of some heterologously expressed nAChR subtypes. By contrast, coexpression with hβ3 subunits having a "reporter" mutation (valine 273 to serine at the M2 second transmembrane domain 9′ position, $h\beta3^{V273S} = h\beta3^{V9'S}$) has a "gain-of-function" effect, potentiating the function of hα6hβ2- or hα6hβ4-nAChR (17), of nAChR containing mouse (m) α6 subunits and hβ2 subunits, of mα6hβ4-nAChR, or of nAChR containing mutant hα6 ($h\alpha6^{N143D+M145V}$) and hβ2 subunits (17, 18). Building on these findings, we hypothesized that hα5 subunits may also exert a dominant-negative effect on hα6hβ2- and hα6hβ4-nAChR function, in part because α5 and β3 subunits are phylogenetically similar, highly homologous, and share service as accessory subunits in several nAChR subtypes. Acutely aware that coexpression with mutated hα5 subunits (M2 second transmembrane domain 9′ position, $h\alpha5^{V290S} = h\alpha5^{V9'S}$) do not induce spontaneous opening or increased agonist sensitivity of the nAChR subtypes tested (19) we further hypothesized that recombinant, chimeric $h\alpha5/h\beta3^{V273S}$ subunits (in which the N-terminal, large extracellular domain of the hα5 subunit is linked to the remaining domains of the gain-of-function $h\beta3^{V9'S}$ subunit) would serve as a reporter mutation and, upon integration into α6*-nAChR, would increase their agonist sensitivity.

Here we report findings using a variety of wild-type, reporter mutant, or chimeric nAChR subunits, hybrid nAChR containing subunits from different species, and the oocyte expression system. We provide evidence that wild-type α5 or reporter mutant $h\alpha5^{V9'S}$ subunits are incorporated into complexes also containing either hα6 or mα6 plus either hβ2 or hβ4 subunits, but have a null or dominant-negative effect on the function of those α6*-nAChR. We also show that chimeric hα5/hβ3 subunits have similar effects, but that reporter mutant chimeric $h\alpha5/h\beta3^{V9'S}$ subunits allow for agonist-activated and some spontaneous function of hα6hβ4*- or mα6hβ4*-nAChR. Moreover, we demonstrate that chimeric hα6/hα3 subunits (10) expressed in combination with hβ2 or hβ4 subunits produce functional α6*-nAChR that are sensitive to dominant-negative effects of coexpression with hα5 or hα5/hβ3 subunits, but also become more sensitive to agonists and have much higher function when coexpressed instead with $h\alpha5^{V9'S}$ or chimeric, reporter mutant $h\alpha5/h\beta3^{V9'S}$ subunits. Finally we show that mutations in the N-terminal domain of the hα6 subunit (N143D and M145V) enable ($h\alpha5/h\beta3^{V9'S}$) subunits to have a gain-of-function effect at hα6hβ2*-nAChR. These findings provide insight into the assembly, structure, and function of functional α6α5*-nAChR, which could be exploited as models for development of new ligands to affect mood and drug dependence.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals for electrophysiology were obtained from Sigma. Fresh nicotine and mecamylamine stock solutions were made daily in Ringer solution and diluted as needed.

nAChR Subunits: Mutants, Chimeras, and in Vitro Transcription—cDNAs corresponding to human nAChR α6 (hα6), hα5, hβ2, hβ3, or hβ4 subunits were excised from vectors containing them and subcloned into the oocyte expression vector pGEMHE. Similarly, cDNA representing mouse nAChR α6 (mα6) subunit (kind gift from Dr. Jerry A. Stitzel, Department of Integrative Physiology, Institute for Behavioral Genetics, University of Colorado, Boulder, CO) was subcloned into pGEMHE. Construct integrity was confirmed by sequencing.

Point Mutants—Some specific mutations have been made in residues present in the second transmembrane domain (M2) of several nAChR subunits (*e.g.* at the so-called 9′ or 13′ positions, counting residues starting at the presumed, first residue in M2). Residues modified typically are thought to line the ion channel. Many studies, for example (20), indicate that some of these mutations can be used to monitor the incorporation of mutant subunits into nAChR assemblies, because they sometimes produce incremental increases in agonist sensitivity (*i.e.* they lower EC_{50} values for stimulation of nAChR function by a fixed degree, which is proportional to the number of mutant subunits incorporated into the pentamer). Thus, these altered subunits can serve as "reporter" mutants, because they can report when and how many mutant subunits are present in a functional nAChR. To the extent that they increase the apparent function of the complex, typically by increasing the magnitude of the peak whole cell current response to agonists relative to levels of function seen in nAChR lacking those subunits or when wild-type subunits are incorporated into complexes, but also by increasing agonist sensitivity as described just above, they are also called gain-of-function mutants. It is difficult to say whether these mutations might be physiologically relevant. The M2 9′ residue gain-of-function mutations in nAChR α7 subunits are lethal in transgenic mice (21), and to human, neuron-like cell lines (22), and when present in a similar subunit, they produce late-onset death in a subset of *Caenorhabditis elegans* neurons (23). Thus, they may not be observed naturally in mammals if they are embryonically lethal. However, these mutations are very useful as experimental tools.

Gain-of-function/reporter mutations in hα5 (V290S) or hβ3 (V273S) subunits were introduced into the pGEMHE background using the QuikChange II Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) and also confirmed. Similarly, mutations in the N-terminal domain of the nAChR hα6 subunit (*i.e.* N143D + M145V) were introduced using the QuikChange II Site-directed Mutagenesis Kit. Primers used for mutagenesis are listed in Table 1.

(hα5/hβ3) Subunit Chimera—To substitute the nAChR hα5 subunit large, N-terminal, extracellular domain (E1) for the equivalent segment of the hβ3 subunit cDNA, a cDNA corresponding to amino acid residues 1 to 246 of hα5 subunit cDNA was first PCR-amplified using primers 5′-taatacgactcactataggg-3′ (forward; T7: corresponding to the pGEMHE sequence) and 5′-ggcagcc**tccgga**ttacaaatgag-3′ (reverse; corresponding to the nAChR hα5 subunit sequence and containing a BspEI site (underlined)). The PCR-amplified hα5 subunit fragment was subcloned into the pCR®2.1-TOPO® vector (Invitro-

TABLE 1

Primers used in mutagenesis

For mutants, the first amino acid (single letter code) designates the wild-type residue at the numbered location (translation start methionine at position 1) that is replaced with the second amino acid. Capitalized nucleotide(s) denote(s) those are different from the wild-type nAChR α6 subunit to create the designated replacement. F, forward primer; *R,* reverse primer.

Mutant	Primer sequences (5′ → 3′)
hα5(V290S)-F	5′-gtctctgcacttcagtacttTCgtctttgactgtcttccttc-3′
hα5(V290S)-R	5′-gaaggaagacagtcaaagacGAaagtactgaagtgcagagac-3′
hβ3(V273S)-F	5′-cattatccacatcggTCttggtttctctgacagttttcc-3′
hβ3(V273S)-R	5′-ggaaaactgtcagagaaaccaaGAccgatgtggataatg-3′
hα6(N143D+M145V)-F	5′-ggcaaaacaaaagctcttcttaaatacGatggcGtgataacctggactccaccagctattt-3′
hα6(N143D+M145V)-R	5′-aaatagctggtggagtccaggttatcaCgccatCgtatttaagaagagcttttgttttgcc-3′

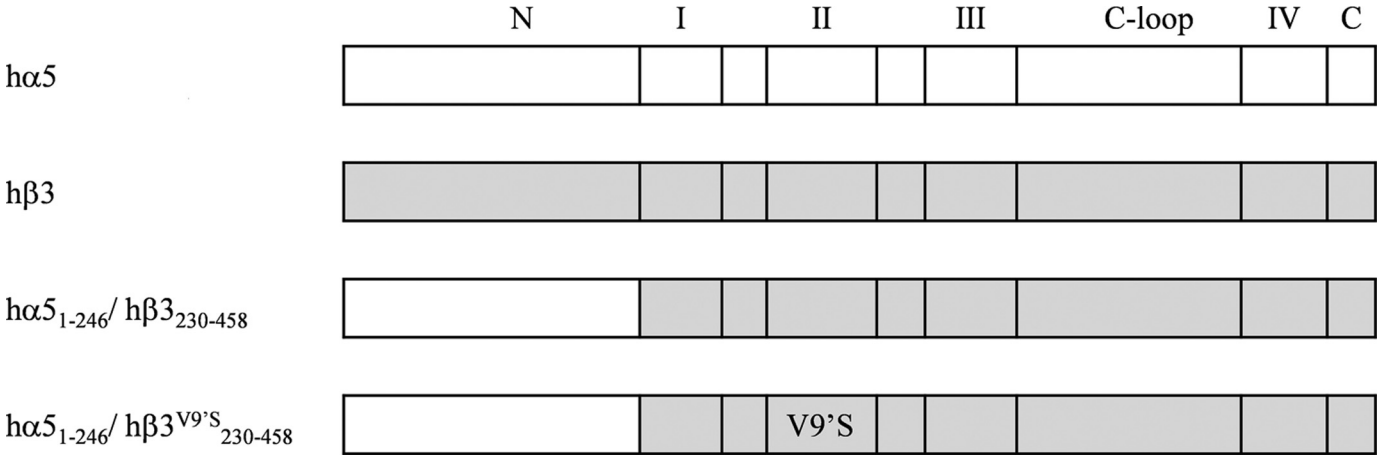


FIGURE 1. **Schematic diagrams of wild-type and chimeric human α5 and β3 subunits.** The notations are: *N* = N-terminal, large extracellular domain; *I, II, III,* and *IV* are the four transmembrane domains; *C-loop*, second, large cytoplasmic loop; *C*, C terminus. The hα5$_{1-246}$/hβ3$_{230-458}$ chimera was constructed using a BspEI restriction enzyme site engineered common to each subunit cDNA resulted in having a serine at position 231 instead of an arginine present in the wild-type hβ3 subunit. The chimera was mutated back to have its wild-type amino acid arginine. The figure is not drawn to scale.

gen), *in vivo* amplified, and digested with BamHI and BspEI. A BspEI restriction site (underlined) was created in the pGEMHE-hβ3 construct by site-directed mutagenesis at position Arg^{230} by using primers: 5′- cacgtattccttcgtcc**tccgga**ggctgcctttattctatacc-3′ (forward) and 5′-ggtatagaataaaggcagcc**tccgga**ggacgaaggaatacgtg-3′ (reverse). The mutated pGEMHE-hβ3 plasmid was digested with BamHI and BspEI, where the BamHI site is located upstream of the N-terminal end of the hβ3 subunit cDNA and in the multiple cloning site. The pGEMHE plasmid devoid of the hβ3 subunit N-terminal fragment was gel purified. The restriction-digested hα5 and pGEMHE-hβ3 fragments were ligated, producing the chimera (hα5/hβ3) (hα5(Met^1-Ile^{246})/hβ3(Arg^{230}-His^{458})) (Fig. 1), which includes a total of 475 amino acid residues, and the final product was checked for integrity by restriction enzyme analysis and DNA sequencing prior to cRNA preparation.

hα5/hβ3$^{V9'S}$ Reporter Mutant Subunit Chimera—Similarly, to create the hα5/hβ3$^{V9'S}$ subunit chimera containing a reporter mutation, a BspEI restriction site was created in the pGEMHE-hβ3$^{V9'S}$ construct by site-directed mutagenesis at position Arg^{230} using the same pair of primers as above, and the rest of the procedures were the same as for creating the hα5/hβ3 subunit chimera. This resulted in a chimeric hα5/hβ3$^{V9'S}$ subunit (hα5(Met^1-Ile^{246})/hβ3^{V273S}(Arg^{230}-His^{458})) (Fig. 1) that includes a total of 475 amino acid residues and was checked for integrity by restriction enzyme analysis and DNA sequencing prior to cRNA preparation.

hα6/hα3 Subunit Chimera—The hα6/hα3 subunit chimera plasmid ((containing the N-terminal, large extracellular domain, E1) of the hα6 subunit fused to domains from transmembrane region 1 through to the C terminus of the hα3 subunit) was a kind gift from Dr. John Lindstrom (10).

In Vitro Transcription—All pGEMHE plasmids were linearized immediately downstream of the 3′-polyadenylation sequence. NheI was used to linearize mβ4, hα6, hα5, hα5$^{V9'S}$, hβ3, hβ3$^{V9'S}$, hα5/hβ3, hα5/hβ3$^{V9'S}$ and hβ4 subunit-containing plasmids. SbfI was used to linearize the hβ2 subunit cDNA. The hα6/hα3 subunit chimera plasmid was linearized using EcoRI. Capped mRNA was transcribed from linearized plasmids in a reaction mixture (25 μl) containing 1× transcription buffer, 1.6 mM rNTPs (Promega), 0.5 mM 7m-CAP (New England Biolabs), 1 μl of RNasin plus (New England Biolabs), and 1 μl of T7 RNA polymerase (New England Biolabs) with the exception that the linearized hα6/hα3 subunit chimera was transcribed using SP6 RNA polymerase (New England Biolabs) following a standard protocol. Integrity and quality of the cRNA was checked by electrophoresis and UV-spectroscopy.

Oocyte Preparation and cRNA Injection—Female *Xenopus laevis* (Xenopus I, Ann Arbor, MI) were anesthetized using 0.2% tricaine methanesulfonate (MS-222). Ovarian lobes were surgically removed from the frogs and placed in an incubation solution that consisted of (in mM) 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 1 Na_2HPO_4, 0.6 theophylline, 2.5 sodium pyruvate, and 5 HEPES, plus 50 mg/ml of gentamycin, 50 units/ml of penicillin, and 50 μg/ml of streptomycin, pH 7.5. The frogs were allowed to recover from surgery before being returned to the incubation tank. The lobes were cut into small pieces and digested with 0.08 Wunsch units/ml of liberase blendzyme 3

(Roche Applied Science) with constant stirring at room temperature for 1.5–2 h. The dispersed oocytes were thoroughly rinsed with incubation solution. Stage VI oocytes were selected and incubated at 16 °C before injection. Micropipettes used for injection were pulled from borosilicate glass (Drummond Scientific, Broomall, PA) using a Sutter P87 horizontal puller, and the tips were broken with forceps to ~40 μm in diameter. cRNA was drawn up into the micropipette and injected into oocytes using a Nanoject microinjection system (Drummond Scientific) at a total volume of ~60 nl. To express nAChR in oocytes, about 4 ng of cRNA corresponding to each nAChR subunit was injected. Lacking *a priori* knowledge about levels of mRNA for specific subunits present in neurons, and not wishing to bias results by injecting disparate amounts of nAChR subunit cRNAs into oocytes, we chose to introduce identical amounts of cRNA, presumably producing equal amounts of each subunit protein, into oocytes. For several reasons as explained under "Discussion," we did not define subunit ratios in the cell surface, functional nAChR in oocytes. Instead, we provisionally assumed that α6 and β2 or β4 subunits would form complexes having 2:3 and/or 3:2 ratios of the indicated subunits and that oocytes also injected with α5 or β3 subunits or variants would express nAChR with 2:2:1 ratios of α6:β2 or β4:α5 or β3 subunits.

Oocyte Electrophysiology—Two to 7 days after injection, oocytes were placed in a small volume chamber and continuously perfused with oocyte Ringer solution, which consisted of (in mM) 92.5 NaCl, 2.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES, pH 7.5. The chamber was grounded through an agarose bridge. The oocytes were voltage-clamped at −70 mV (unless otherwise noted) to measure agonist-induced currents using AxoClamp 900A and pClamp 10.2 software (Axon Instruments). The current signal was low-pass filtered at 10 Hz with the built-in low-pass Bessel filter in the Axoclamp 900A and digitized at 20 Hz with Axon Digidata 1440A and pClamp 10.2. Electrodes contained 3 M KCl and had a resistance of 1–2 megohms. Drugs (agonists and antagonists) were prepared daily in bath solution. Drugs were applied using a Valvelink 8.2 perfusion system (Automate Scientific, Berkeley, CA). All electrophysiological measurements were conducted or checked in at least two batches of oocytes.

Experimental Controls—Injection of water or empty vector (used as two forms of negative controls) or of cRNA corresponding to one subunit alone or pairwise combinations of nAChR hα5, h$\alpha5^{V9'S}$, (hα5/hβ3), or (hα5/h$\beta3^{V9'S}$) subunits with either an α6 subunit or β2 or β4 subunits (10–12 ng of total cRNA) did not result in the expression of functional nAChR. Current responses to 100 μM nicotine were less than 5–10 nA (data not shown).

Data Analyses—Raw data were collected and processed in part using pClamp 10.2 (Molecular Devices, Sunnyvale, CA) and a spreadsheet (Excel, Microsoft, Bellevue, WA), using peak current amplitudes (I_{max}) as measures of functional nAChR expression and results were pooled across experiments (mean ± S.E. for data from at least three oocytes). In some cases, mean peak current amplitudes in response to a single concentration of an agonist were compared across different subunit combinations. However, assessment of true I_{max} values for different nAChR subunit combinations required evaluations based on more complete concentration-response relationships, in which mean peak current amplitudes at specified ligand concentrations were fit to the Hill equation or its variants using Prism 4 (GraphPad Software, San Diego, CA). *F*-tests ($p < 0.05$ to define statistical significance) were carried out to compare the best fit values of log molar EC_{50} values across specific nAChR subunit combinations. There are limitations in the ability to compare levels of functional nAChR expression, even though we injected similar amounts of RNA for all constructs. This is because expression levels assessed as peak current amplitudes are affected by batch-to-batch variations in oocytes, time between cRNA injection and recording, and subunit combination-specific parameters, such as channel open probability (influenced by gating rate constants, rates and extents of desensitization), single channel conductance, subunit assembly efficiency, and efficiency of receptor trafficking to the cell surface (19). We made no attempt to measure or control for subunit combination-specific effects, but whenever preliminary studies revealed possible differences in peak current amplitudes, findings were further confirmed across different subunit combinations using the same batch of oocytes and the same time between cRNA injection and recording. Peak current amplitudes are shown from representative traces in some figures presented below, pooled data from limited sets of studies, and mean peak current amplitudes across all studies for a given combination of subunits as presented in the tables sometimes differ. However, when we make statements about results comparing ligand potencies and efficacies across subunit combinations, the observations are clear, significant, and in agreement whether for pooled data or for results from smaller sets of studies (one-way analyses of variance followed by Tukey's multiple comparison tests).

RESULTS

Studies of Incorporation of Human nAChR α5 Subunits into hα6hβ4- or mα6hβ4*-nAChR1*

Human nAChR Chimeric α5/β3$^{V9'S}$ Subunits (but Not Human Wild-type α5, α5$^{V9'S}$, or α5/β3 subunits) Form Functional Receptors When Coassembled with hα6hβ4- or mα6hβ4*-nAChR and Increase Agonist Sensitivity of Expressed Receptors*—In initial studies, coexpression of nAChR wild-type hα6 and hβ4 subunits produced nicotinic responses manifest as inward currents in only about 3–5% of injected oocytes. Functional responses to 100 μM ACh when present were very modest (22 ± 3 nA) (data not shown). It was difficult to obtain measurable and reproducible responses to nicotine. Coexpression with presumed accessory, wild-type hα5, mutant h$\alpha5^{V9'S}$ subunits, or chimeric hα5/hβ3 subunits along with wild-type hα6 and hβ4 subunits did not produce any response to nicotine. However, coexpression with chimeric mutant hα5/h$\beta3^{V9'S}$ subunits as accessory partners significantly increased nicotinic responses to 10 μM nicotine (225 ± 31 nA), and nearly every oocyte injected with nAChR hα6, hβ4, and mutant hα5/h$\beta3^{V9'S}$ subunits expressed functional nAChR (Fig. 2, Table 2). These results at least indicated that both hα5/hβ3 and mutant

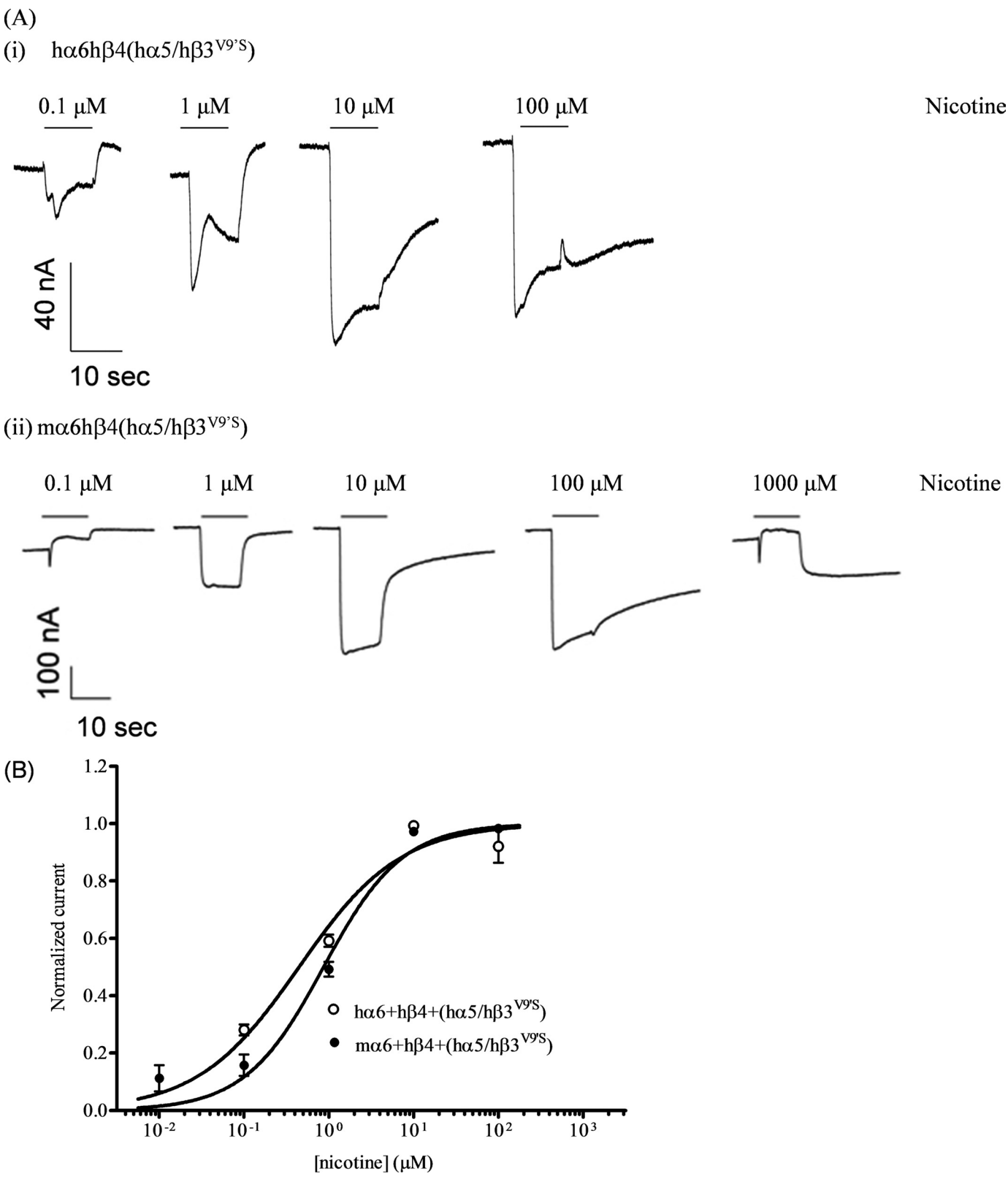


FIGURE 2. **Functional properties of α6β4*-nAChR.** *A*, representative traces are shown for inward currents in oocytes held at −70 mV, responding to application at the indicated concentrations of nicotine (shown with the duration of drug exposure as *black bars above* the traces), and expressing (*i*) nAChR hα6, hβ4, and hα5/hβ3$^{V9'S}$ subunits or (*ii*) nAChR mα6, hβ4, and hα5/hβ3$^{V9'S}$ subunits. *Calibration bars* are for (*i*) 40 or (*ii*) 100 nA currents (vertical) or 10 s (horizontal). Note the differences in inward current kinetics. *B*, results for these and other studies averaged across experiments were used to produce concentration-response curves (*ordinate*, mean normalized current ± S.E.; *abscissa*, ligand concentration in log μM) for inward current responses to nicotine as indicated, where current amplitudes are represented as a fraction of the peak inward current amplitude in response to the most efficacious concentration of nicotine (hα6hβ4(hα5/hβ3$^{V9'S}$)-nAChR, ◇; mα6hβ4(α5/β3$^{V9'S}$)-nAChR, ◆). Much higher levels of evoked currents are evident for functional nAChR containing hα5/hβ3$^{V9'S}$ subunits compared with receptors lacking or also containing altered forms of wild-type or chimeric α5 subunits, none of which gave substantial or consistent currents responses (not shown; $p < 0.0001$). See Table 2 for parameters.

TABLE 2

Parameters for ligand action at nAChR containing α6 nAChR subunits

Agonist potencies (micromolar EC_{50} values with 95% confidence intervals), Hill coefficients ($n_H \pm$ SE), mean ± S.E. peak responses (I_{max} in nanoamps) and concentrations, where I_{max} is achieved (μM) are provided for nicotine acting at nAChR composed of the indicated subunits derived from the specified species and from the indicated number of independent experiments (*n*) based on studies as shown in Figs. 2–5. For the indicated potency of the agonist or peak current responses when acting at the specified nAChR subtype composed of the indicated subunits, significant ($p < 0.05$) increases or decreases, respectively, are indicated relative to the α6β2- or α6β4-nAChR subtype alone (↑ or ↓) or in the presence of wild-type α5 subunits (▲ or ▼), $\alpha5^{V9'S}$ subunits (△ or ▽), or (α5/β3) subunits (⇧ or ⇩). Note that no or very rare and then small responses to nicotine were seen for the following subunit combinations ($n = 4–9$ each): hα6 + hβ2 alone or with hα5, $h\alpha5^{V9'S}$, hα5/hβ3, or hα5/$h\beta3^{V9'S}$; or hα6+ hβ4 alone or with hα5, $h\alpha5^{V9'S}$, or hα5/hβ3; mα6 + hβ2 alone or with hα5, $h\alpha5^{V9'S}$, hα5/hβ3, or hα5/$h\beta3^{V9'S}$; mα6 + hβ4 a lone or with hα5, $h\alpha5^{V9'S}$, or hα5/hβ3; and hα6(N143D+M145V) + hβ2 alone or with hα5, $h\alpha5^{V9'S}$, or hα5/hβ3. No or very rare and then small responses were seen for the subunit combinations ($n = 6–9$ each) where data are entered as dashes.

nAChR subunit combinations	Nicotine					
	Potency			Peak response		
	n	EC_{50} (μM) (95 % CI)	$n_H \pm$ SE	n	Mean I_{max} ± SE (nA)	I_{max} Conc. (μM)
hα6 + hβ4 + hα5/$h\beta3^{V9'S}$	6	0.45 (0.28-0.72) ↑▲△⇧	0.73± 0.11	4	225±31	10 ↑▲△⇧
mα6 + hβ4 + hα5/$h\beta3^{V9'S}$	3	0.87 (0.67-1.1) ↑▲△⇧	0.94±0.11	4	154±81 ↑▲△⇧	10 ↑▲△⇧
hα6/hα3 + hβ2	6	13 (5.6 - 28)	1.2±0.43	3	48±12	100
hα6/hα3 + hβ2 + hα5	9	-	-	9	20±6	10
hα6/hα3 + hβ2 + $h\alpha5^{V9'S}$	6	1.1 (0.6 - 1.2) ↑▲	1.0±0.2	4	252±99 ↑▲	10 ↑▲
hα6/hα3 + hβ2 + hα5/hβ3	3	-	-	3	25±8	10
hα6/hα3 + hβ2 + hα5/$h\beta3^{V9'S}$	6	0.03 (0.02 - 0.07) ↑▲△⇧	0.49±0.08	4	256±16 ↑▲	10 ↑▲⇧
hα6/hα3 + hβ4	3	25 (20-30)	1.73±0.15	5	224±28	1000
hα6/hα3 + hβ4 + hα5	3	9.1 (5.3-16) ↑	0.91±0.22	3	45±22	100↑
hα6/hα3 + hβ4 + $h\alpha5^{V9'S}$	6	2.9 (2.5-3.2) ↑▲	1.5±0.09	6	649±244	100↑
hα6/hα3 + hβ4 + hα5/hβ3	3	6.1 (4.2-8.8) ↑	1.4±0.32	3	1969±151↑▲△	100↑
hα6/hα3 + hβ4 + hα5/$h\beta3^{V9'S}$	4	0.03 (0.02-0.07) ↑▲△⇧	0.49±0.08	4	2988±717 ↑▲△	10 ↑▲△⇧
hα6(N143D+M145V) + hβ2 + hα5/$h\beta3^{V9'S}$	3	0.06 (0.02-0.15) ↑▲△⇧	0.7±0.2	4	132±77 ↑▲△⇧	10 ↑▲△⇧

hα5/$h\beta3^{V9'S}$ subunits incorporate into at least some complexes containing hα6 and hβ4 subunits, because agonist sensitivities (EC_{50} values) that could be assessed changed as a function of subunits injected into oocytes. Moreover, the results also show that incorporation of nAChR hα5/hβ3 subunits into hα6hβ4*-nAChR has a dominant-negative effect, reflected by lack of functional receptors (again, assuming that peak current amplitudes are legitimate proxies for functional nAChR expression levels, with the caveats about this interpretation mentioned under "Experimental Procedures," "Data Analyses"). By contrast, incorporation of nAChR hα5/$h\beta3^{V9'S}$ subunits produces a gain-of-function effect reflected by an increase in agonist sensitivity and in absolute levels of functional receptor expression.

Knowing that at least some α6*-nAChR are functional in mice, and but lacking such documentation of native, all-human α6*-nAChR function, we had previously extended our work to mouse nAChR subunits and found that coexpression of hβ3 subunits with mα6 and hβ4 nAChR subunits form hybrid (*i.e.* composed of subunits from two different species), functional mα6hβ4hβ3-nAChR (18). This and the findings just above that functional nAChR hα6hβ4(hα5/$h\beta3^{V9'S}$) could be formed led us to explore effects of the incorporation of hα5 or hα5/hβ3 subunits on function of the hybrid mα6hβ4*-nAChR. Oocytes expressing nAChR mα6 and hβ4 alone or with wild-type hα5 subunits from co-injected cRNAs failed to respond to nicotine (Table 2). Substitution for wild-type hα5 subunits with hα5/$h\beta3^{V9'S}$ subunits, but not with $h\alpha5^{V9'S}$ or hα5/hβ3 subunits, yielded oocytes in which maximal responses were achieved in the presence of 10 μM nicotine (Fig. 2, Table 2). Nicotine concentration-response relationships yielded EC_{50} values of 0.87 μM for mα6hβ4(hα5/$h\beta3^{V9'S}$)-nAChR (Table 2). These findings suggest that mutant hα5/$h\beta3^{V9'S}$ subunits also serve as gain-of-function partners for mα6hβ4*-nAChR.

Human nAChR α5, $\alpha5^{V9'S}$, α5/β3, or $\alpha5/\beta3^{V9'S}$ Subunits Do Not Produce Functional Receptors When Coexpressed with hα6 or mα6 Plus hβ2 Subunits—Coexpression of nAChR wild-type hα6 or mα6 plus hβ2 subunits did not yield oocytes responding to nicotine (data not shown). Additional expression of wild-type hα5 or hα5/hβ3 subunits does not alter this circumstance, nor does additional expression of mutant $h\alpha5^{V9'S}$ or hα5/$h\beta3^{V9'S}$ subunits (Table 2). These results indicate that although the nAChR mutant hα5/$h\beta3^{V9'S}$ subunits exert gain-of-function effects on hα6hβ4*- or mα6hβ4*-nAChR, they are unable to activate the function of hα6hβ2*- or mα6hβ2*-nAChR expressed in oocytes.

Studies of Human nAChR α5 Subunit Incorporation into Chimeric (hα6/hα3)-nAChR*

Coexpression of $h\alpha5^{V9'S}$ or hα5/$h\beta3^{V9'S}$ Subunits with Chimeric hα6/hα3 and hβ2 nAChR Subunits Produce Functional

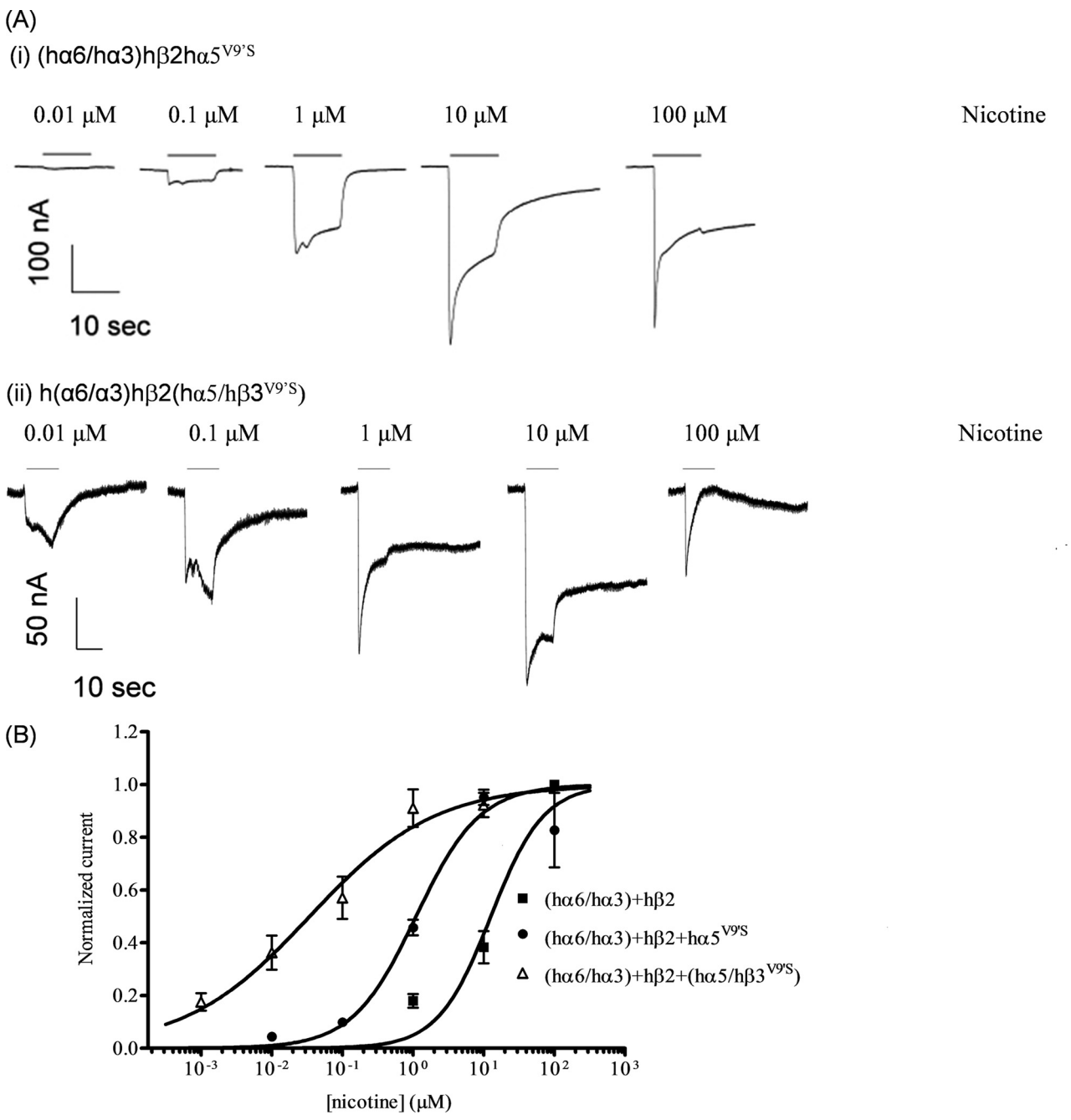
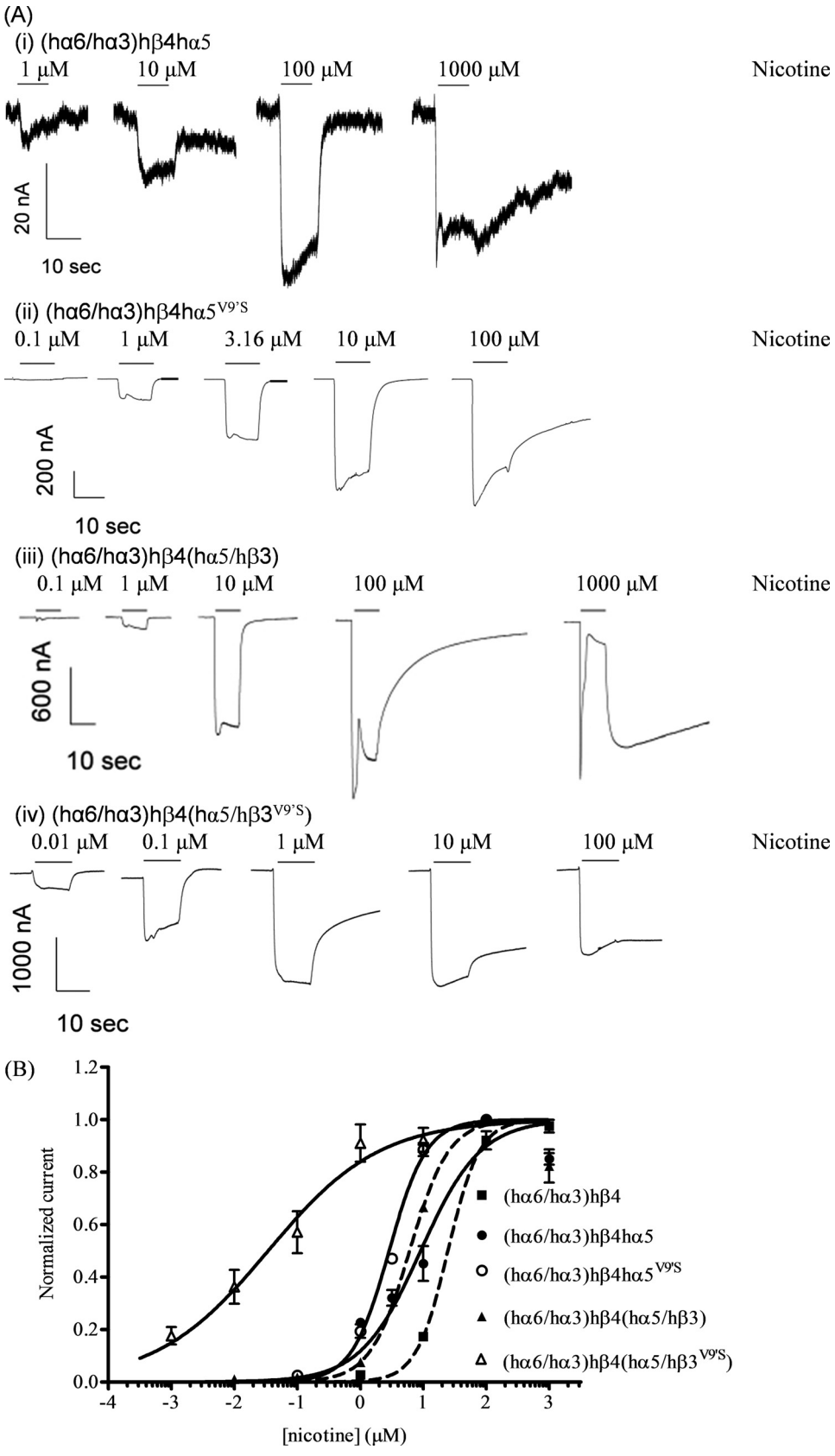


FIGURE 3. **Functional properties of (hα6/hα3)hβ2*-nAChR.** *A*, representative traces are shown for inward currents in oocytes held at −70 mV, responding to application at the indicated concentrations of nicotine (shown with the duration of drug exposure as *black bars above* the traces), and expressing nAChR hα6/hα3, hβ2, and either (*i*) hα5$^{V9'S}$ or (*ii*) hα5/hβ3$^{V9'S}$ subunits. *Calibration bars* are 100 (*i*) or 50 nA (*ii*) currents (vertical) or 10 s (horizontal). Note the differences in inward current kinetics. *B*, results for these and other studies averaged across experiments were used to produce concentration-response curves (*ordinate,* mean normalized current ± S.E.; *abscissa*, ligand concentration in log μM) for responses to nicotine as indicated for oocytes expressing nAChR hα6/hα3 and hβ2 subunits alone (■), with hα5$^{V9'S}$ subunits (●), or with hα5/hβ3$^{V9'S}$ subunits (△). Current amplitudes are represented as a fraction of the peak inward current amplitude in response to the most efficacious concentration of nicotine. Leftward shifts in nicotine concentration-response curves are evident for functional nAChR containing hα5$^{V9'S}$ or hα5/hβ3$^{V9'S}$ subunits ($p < 0.0001$; ~12- and 433-fold lower EC_{50} values, respectively). See Table 2 for parameters.

Receptors with Increased Sensitivity to Agonists—Oocytes expressing chimeric hα6/hα3 and hβ2 subunits occasionally displayed modest function that was reduced by coexpression with hα5 or hα5/hβ3 subunits (Table 2). By contrast, nearly all oocytes injected with hα5$^{V9'S}$ or hα5/hβ3$^{V9'S}$ subunit cRNAs along with nAChR ha6/ha3 and hβ2 subunit cRNAs yielded functional nicotinic responses (Fig. 3, Table 2). Oocyte-expressed (hα6/hα3)hβ2(hα5/hβ3$^{V9'S}$)-nAChR had responses to nicotine at lower concentrations (0.03 μM EC_{50}) than seen for receptors containing hα5/hβ3 subunits or no α5 subunit (13 μM EC_{50}), and there also was higher sensitivity to nicotine for (hα6/hα3)hβ2hα5$^{V9'S}$-nAChR (1.1 μM EC_{50}; Table 2). The findings for oocytes expressing hα6/hα3 and hβ2 subunits alone or with hα5 subunits are consistent with earlier observations by Kuryatov *et al.* (10). Interestingly, Hill coefficients for agonist responses of (hα6/

hα3)hβ2(hα5/hβ3$^{V9'S}$)-nAChR are shallow (n_H = 0.49) and smaller than those for other functional (hα6/hα3)β2*-nAChR, perhaps suggesting heterogeneity or negative cooperativity in the complexes formed. Moreover, agonist EC_{50} values are ~37-fold lower for (hα6/hα3)hβ2(hα5/hβ3$^{V9'S}$)-nAChR than for (hα6/hα3)hβ2(hα5$^{V9'S}$)-nAChR.



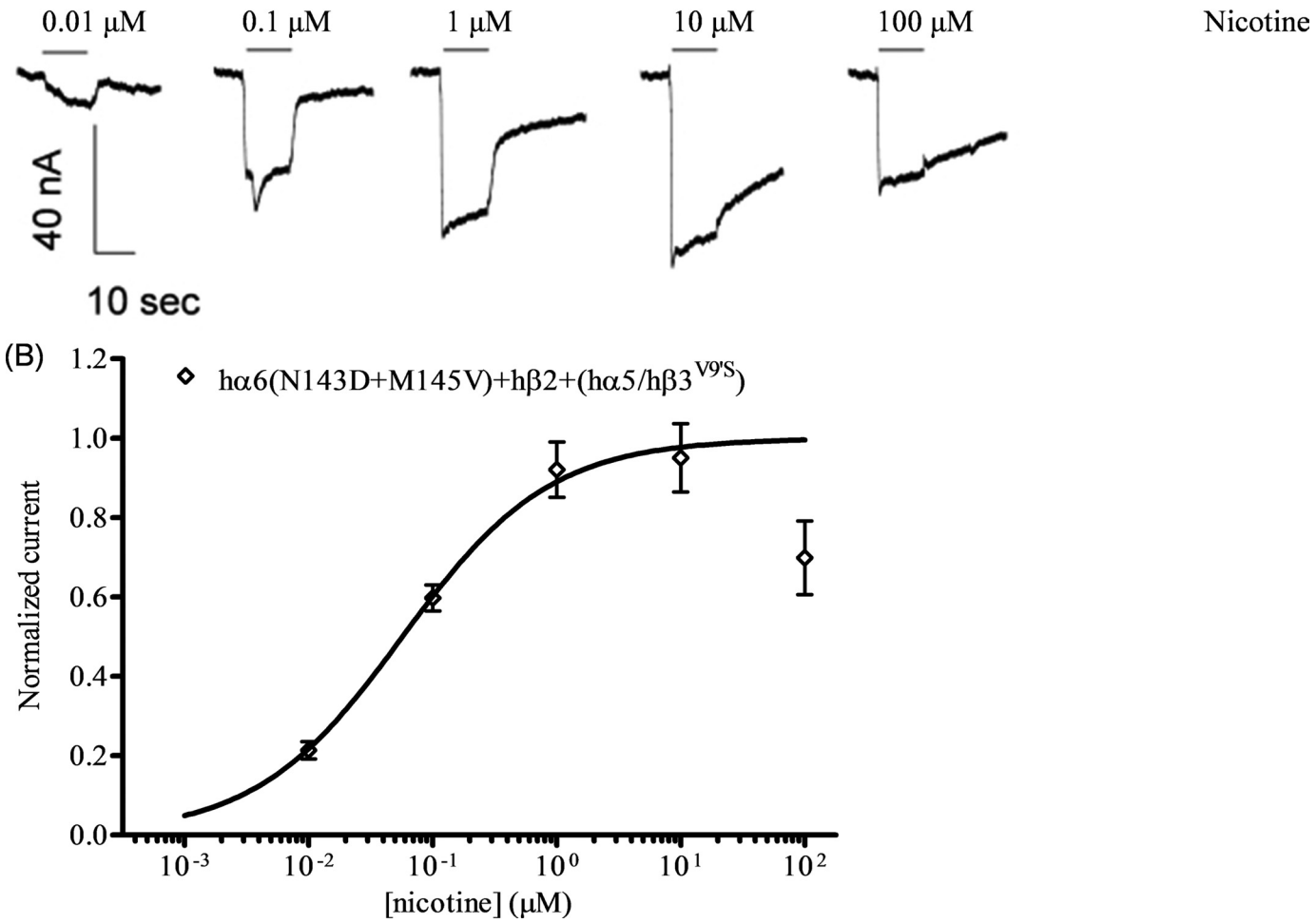


FIGURE 5. **Functional properties of hα6(N143D+M145V)hβ4*-nAChR.** *A*, representative traces are shown for inward current responses in oocytes held at −70 mV, responding to application of nicotine at the indicated concentrations (shown with the duration of drug exposure as *black bars above* the traces), and expressing nAChR hα6(N143D+M145V), hβ4, and (hα5/hβ3$^{V9'S}$) subunits. *Calibration bars* are 40 nA current (vertical) or 10 s (horizontal). *B*, results for these and other studies averaged across experiments were used to produce concentration-response curves (*ordinate*, mean normalized current ± S.E.; *abscissa*, ligand concentration in log μM) for inward current responses to nicotine, where current amplitudes are represented as a fraction of the peak inward current amplitude in response to the most efficacious concentration of nicotine. See Table 2 for parameters.

Coexpression of hα5, hα5$^{V9'S}$, hα5/hβ3, or hα5/hβ3$^{V9'S}$ Subunits with Chimeric hα6/hα3 and hβ4 nAChR Subunits Produces Functional Receptors with Increased Sensitivity for Agonists—Nearly all oocytes injected with hα5, hα5$^{V9'S}$, hα5/hβ3, or hα5/hβ3$^{V9'S}$ subunit cRNAs along with nAChR (hα6/hα3) and hβ4 subunit cRNAs yielded functional nicotinic responses (Table 2, Fig. 4). Oocytes expressing nAChR hα6/hα3 and hβ4 subunits along with hα5 subunits generally had 5-fold lower levels of function (although higher agonist sensitivity; 9.1 μM nicotine EC_{50} values) than oocytes expressing nAChR hα6/hα3 and hβ4 subunits alone (25 μM nicotine EC_{50}: Table 2). However, oocytes expressing nAChR hα6/hα3 and hβ4 subunits along with hα5/hβ3 subunits had ~9-fold higher levels of function (also higher agonist sensitivity; 6.1 μM nicotine EC_{50} values) than oocytes expressing nAChR hα6/hα3 and hβ4 subunits alone. By contrast, both levels of functional receptor expression (3–13-fold higher than for (hα6/hα3)hβ4-nAChR) and agonist sensitivity (2.9 and 0.03 μM nicotine EC_{50} values, respectively; Fig. 4 and Table 2) are increased when hα6/hα3 and hβ4 subunits are

FIGURE 4. **Functional properties of (hα6/hα3)hβ4*-nAChR.** *A*, representative traces are shown for inward currents in oocytes held at −70 mV, responding to application at the indicated concentrations of nicotine (shown with the duration of drug exposure as *black bars above* the traces), and expressing nAChR hα6/hα3, hβ4 and hα5 (*i*), hα5$^{V9'S}$ (*ii*), hα5/hβ3 (*iii*), or hα5/hβ3$^{V9'S}$ (*iv*) subunits. *Calibration bars* are for 20 (*i*), 200 (*ii*), 600 (*iii*), or 1000 nA (*iv*) currents (vertical) or 10 s (horizontal). Note the differences in inward current kinetics. *B*, results for these and other studies averaged across experiments were used to produce concentration-response curves (*ordinate*, mean normalized current ± S.E.; *abscissa*, ligand concentration in log μM) for responses to nicotine as indicated for oocytes expressing nAChR hα6/hα3 and hβ4 subunits alone (■), with wild-type hα5 subunits (♦), hα5$^{V9'S}$ subunits (○), hα5/hβ3 subunits (▲), or hα5/hβ3$^{V9'S}$ subunits (△). Current amplitudes are represented as a fraction of the peak inward current amplitude in response to the most efficacious concentration of nicotine. Leftward shifts in nicotine concentration-response curves are evident for functional nAChR containing hα5, hα5$^{V9'S}$, hα5/hβ3, or hα5/hβ3$^{V9'S}$ subunits ($p < 0.0001$; ~3-, ~9-, ~3-, and 833-fold, respectively). See Table 2 for parameters.

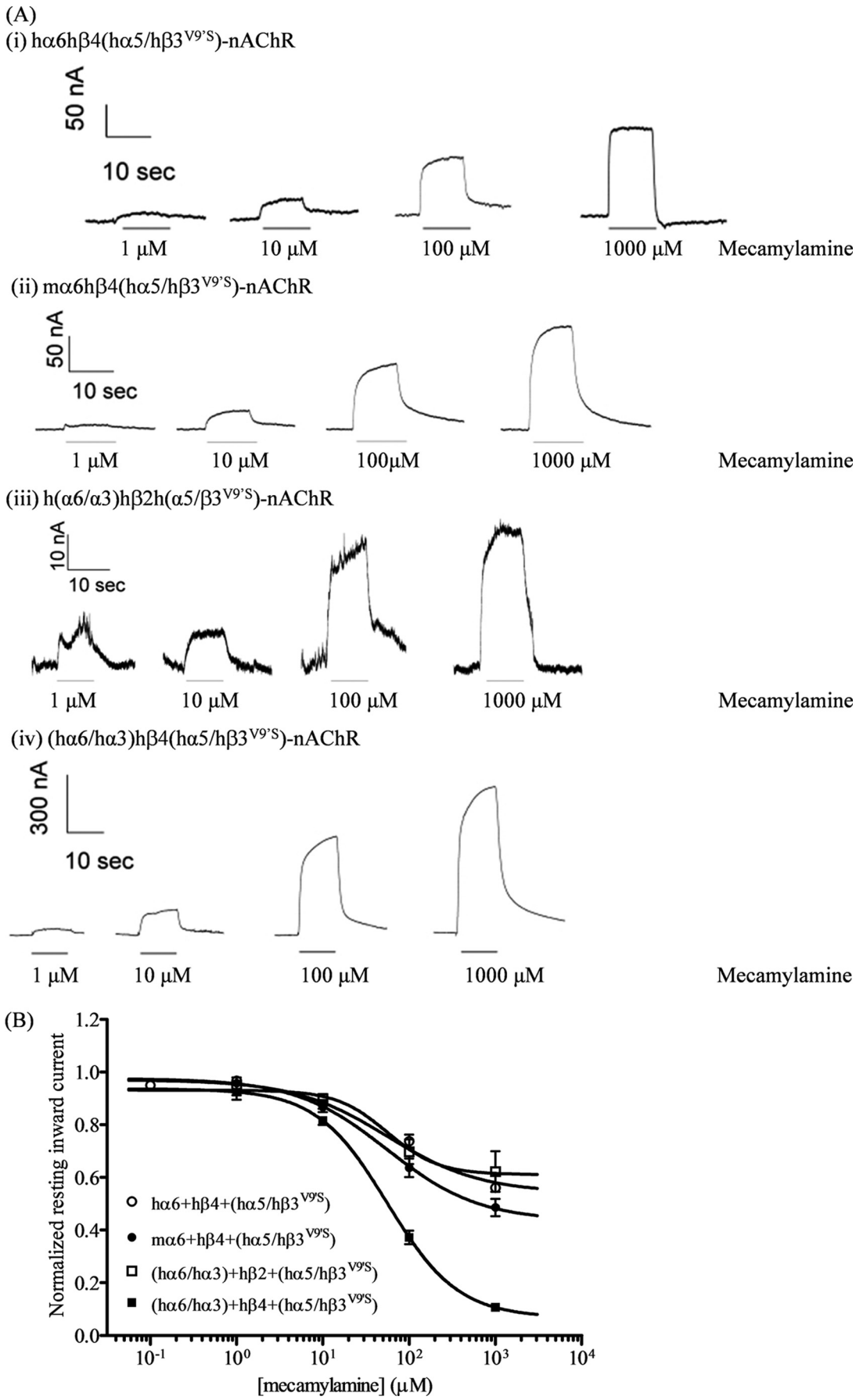


TABLE 3

Parameters for antagonist action at α6*-nAChR containing hα5/hβ3$^{V9'S}$ nAChR subunits

Inhibitor potencies (micromolar IC_{50} values with 95% confidence intervals), Hill coefficents ($n_H \pm$ S.E.), mean ± S.E. peak responses (I_{max} in nanoamps), and concentrations where I_{max} is achieved (μM) are provided for mecamylamine acting at nAChR composed of the indicated subunits derived from the specified species and from the indicated number of independent experiments (*n*) based on studies as shown in Fig. 6. Note that no or very rare and then small responses to mecamylamine were seen for the following subunit combinations ($n = 9$ each): hα6 + hβ2 with hα5$^{V9'S}$ or hα5/hβ3$^{V9'S}$; hα6 + hβ4 with hα5$^{V9'S}$; mα6 + hβ2 with hα5$^{V9'S}$ or hα5/hβ3$^{V9'S}$; mα6 + hβ4 with hα5$^{V9'S}$; hα6/hα3 + hβ2 with hα5$^{V9'S}$; and hα6/hα3 + hβ4 with hα5$^{V9'S}$.

	Mecamylamine					
	Potency			Peak response		
nAChR subunit combinations	*n*	IC_{50} (95% CI)	$n_H \pm$ S.E.	*n*	Mean $I_{max} \pm$ S.E.	I_{max} concentration
		μM			nA	μM
hα6 + hβ4 + hα5/hβ3$^{V9'S}$	3	135 (23–779)	−0.65 ± 0.23	3	150 ± 21	1000
mα6 + hβ4 + hα5/hβ3$^{V9'S}$	4	50 (20–125)	−0.84 ± 0.29	4	125 ± 35	1000
hα6/hα3 + hβ2 + hα5/hβ3$^{V9'S}$	3	30 (4.6–196)	−0.86 ± 0.45	3	67 ± 22	1000
hα6/hα3 + hβ4 + hα5/hβ3$^{V9'S}$	3	56 (40–78)	−1.1 ± 0.16	3	695 ± 95	1000

expressed with hα5$^{V9'S}$ or hα5/hβ3$^{V9'S}$ subunits. The findings for oocytes expressing hα6/hα3 and hβ4 subunits alone or with hα5 subunits are consistent with earlier observations by Kuryatov *et al.* (10). Once again, Hill coefficients for agonist responses of (hα6/hα3)hβ4*-nAChR were lower (n_H = 0.49) in the presence of coexpressed hα5/hβ3$^{V9'S}$ subunits, even in the presence of hα5$^{V9'S}$ subunits, and smaller than those for other functional (hα6/hα3)β2*-nAChR, a classic indication of negative cooperativity but also possibly of heterogeneity in complexes formed. Moreover, agonist EC_{50} values are ~100-fold lower for (hα6/hα3)hβ4(hα5/hβ3$^{V9'S}$)-nAChR than for (hα6/hα3)hβ42(hα5$^{V9'S}$)-nAChR.

Coexpression of hα5/hβ3$^{V9'S}$ Subunits with hα6(N143D + M145V) and hβ2 nAChR Subunits Produces Functional Receptors—Earlier, we had shown (18) that mutations in the N-terminal domain of the nAChR hα6 subunit enable nAChR hβ3$^{V9'S}$ subunits to exert a gain-of-function effect at hα6(N143D+M145V)hβ2*-nAChR (*i.e.* hα6(N143D+M145V)hβ2hβ3$^{V9'S}$-nAChR are functional). This finding led us to explore effects of incorporation of hα5 or hα5/hβ3 subunits into hα6(N143D+M145V)hβ2*-nAChR.

Oocytes injected with hα5, hα5$^{V9'S}$, or hα5/hβ3 subunit cRNAs along with nAChR hα6(N143D+M145V) and hβ2 subunit cRNAs did not yield functional nicotinic responses (Table 2). However, oocytes injected with hα5/hβ3$^{V9'S}$ subunit cRNAs along with nAChR hα6(N143D+M145V) and hβ2 subunit cRNAs yielded functional responses with an EC_{50} value of 0.06 μM for nicotine (Table 2, Fig. 5). The maximal current response was 132 ± 77 nA (mean ± S.E.) for 10 μM nicotine.

Exposure to Mecamylamine Reveals Spontaneous Channel Opening of nAChR Containing hα5/hβ3$^{V9'S}$ Subunits—Specificity of nicotine effects were routinely assessed based on the ability of the noncompetitive nAChR antagonist, mecamylamine, to block agonist-induced inward currents, but in doing so, we observed reversal of currents at very high concentrations of mecamylamine in the presence of nicotine. Effects of mecamylamine alone were absent when assessed using oocytes expressing any combination of wild-type nAChR subunits (data not shown). However, exposure to mecamylamine alone produced concentration-dependent, reversible, outward currents in oocytes expressing hα6 and hβ4 subunits in combination with hα5/hβ3$^{V9'S}$ subunits but not with hα5$^{V9'S}$ subunits (Fig. 6, Table 3). These results suggest that there is spontaneous opening of α6*-nAChR containing mutant hα5/hβ3$^{V9'S}$ subunits but not with hα5$^{V9'S}$ subunits, and that mecamylamine mediates the open channel block with an IC_{50} value of 135 μM at these spontaneously opening hα6hβ4(hα5/hβ3$^{V9'S}$)-nAChR (Fig. 6, Table 3). Amplitudes of the mecamylamine-induced outward currents are about 66% of the amplitudes of agonist-induced inward currents, suggesting that a substantial percentage (~40%; maximum outward current of 150 nA divided by the sum of that figure plus the maximum inward current of 225 nA; Table 3; see Fig. 6) of hα6hβ4*-nAChR containing mutant hα5/hβ3$^{V9'S}$ subunits are spontaneously open at any time.

As was the case for oocytes expressing hα6, hβ4, and mutant hα5/hβ3$^{V9'S}$ subunits or expressing mα6, mβ4, and mutant hα5/hβ3$^{V9'S}$ subunits, oocytes co-injected with cRNAs for mα6, hβ4, and hα5/hβ3$^{V9'S}$ subunits gave outward current responses to mecamylamine in a concentration-dependent, reversible manner (Fig. 6, Table 3). This inhibition of spontaneous channel opening occurred with an IC_{50} value of 50 μM mecamylamine. Estimates are that ~40% of these receptors are spontaneously open at any one time.

Similarly, oocytes expressing (hα6/hα3)hβ2(hα5/hβ3$^{V9'S}$)- or (hα6/hα3)hβ4(hα5/hβ3$^{V9'S}$)-nAChR gave outward current responses to mecamylamine in a concentration-dependent, reversible manner (Fig. 6, Table 3). This inhibition of spontaneously opening (hα6/hα3)hβ2(hα5/hβ3$^{V9'S}$)- and (hα6/hα3)hβ4(hα5/hβ3$^{V9'S}$)-nAChR occurred with an IC_{50} value of 30 and 56 μM, respectively. Estimates are that ~19–20% of these receptors are spontaneously open at any one time.

FIGURE 6. **Functional properties of antagonist action at α6*-nAChR containing hα5/hβ3$^{V9'S}$ subunits.** *A*, representative traces are shown for outward currents in oocytes held at −70 mV, responding to application at the indicated concentrations of mecamylamine (shown with the duration of drug exposure as *black bars above* the traces), and expressing nAChR containing hα6 and hβ4 (*i*), mα6 and hβ4 (*ii*), hα6/α3 and hβ2 (*iii*), or hα6/α3 and hβ4 subunits (*iv*), all along with hα5/hβ3$^{V9'S}$ subunits. *Calibration bars* are 50 (*i* and *ii*), 10 (*iii*), or 300 (*iv*) nA currents (vertical) or 10 s (horizontal). Note the differences in inward current kinetics. *B*, results for these and other studies averaged across experiments were used to produce concentration-response curves (*ordinate,* mean normalized current ± S.E.; *abscissa*, ligand concentration in log μM) for outward current responses to mecamylamine as indicated, for hα6hβ4(hα5/hβ3$^{V9'S}$)-(○), mα6hβ4(hα5/hβ3$^{V9'S}$)- (●), (hα6/α3)hβ2(hα5/hβ3$^{V9'S}$)- (□), or (hα6/α3)hβ4(hα5/hβ3$^{V9'S}$)-nAChR (■), where current amplitudes are represented as a fraction of the resting current amplitude. See Table 3 for parameters.

DISCUSSION

Although excellent studies have determined the distribution of nAChR α5 subunit messages in the rodent brain, but left largely uninvestigated is how α5 subunits might incorporate as accessory partners into nAChR subtypes, specifically into α6*-nAChR. To understand how α5 subunits might incorporate into α6*-nAChR, we exploited the reporter or gain-of-function mutant strategy (20) to reveal whether α5 subunits or their variants integrate into α6*-nAChR complexes that are on the cell surface and functional. This approach allows focus on cell surface, functional receptors without complications due to ambiguities of protein chemical or immunochemical studies confounded by the prevalent expression of intracellular and perhaps partially assembled receptor complexes, and the unreliable quality and/or availability of most anti-nAChR antibodies for use in immunoprecipitation and/or immunoblot studies. Furthermore, we used chimeric subunits and hybrid nAChR as tools. In addition, we based the current studies on our findings (18) that (i) incorporation of nAChR β3 subunits into α6*-nAChR has a dominant-negative effect that can be overcome by introducing a gain-of-function mutation into a key residue in the M2 second transmembrane domain of the β3 (or other) subunit, (ii) incorporation of nAChR hβ3 subunits into mα6hβ4*-nAChR leads to formation of functional nAChR, and (iii) mutations in the E1 N-terminal domain of the nAChR hα6 subunit are essential for successful assembly and formation of functional hα6(N143D+M145V)hβ2hβ3$^{V9'S}$-nAChR.

The principal observations of the current study are as follows. Coexpression in *Xenopus* oocytes of nAChR hα6 subunits, hβ4 subunits, and wild-type hα5 subunits fails to produce functional receptors (*i.e.* significant and reproducible, inward current response to nicotine), eliminating the small amount of function seen in some cases when oocytes express just hα6 plus hβ4 subunits. No function is seen in the absence or presence of wild-type hα5 subunits in oocytes expressing hα6 and hβ2 subunits. Moreover, no function is seen for hybrid (a mixture of subunits from different species) mα6hβ2*- or mα6hβ4*-nAChR when those subunits are coexpressed with hα5$^{V9'S}$ subunits or when α6 and either β2 or β4 subunits are coexpressed with chimeric hα5/hβ3 subunits. By contrast, there is very clear evidence for incorporation of chimeric, mutant hα5/hβ3$^{V9'S}$ subunits containing a valine 273 to serine mutation into α6β4*-nAChR, because oocytes expressing the indicated subunits have large, agonist-induced, peak inward current responses. Moreover, agonist sensitivity of α6β4*-nAChR containing mutant hα5/hβ3$^{V9'S}$ subunits is higher than for the equivalent receptors expressed in the absence of mutant subunits. Chimeric hα6/hα3 subunits (10) form functional nAChR when combined with either nAChR hβ2 or hβ4 subunits. Further incorporation of hα5 or hα5/hβ3 subunits diminishes functional responsiveness, but levels of functional expression and sensitivity to nicotine are increased when hα6/hα3 and either hβ2 or hβ4 subunits are coexpressed with hα5$^{V9'S}$ or hα5/hβ3$^{V9'S}$ subunits. Interestingly, (hα6/hα3)β2*- or (hα6/hα3)hβ4*-nAChR complexes in oocytes coexpressing hα5/hβ3$^{V9'S}$ subunits have higher agonist sensitivity than in the presence of coexpressed hα5$^{V9'S}$ subunits. When there is expression of functional receptors containing hα5/hβ3$^{V9'S}$ subunits, but not hα5$^{V9'S}$ subunits, mecamylamine exposure produces outward currents, indicative of spontaneous channel opening in the presence of hα5/hβ3$^{V9'S}$ subunits, and that mecamylamine blocks spontaneously open channels and agonist-induced open channels. Finally, there is expression of functional receptors when hα5/hβ3$^{V9'S}$ subunits (but not hα5, hα5$^{V9'S}$, or hα5/hβ3 subunits) are coexpressed with hα6(N143D+M145V) and hβ2 subunits.

We derive several conclusions from these findings. One is that nAChR α5 subunits or their variants can incorporate into heterologously expressed α6β4*-nAChR, where they exert dominant-negative (wild-type hα5 or hα5/hβ3 subunits) or gain-of-function (mutant hα5/hβ3$^{V9'S}$ subunits) effects. The inability of wild-type, mutant, chimeric, or mutant chimeric α5 subunit variants to influence function of α6β2*-nAChR confounds the ability to make inferences about assembly of the indicated subunits. However, we also conclude from studies with hα6(N143D+M145V) subunits that the N-terminal domain of α6 subunits influences the ability to see a mutant α5/β3$^{V9'S}$ subunit-mediated, gain-of-function effect at α6β2*-nAChR, consistent with earlier findings that hβ3$^{V9'S}$ subunits have a gain-of-function effect when expressed as part of hα6(N143D+M145V)hβ2hβ3$^{V9'S}$-nAChR. Effects of mecamylamine are interpreted as demonstrating that α6*-nAChR that show gain-of-function have a significant probability of spontaneous channel opening, consistent with the observation that other receptors containing subunits with second transmembrane domain, gain-of-function mutations can open spontaneously in a way sensitive to open channel blockers (24, 25). Gain-of-function effects found for the α5/β3$^{V9'S}$ mutant chimeric subunit are largely similar to those found for β3$^{V9'S}$ mutant subunits, but in some cases yield functional receptors with different agonist sensitivities, suggesting subunit-specific subtleties in coupling between ligand binding and channel opening. The studies using nAChR containing chimeric α6/α3 subunits provide a more reliable functional background when expressed as binary complexes with β2 or β4 subunits and underscore the interpretation that wild-type α5 or chimeric α5/β3 subunits have a dominant-negative effect and that α5$^{V9'S}$, in addition to α5/β3$^{V9'S}$ subunits, exert gain-of-function effects. However, magnitudes of nicotine-evoked currents, sensitivity to nicotine, and the extent of spontaneous opening differ for receptors containing α5$^{V9'S}$ or α5/β3$^{V9'S}$ subunits, thus providing additional evidence that fine features of subunits influence their effects on α6*-nAChR function. In the aggregate, although we cannot discount the possibility that inclusion of mutant subunits increases the efficiency of incorporation of functional receptors into the cell surface, we think that inclusion of these reporter mutant subunits enhances the functional gain of receptors (20, 26) expressed at about the same level, in part as evident by changes in agonist sensitivity, increases in peak inward current magnitude, and increased susceptibility to spontaneous channel opening. This lessens epistemological constraints on experiments by allowing more ready detection of functional receptors, as do studies of nAChR containing chimeric hα6/hα3 subunits.

The current findings are in general agreement with and extend upon recent experimental successes demonstrating that nAChR α6 subunits, long refractory to heterologous expression (27, 28) in functional nAChR assemblies with other subunits, indeed can

interact with β2 or β4 subunits to form functional receptors in oocytes. The current studies have overcome difficulties in expressing and characterizing α6α5*-nAChR, in part by demonstrating that (i) a dominant-negative effect of wild-type α5 subunits on α6*-nAChR can be counteracted using a gain-of-function strategy and (ii) mutations in the N-terminal domain of α6 subunit are essential for a gain-of-function effect at α6β2*-nAChR.

Recently, it was observed (17, 18) that incorporation of the human nAChR β3 subunit has a dominant-negative effect on the function of specific nAChR subtypes including hα6hβ4*- and hα6hβ2*-nAChR heterologously expressed in oocytes. The current findings extend upon these previous findings and reinforce the perspective that β3 and α5 subunits are highly homologous and serve as auxiliary subunits in formation of functional nAChR. Contrary to the gain-of-function observed for $\beta3^{V9'S}$ subunits, $\alpha5^{V9'S}$ subunits did not exert any gain-of-function in combination with wild-type α6 and β2 or β4 subunits. However, we have extended the work to show that gain-of-function occurs for hα5/h$\beta3^{V9'S}$ subunits. Moreover, wild-type α5 subunits show a dominant-negative effect, and $\alpha5^{V9'S}$ subunits do exert a gain-of-function effect when coexpressed with chimeric hα6/hα3 and β2 or β4 subunits, further reinforcing our interpretation.

Lack of or low level of function compromised our ability to definitively generate EC_{50} values for agonists at hα6hβ2hα5-, mα6mβ2hα5-, mα6hβ2hα5-, hα6hβ4hα5-, mα6mβ4hα5-, mα6hβ4hα5-, (hα6/hα3)hβ2hα5-nAChR, etc., but an EC_{50} value of 9.1 μM nicotine for (hα6/hα3)hβ4hα5-nAChR is from our studies. Nicotine EC_{50} values generated from the current study (0.03–2 μM) for receptors containing (hα5/h$\beta3^{V9'S}$) subunits reflect the increased agonist sensitivity of receptors harboring gain-of-function mutant subunits as has been seen for other nAChR subunits mutated the same way at equivalent residues (20, 29).

One of the more interesting but mysterious observations is the lack-of-function in oocytes expressing many combinations of α6 or even hα6(N143D+M145V) subunits plus β2 or β4 subunits in the presence of h$\alpha5^{V9'S}$ subunits when previous studies (17, 18) demonstrated function of those combinations in the presence of h$\beta3^{V9'S}$ subunits, even though β3 and α5 subunits are highly, but not perfectly, homologous. The fact that function is expressed in the presence of chimeric hα5/h$\beta3^{V9'S}$ subunits suggests that features in the β3 subunit as opposed to in the α5 subunit C-terminal to E1 (including the second, large cytoplasmic loop and all four transmembrane domains, but also containing a small cytoplasmic loop between the first and second transmembrane domains, an extracellular linker between the second and third transmembrane domains, and the extracellular C-terminal tail) facilitate assembly of functional complexes and the gain-of-function behavior of hα5/h$\beta3^{V9'S}$ subunits. Studies of (hα6/hα3)hβ2*- or (hα6/hα3)hβ4*-nAChR showing more evident dominant-negative effects of α5 subunits and gain-of-function effects of $\alpha5^{V9'S}$ subunits also support these suggestions. This is because domains C-terminal to E1 from the α3 subunit might be more compatible than those from the α6 subunit with regard to productive assembly of functional nAChR in combination with α5 subunits, overcoming possible incompatibilities in those domains between α6 and α5 subunits. Moreover, the potentiation of chimeric α5/β3 subunits rather than the dominant-negative influence of α5 subunits on function of (hα6/hα3)β4*-nAChR also is consistent with heretofore unrecognized roles of α5 or β3 domains C-terminal to E1 (and other than an M2 9′ mutation itself) in formation of functional α6*-nAChR and agonist sensitivity.

Because there are indications that naturally expressed, rodent α6β2*-nAChR seem to be functional, perhaps when combined with α5 subunits, and given the difficulties in heterologously expressing human α6β2*-nAChR, we wondered whether there simply might be species-specific differences in the ability to heterologously express α6β2α5*-nAChR, and so we chose to see if mα6 and hβ2 nAChR subunits could be functionally expressed when combined with hα5 subunits or their variants. However, we realized very similar outcomes in our studies of all human, all mouse, or hybrid α6β2α5*-nAChR, even when α5 or (α5/β3) subunits had gain-of-function mutations and despite success of the (α5/β3) subunit gain-of-function strategy when applied to α6β4*-nAChR. It is possible that a key assembly partner, such as α4 or α3 subunits, are required for formation of naturally expressed, functional α6β2α5*-nAChR, and we are involved in studies to test this hypothesis. Another possibility that would be much more difficult to test is that oocytes, but not the right kinds of nerve cells, lack chaperones that facilitate assembly and functional expression of α6β2α5*-nAChR.

Some of our other site-directed mutagenesis work implicated α6 residues 143 and 145 in the ability of β3 subunits to affect α6β2*-nAChR function (18). The hα6(N143D+M145V) mutations change the indicated residues to those that are in the mα6 subunit and permits mutated hα6 subunits to show function when coexpressed with hβ2 and either h$\beta3^{V9'S}$ or hα5/h$\beta3^{V9'S}$ subunits when wild-type hα6 subunit do not. This is very interesting for two reasons. First, agonist binding domains are thought to be present at the interface between E1 domains of specific nAChR subunits, where the principal or (+) face is contributed by one subunit via the so-called A, B, and C loops, which are apposed to the D, E, and F loops in the (−) or complementary face of the neighboring subunit. The consensus perspective would predict that the α6(+)/(−)β2 or α6(+)/(−)β4 subunit interfaces are where there would be productive and functionally relevant agonist binding. That is, they would be at the two α6:β2 interfaces in a complex with the arrangement, counterclockwise when viewed from the extracellular space, of β3 (or α5):α6:β2:α6:β2. However, α6 residues 143 and 145 are in the E1 domain, in loop E, on the (−) or complementary face of the subunit. This means, unexpectedly, that mutations in the α6 subunit interface thought not to engage in agonist binding influences formation of functional receptors with β2 subunits and with subunits that contain either β3 or α5 E1 domains coupled to β3 subunit domains C-terminal to E1 (in addition to the reporter V293S mutation). This suggests that interactions between the α6 subunit (−) face with the (+) face from either β2 subunits or from either β3 or α5 subunits are important for functional α6*-nAChR expression, raising the question as to whether agonists also bind at that interface to play allosteric or co-agonist roles. At a finer detail level, one might hypothesize that the introduction of a negatively charged side chain at position 143, a modest increase in hydrophobicity index (from 74 to 79) at residue 145, and/or a difference in side chain volume at position 145 might enhance interactions between α6 and β2, β3 or α5 subunits, and the ability of ligands to bind at that interface. Second, because the α6 subunit (−) face mutations affect functional nAChR forma-

tion with β2 subunits and either hβ3$^{V9'S}$ or hα5/hβ3$^{V9'S}$ subunits, it does not seem that the α5(+)/(−)α6 or β3(+)/(−)α6 E1 interfaces are critical, but rather that the β2(+)/(−)α6 E1 interface is important. The analogous interface in α4β2-nAChR (β2(+)/(−)α4) is an allosteric site of action of Zn^{2+} (30). β2:α6 E1 subunit interfaces in the vicinity of loop E could play roles in subunit assembly leading to closure of functional, cell-surface α6β2β3$^{V9'S}$-nAChR, although distal involvement of β2:α6 subunit interfaces in ligand binding or transduction of ligand binding to channel gating cannot be discounted. The lack of function of hα6(N143D+M145V)hβ2hα5$^{V9'S}$-nAChR again is consistent with limited compatibility between α6 and α5 subunit domains C-terminal to E1.

In conclusion, our results provide evidence that wild-type or mutant α5 subunits can incorporate into and either suppress/abolish or enhance, respectively, function of α6β4*- (although low levels of function make this conclusion more tenuous), (hα6/hα3)hβ2*-, or (hα6/hα3)hβ4*-nAChR, but that effects are more evident in the presence of chimeric α5/β3 subunits. These studies using the oocyte expression system have been useful in illuminating the α6*-nAChR structure and function, and there is the prospect that cell lines containing the same assemblies could be generated in part to serve the same purpose. With the caveat that α6*-nAChR may have different sensitivities for agonists or perhaps other types of ligands depending on whether fully wild-type or one or more gain-of-function subunits are in the complex, the strategies demonstrated here to increase function of α6*-nAChR to levels compatible with drug screening, perhaps using high throughput methods, could facilitate the development of new drugs selective for α6*-nAChR. This is of increasing importance given the potentially important roles for α6*-nAChR in movement and movement disorders, mood disorders, and drug reinforcement (6, 7, 14–16).

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Reporter Mutation Studies Show That Nicotinic Acetylcholine Receptor (nAChR) α5 Subunits and/or Variants Modulate Function of α6*-nAChR

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