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Reporter Mutation Studies Show That Nicotinic Acetylcholine Receptor (nAChR) $\alpha 5$ Subunits and/or Variants Modulate Function of $\alpha 6^*$ -nAChR^{*}

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Background: We asked how partner subunits influence $\alpha 6^*$ -nicotinic receptor (nAChR) function.

Results: We found several, novel ways to manipulate effects of $\alpha 5$ subunits on $\alpha 6^*$ -nAChR function.

Conclusion: Extracellular domains in $\alpha 6$ and cytoplasmic/transmembrane domains in $\alpha 6/\alpha 3/\alpha 5/\beta 3$ subunits have unexpected influences on $\alpha 6^*$ -nAChR function.

Significance: We found factors that influence assembly and function of $\alpha 6^*$ -nAChRs, which play important roles in mood, reward, and nicotine dependence.

To further the understanding of functional $\alpha 6\alpha 5^*$ -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 $\alpha 5$ subunits or chimeric $\alpha 5/\beta 3$ subunits (in which the human $\alpha 5$ subunit N-terminal, extracellular domain is linked to the remaining domains of the human $\beta 3$ subunit) almost completely abolishes the very small amount of function seen for $\alpha 6\beta 4^*$ -nAChR and does not induce function of $\alpha 6\beta 2^*$ -nAChR. Coexpression with human $\alpha 5^{V9/S}$ subunits bearing a valine 290 to serine mutation in the 9' position of the second transmembrane domain does not rescue the function of $\alpha 6\beta 4^*$ -nAChR or induce function of $\alpha 6\beta 2^*$ -nAChR. However, coexpression with mutant chimeric $\alpha 5/\beta 3^{V9/S}$ subunits has a gain-of-function effect (higher functional expression and agonist sensitivity and spontaneous opening inhibited by mecamylamine) on $\alpha 6\beta 4^*$ -nAChR. Moreover, N143D + M145V mutations in the $\alpha 6$ subunit N-terminal domain enable $\alpha 5/\beta 3^{V9/S}$ subunits to have a gain-of-function effect on $\alpha 6\beta 2^*$ -nAChR. nAChR containing chimeric $\alpha 6/\alpha 3$ subunits plus either $\beta 2$ or $\beta 4$ subunits have some function that is modulated in the presence of $\alpha 5$ or $\alpha 5/\beta 3$ subunits. Coexpression with $\alpha 5/\beta 3^{V9/S}$ subunits has a gain-of-function effect more pronounced than that in the presence of $\alpha 5^{V9/S}$ 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 $\alpha 6\alpha 5^*$ -nAChR and provide tools for development of $\alpha 6^*$ -

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 $\alpha 2$ - $\alpha 7$ and $\beta 2$ - $\beta 4$ subunits being expressed in the nervous system (1). Some of these subunits form homopentameric receptors when expressed in heterologous expression systems ($\alpha 7$, $\alpha 8$, and $\alpha 9$), 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 $\alpha 3$ and $\beta 4$ subunits, work on the $\alpha 5$ 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 $\alpha 5$ 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 $\alpha 6$ and $\alpha 5$ subunit messages share very similar expression patterns, and some studies suggest that the $\alpha 5$ subunit may participate in formation of functional $\alpha 6^*$ -nAChR (where the asterisk (*) indicates the known or possible presence of additional subunits in the complex), perhaps promoting assembly and stability of mature $\alpha 6^*$ -nAChR (10, 13). Interest in $\alpha 6\alpha 5^*$ -nAChR is increasing because $\alpha 6\alpha 5^*$ -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). $\alpha 6^*$ -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.

Modulation of $\alpha 6^*$ -Nicotinic Receptor Function: $\alpha 5$ Subunits

has been difficult to demonstrate the function of heterologously expressed nAChR containing $\alpha 6$ and $\alpha 5$ subunits.

Recently Broadbent *et al.* (17) and we (18) observed that incorporation of the human (h) nAChR $\beta 3$ ($h\beta 3$) subunit has a dominant-negative effect on the function of some heterologously expressed nAChR subtypes. By contrast, coexpression with $h\beta 3$ subunits having a “reporter” mutation (valine 273 to serine at the M2 second transmembrane domain 9' position, $h\beta 3^{V273S} = h\beta 3^{V9'S}$) has a “gain-of-function” effect, potentiating the function of $h\alpha 6h\beta 2$ - or $h\alpha 6h\beta 4$ -nAChR (17), of nAChR containing mouse (m) $\alpha 6$ subunits and $h\beta 2$ subunits, of $m\alpha 6h\beta 4$ -nAChR, or of nAChR containing mutant $h\alpha 6$ ($h\alpha 6^{N143D+M145V}$) and $h\beta 2$ subunits (17, 18). Building on these findings, we hypothesized that $h\alpha 5$ subunits may also exert a dominant-negative effect on $h\alpha 6h\beta 2$ - and $h\alpha 6h\beta 4$ -nAChR function, in part because $\alpha 5$ and $\beta 3$ subunits are phylogenetically similar, highly homologous, and share service as accessory subunits in several nAChR subtypes. Acutely aware that coexpression with mutated $h\alpha 5$ subunits (M2 second transmembrane domain 9' position, $h\alpha 5^{V290S} = h\alpha 5^{V9'S}$) do not induce spontaneous opening or increased agonist sensitivity of the nAChR subtypes tested (19) we further hypothesized that recombinant, chimeric $h\alpha 5/h\beta 3^{V273S}$ subunits (in which the N-terminal, large extracellular domain of the $h\alpha 5$ subunit is linked to the remaining domains of the gain-of-function $h\beta 3^{V9'S}$ subunit) would serve as a reporter mutation and, upon integration into $\alpha 6^*$ -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 $\alpha 5$ or reporter mutant $h\alpha 5^{V9'S}$ subunits are incorporated into complexes also containing either $h\alpha 6$ or $m\alpha 6$ plus either $h\beta 2$ or $h\beta 4$ subunits, but have a null or dominant-negative effect on the function of those $\alpha 6^*$ -nAChR. We also show that chimeric $h\alpha 5/h\beta 3$ subunits have similar effects, but that reporter mutant chimeric $h\alpha 5/h\beta 3^{V9'S}$ subunits allow for agonist-activated and some spontaneous function of $h\alpha 6h\beta 4^*$ - or $m\alpha 6h\beta 4^*$ -nAChR. Moreover, we demonstrate that chimeric $h\alpha 6/h\alpha 3$ subunits (10) expressed in combination with $h\beta 2$ or $h\beta 4$ subunits produce functional $\alpha 6^*$ -nAChR that are sensitive to dominant-negative effects of coexpression with $h\alpha 5$ or $h\alpha 5/h\beta 3$ subunits, but also become more sensitive to agonists and have much higher function when coexpressed instead with $h\alpha 5^{V9'S}$ or chimeric, reporter mutant $h\alpha 5/h\beta 3^{V9'S}$ subunits. Finally we show that mutations in the N-terminal domain of the $h\alpha 6$ subunit (N143D and M145V) enable ($h\alpha 5/h\beta 3^{V9'S}$) subunits to have a gain-of-function effect at $h\alpha 6h\beta 2^*$ -nAChR. These findings provide insight into the assembly, structure, and function of functional $\alpha 6\alpha 5^*$ -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 $\alpha 6$ ($h\alpha 6$), $h\alpha 5$, $h\beta 2$, $h\beta 3$, or $h\beta 4$ subunits were excised from vectors containing them and subcloned into the oocyte expression vector pGEMHE. Similarly, cDNA representing mouse nAChR $\alpha 6$ ($m\alpha 6$) 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 $\alpha 7$ 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 $h\alpha 5$ (V290S) or $h\beta 3$ (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 $h\alpha 6$ subunit (*i.e.* N143D + M145V) were introduced using the QuikChange II Site-directed Mutagenesis Kit. Primers used for mutagenesis are listed in Table 1.

($h\alpha 5/h\beta 3$) Subunit Chimera—To substitute the nAChR $h\alpha 5$ subunit large, N-terminal, extracellular domain (E1) for the equivalent segment of the $h\beta 3$ subunit cDNA, a cDNA corresponding to amino acid residues 1 to 246 of $h\alpha 5$ subunit cDNA was first PCR-amplified using primers 5'-taatacagctacataggg-3' (forward; T7: corresponding to the pGEMHE sequence) and 5'-ggcagcctccggattacaatgag-3' (reverse; corresponding to the nAChR $h\alpha 5$ subunit sequence and containing a BspEI site (underlined)). The PCR-amplified $h\alpha 5$ 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 $\alpha 6$ subunit to create the designated replacement. F, forward primer; R, reverse primer.

Mutant	Primer sequences (5' → 3')
$\alpha 5$ (V290S)-F	5'-gtctctgcacttcagtaacttTCgtctttgactgtcttctcttc-3'
$\alpha 5$ (V290S)-R	5'-gaaggaagacagtcGaaagacGaaagactgaagtcagagac-3'
$\beta 3$ (V273S)-F	5'-cattatccacatcggTCTtggtttctctgacagttttcc-3'
$\beta 3$ (V273S)-R	5'-ggaaaactgtcagagaaaccaaGAccgatgtggataatg-3'
$\alpha 6$ (N143D+M145V)-F	5'-ggcaaaacaaaagctcttcttaataacGatggcGtgataaacctggactccaccagctattt-3'
$\alpha 6$ (N143D+M145V)-R	5'-aaatagctggaggagtcagggtatcaCgccatCgtatttaagaagagcttttgttttggcc-3'

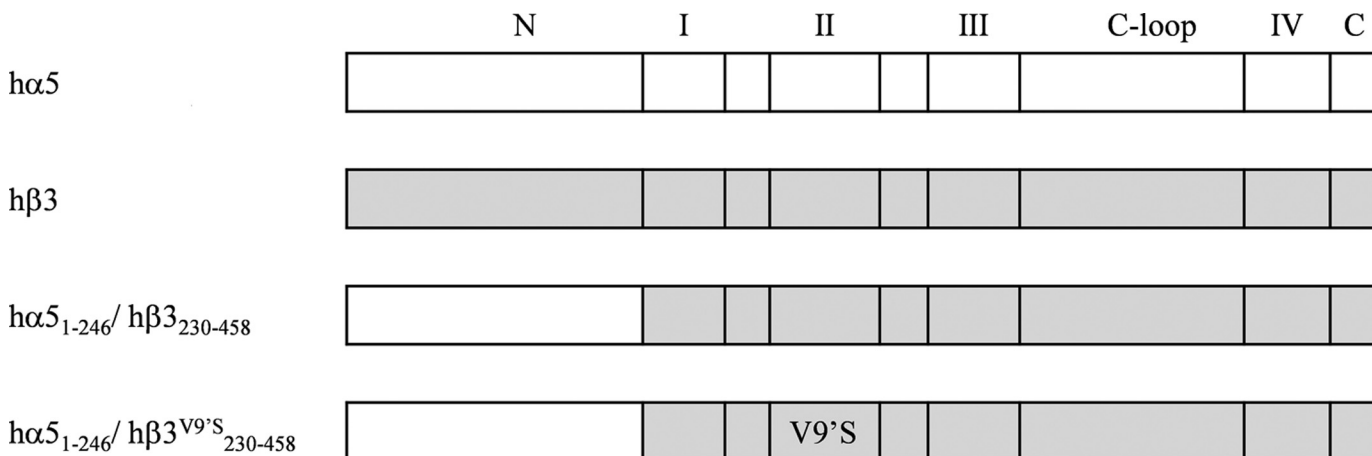


FIGURE 1. Schematic diagrams of wild-type and chimeric human $\alpha 5$ and $\beta 3$ subunits. The notations are: *N* = N-terminal, large extracellular domain; *I*, *II*, *III*, and *IV* are the four transmembrane domains; *C-loop*, second, large cytoplasmic loop; *C*, C terminus. The $\alpha 5_{1-246}/\beta 3_{230-458}$ 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 $\beta 3$ 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- $\beta 3$ construct by site-directed mutagenesis at position Arg²³⁰ by using primers: 5'-cagctattcctctgcctcccggagggcctttattctataacc-3' (forward) and 5'-ggtagaataaaggcagcctccggaggcagaaggaatacgtg-3' (reverse). The mutated pGEMHE- $\beta 3$ plasmid was digested with BamHI and BspEI, where the BamHI site is located upstream of the N-terminal end of the $\beta 3$ subunit cDNA and in the multiple cloning site. The pGEMHE plasmid devoid of the $\beta 3$ subunit N-terminal fragment was gel purified. The restriction-digested $\alpha 5$ and pGEMHE- $\beta 3$ fragments were ligated, producing the chimera ($\alpha 5/\beta 3$) ($\alpha 5$ (Met¹-Ile²⁴⁶)/ $\beta 3$ (Arg²³⁰-His⁴⁵⁸)) (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.

$\alpha 5/\beta 3^{V9'S}$ Reporter Mutant Subunit Chimera—Similarly, to create the $\alpha 5/\beta 3^{V9'S}$ subunit chimera containing a reporter mutation, a BspEI restriction site was created in the pGEMHE- $\beta 3^{V9'S}$ construct by site-directed mutagenesis at position Arg²³⁰ using the same pair of primers as above, and the rest of the procedures were the same as for creating the $\alpha 5/\beta 3$ subunit chimera. This resulted in a chimeric $\alpha 5/\beta 3^{V9'S}$ subunit ($\alpha 5$ (Met¹-Ile²⁴⁶)/ $\beta 3^{V273S}$ (Arg²³⁰-His⁴⁵⁸)) (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.

$\alpha 6/\alpha 3$ Subunit Chimera—The $\alpha 6/\alpha 3$ subunit chimera plasmid ((containing the N-terminal, large extracellular

domain, E1) of the $\alpha 6$ subunit fused to domains from transmembrane region 1 through to the C terminus of the $\alpha 3$ 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 m $\beta 4$, $\alpha 6$, $\alpha 5$, $\alpha 5^{V9'S}$, $\beta 3$, $\beta 3^{V9'S}$, $\alpha 5/\beta 3$, $\alpha 5/\beta 3^{V9'S}$ and $\beta 4$ subunit-containing plasmids. SbfI was used to linearize the $\beta 2$ subunit cDNA. The $\alpha 6/\alpha 3$ subunit chimera plasmid was linearized using EcoRI. Capped mRNA was transcribed from linearized plasmids in a reaction mixture (25 μ l) containing 1 \times transcription buffer, 1.6 mM rNTPs (Promega), 0.5 mM 7m-CAP (New England Biolabs), 1 μ l of RNasin plus (New England Biolabs), and 1 μ l of T7 RNA polymerase (New England Biolabs) with the exception that the linearized $\alpha 6/\alpha 3$ 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 MgCl₂, 1 CaCl₂, 1 Na₂HPO₄, 0.6 theophylline, 2.5 sodium pyruvate, and 5 HEPES, plus 50 mg/ml of gentamycin, 50 units/ml of penicillin, and 50 μ g/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

Modulation of $\alpha 6^*$ -Nicotinic Receptor Function: $\alpha 5$ Subunits

(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 $\alpha 6$ and $\beta 2$ or $\beta 4$ subunits would form complexes having 2:3 and/or 3:2 ratios of the indicated subunits and that oocytes also injected with $\alpha 5$ or $\beta 3$ subunits or variants would express nAChR with 2:2:1 ratios of $\alpha 6$: $\beta 2$ or $\beta 4$: $\alpha 5$ or $\beta 3$ 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 CaCl₂, 1 MgCl₂, 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 $\alpha 5$, $\alpha 5^{V91S}$, ($\alpha 5$ /h $\beta 3$), or ($\alpha 5$ /h $\beta 3^{V91S}$) subunits with either an $\alpha 6$ subunit or $\beta 2$ or $\beta 4$ 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 \pm 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 $\alpha 5$ Subunits into $\alpha 6$ h $\beta 4^*$ - or $\alpha 6$ h $\beta 4^*$ -nAChR1

Human nAChR Chimeric $\alpha 5$ / $\beta 3^{V91S}$ Subunits (but Not Human Wild-type $\alpha 5$, $\alpha 5^{V91S}$, or $\alpha 5$ / $\beta 3$ subunits) Form Functional Receptors When Coassembled with $\alpha 6$ h $\beta 4^*$ - or $\alpha 6$ h $\beta 4^*$ -nAChR and Increase Agonist Sensitivity of Expressed Receptors—In initial studies, coexpression of nAChR wild-type $\alpha 6$ and h $\beta 4$ 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 $\alpha 5$, mutant $\alpha 5^{V91S}$ subunits, or chimeric $\alpha 5$ /h $\beta 3$ subunits along with wild-type $\alpha 6$ and h $\beta 4$ subunits did not produce any response to nicotine. However, coexpression with chimeric mutant $\alpha 5$ /h $\beta 3^{V91S}$ subunits as accessory partners significantly increased nicotinic responses to 10 μM nicotine (225 ± 31 nA), and nearly every oocyte injected with nAChR $\alpha 6$, h $\beta 4$, and mutant $\alpha 5$ /h $\beta 3^{V91S}$ subunits expressed functional nAChR (Fig. 2, Table 2). These results at least indicated that both $\alpha 5$ /h $\beta 3$ and mutant

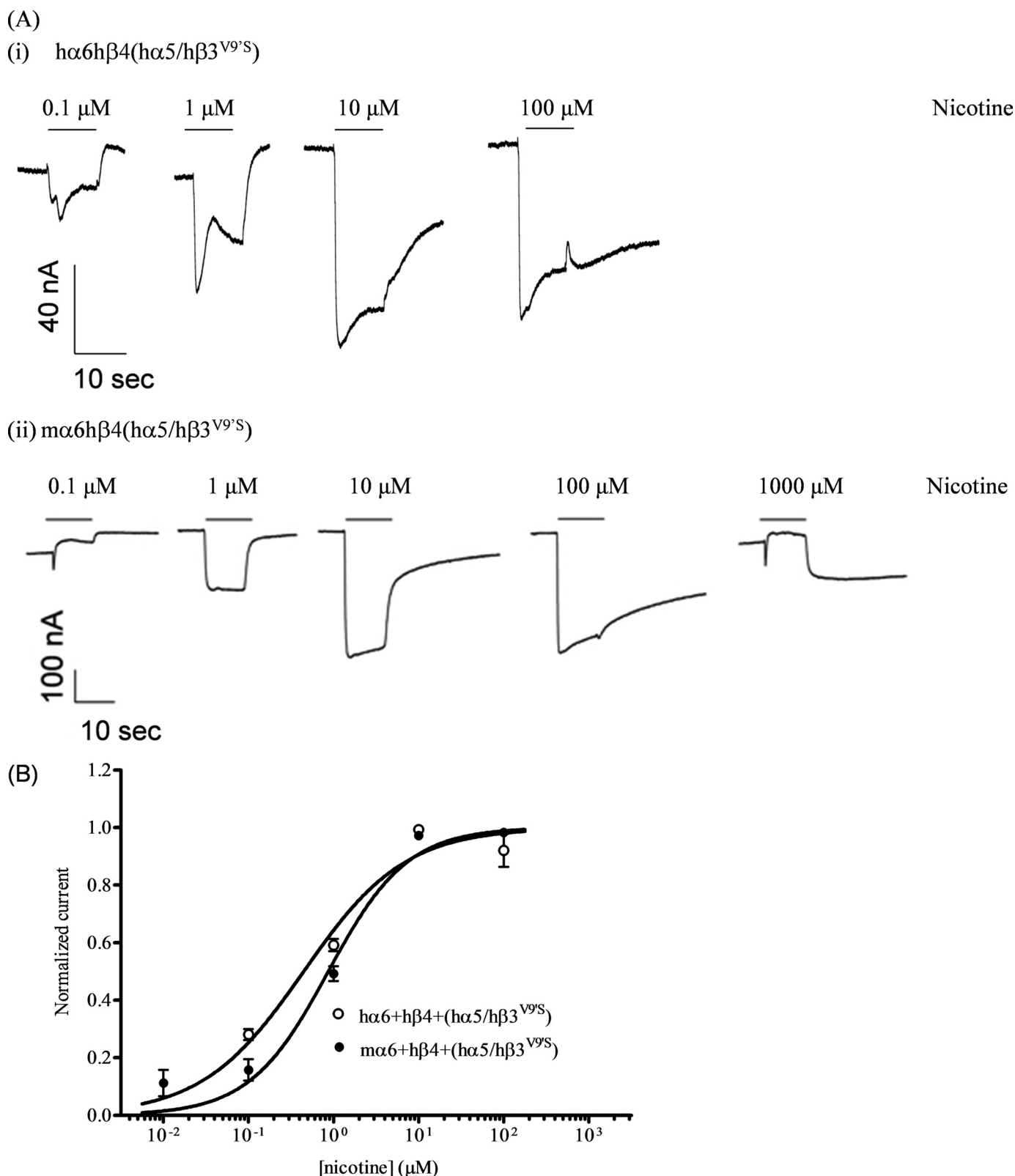


FIGURE 2. **Functional properties of $\alpha 6\beta 4^*$ -nAChR.** *A*, representative traces are shown for inward currents in oocytes held at -70 mV, responding to application at the indicated concentrations of nicotine (shown with the duration of drug exposure as *black bars* above the traces), and expressing (i) nAChR $h\alpha 6$, $h\beta 4$, and $h\alpha 5/h\beta 3^{V97S}$ subunits or (ii) nAChR $m\alpha 6$, $h\beta 4$, and $h\alpha 5/h\beta 3^{V97S}$ 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 \pm S.E.; *abscissa*, ligand concentration in $\log \mu$ M) 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 ($h\alpha 6h\beta 4(h\alpha 5/h\beta 3^{V97S})$ -nAChR, \diamond ; $m\alpha 6h\beta 4(h\alpha 5/h\beta 3^{V97S})$ -nAChR, \blacklozenge). Much higher levels of evoked currents are evident for functional nAChR containing $h\alpha 5/h\beta 3^{V97S}$ subunits compared with receptors lacking or also containing altered forms of wild-type or chimeric $\alpha 5$ subunits, none of which gave substantial or consistent currents responses (not shown; $p < 0.0001$). See Table 2 for parameters.

Modulation of $\alpha 6^*$ -Nicotinic Receptor Function: $\alpha 5$ Subunits

TABLE 2

Parameters for ligand action at nAChR containing $\alpha 6$ nAChR subunits

Agonist potencies (micromolar EC_{50} values with 95% confidence intervals), Hill coefficients ($n_H \pm SE$), mean \pm 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 $\alpha 6\beta 2$ - or $\alpha 6\beta 4$ -nAChR subtype alone (\uparrow or \downarrow) or in the presence of wild-type $\alpha 5$ subunits (\blacktriangle or \blacktriangledown), $\alpha 5^{V91S}$ subunits (\triangle or \triangledown), or ($\alpha 5/\beta 3$) subunits ($\hat{\uparrow}$ or $\hat{\downarrow}$). Note that no or very rare and then small responses to nicotine were seen for the following subunit combinations ($n = 4$ – 9 each): $\alpha 6 + h\beta 2$ alone or with $\alpha 5$, $\alpha 5^{V91S}$, $\alpha 5/h\beta 3$, or $\alpha 5/h\beta 3^{V91S}$; or $\alpha 6 + h\beta 4$ alone or with $\alpha 5$, $\alpha 5^{V91S}$, or $\alpha 5/h\beta 3$; $\alpha 6 + h\beta 2$ alone or with $\alpha 5$, $\alpha 5^{V91S}$, $\alpha 5/h\beta 3$, or $\alpha 5/h\beta 3^{V91S}$; $\alpha 6 + h\beta 4$ alone or with $\alpha 5$, $\alpha 5^{V91S}$, or $\alpha 5/h\beta 3$; and $\alpha 6(N143D+M145V) + h\beta 2$ alone or with $\alpha 5$, $\alpha 5^{V91S}$, or $\alpha 5/h\beta 3$. 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 $I_{max} \pm SE$ (nA)	I_{max} Conc. (μM)
$\alpha 6 + h\beta 4 + \alpha 5/h\beta 3^{V91S}$	6	0.45 (0.28-0.72) $\uparrow\blacktriangle\hat{\uparrow}$	0.73 \pm 0.11	4	225 \pm 31	10 $\uparrow\blacktriangle\hat{\uparrow}$
$\alpha 6 + h\beta 4 + \alpha 5/h\beta 3^{V91S}$	3	0.87 (0.67-1.1) $\uparrow\blacktriangle\hat{\uparrow}$	0.94 \pm 0.11	4	154 \pm 81 $\uparrow\blacktriangle\hat{\uparrow}$	10 $\uparrow\blacktriangle\hat{\uparrow}$
$\alpha 6/\alpha 3 + h\beta 2$	6	13 (5.6 - 28)	1.2 \pm 0.43	3	48 \pm 12	100
$\alpha 6/\alpha 3 + h\beta 2 + \alpha 5$	9	-	-	9	20 \pm 6	10
$\alpha 6/\alpha 3 + h\beta 2 + \alpha 5^{V91S}$	6	1.1 (0.6 - 1.2) $\uparrow\blacktriangle$	1.0 \pm 0.2	4	252 \pm 99 $\uparrow\blacktriangle$	10 $\uparrow\blacktriangle$
$\alpha 6/\alpha 3 + h\beta 2 + \alpha 5/h\beta 3$	3	-	-	3	25 \pm 8	10
$\alpha 6/\alpha 3 + h\beta 2 + \alpha 5/h\beta 3^{V91S}$	6	0.03 (0.02 - 0.07) $\uparrow\blacktriangle\hat{\uparrow}$	0.49 \pm 0.08	4	256 \pm 16 $\uparrow\blacktriangle$	10 $\uparrow\blacktriangle\hat{\uparrow}$
$\alpha 6/\alpha 3 + h\beta 4$	3	25 (20-30)	1.73 \pm 0.15	5	224 \pm 28	1000
$\alpha 6/\alpha 3 + h\beta 4 + \alpha 5$	3	9.1 (5.3-16) \uparrow	0.91 \pm 0.22	3	45 \pm 22	100 \uparrow
$\alpha 6/\alpha 3 + h\beta 4 + \alpha 5^{V91S}$	6	2.9 (2.5-3.2) $\uparrow\blacktriangle$	1.5 \pm 0.09	6	649 \pm 244	100 \uparrow
$\alpha 6/\alpha 3 + h\beta 4 + \alpha 5/h\beta 3$	3	6.1 (4.2-8.8) \uparrow	1.4 \pm 0.32	3	1969 \pm 151 $\uparrow\blacktriangle\hat{\uparrow}$	100 \uparrow
$\alpha 6/\alpha 3 + h\beta 4 + \alpha 5/h\beta 3^{V91S}$	4	0.03 (0.02-0.07) $\uparrow\blacktriangle\hat{\uparrow}$	0.49 \pm 0.08	4	2988 \pm 717 $\uparrow\blacktriangle\hat{\uparrow}$	10 $\uparrow\blacktriangle\hat{\uparrow}$
$\alpha 6(N143D+M145V) + h\beta 2 + \alpha 5/h\beta 3^{V91S}$	3	0.06 (0.02-0.15) $\uparrow\blacktriangle\hat{\uparrow}$	0.7 \pm 0.2	4	132 \pm 77 $\uparrow\blacktriangle\hat{\uparrow}$	10 $\uparrow\blacktriangle\hat{\uparrow}$

$\alpha 5/h\beta 3^{V91S}$ subunits incorporate into at least some complexes containing $\alpha 6$ and $h\beta 4$ 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 $\alpha 5/h\beta 3$ subunits into $\alpha 6h\beta 4^*$ -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 $\alpha 5/h\beta 3^{V91S}$ 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 $\alpha 6^*$ -nAChR are functional in mice, and but lacking such documentation of native, all-human $\alpha 6^*$ -nAChR function, we had previously extended our work to mouse nAChR subunits and found that coexpression of $h\beta 3$ subunits with $\alpha 6$ and $h\beta 4$ nAChR subunits form hybrid (*i.e.* composed of subunits from two different species), functional $\alpha 6h\beta 4h\beta 3$ -nAChR (18). This and the findings just above that functional nAChR $\alpha 6h\beta 4(\alpha 5/h\beta 3^{V91S})$ could be formed led us to explore effects of the incorporation of $\alpha 5$ or $\alpha 5/h\beta 3$ subunits on function of the hybrid $\alpha 6h\beta 4^*$ -nAChR. Oocytes expressing nAChR $\alpha 6$ and $h\beta 4$ alone or with wild-type $\alpha 5$ subunits from co-injected cRNAs failed to respond to nicotine

(Table 2). Substitution for wild-type $\alpha 5$ subunits with $\alpha 5/h\beta 3^{V91S}$ subunits, but not with $\alpha 5^{V91S}$ or $\alpha 5/h\beta 3$ 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 $\alpha 6h\beta 4(\alpha 5/h\beta 3^{V91S})$ -nAChR (Table 2). These findings suggest that mutant $\alpha 5/h\beta 3^{V91S}$ subunits also serve as gain-of-function partners for $\alpha 6h\beta 4^*$ -nAChR.

Human nAChR $\alpha 5$, $\alpha 5^{V91S}$, $\alpha 5/\beta 3$, or $\alpha 5/\beta 3^{V91S}$ Subunits Do Not Produce Functional Receptors When Coexpressed with $\alpha 6$ or $\alpha 6$ Plus $h\beta 2$ Subunits—Coexpression of nAChR wild-type $\alpha 6$ or $\alpha 6$ plus $h\beta 2$ subunits did not yield oocytes responding to nicotine (data not shown). Additional expression of wild-type $\alpha 5$ or $\alpha 5/h\beta 3$ subunits does not alter this circumstance, nor does additional expression of mutant $\alpha 5^{V91S}$ or $\alpha 5/h\beta 3^{V91S}$ subunits (Table 2). These results indicate that although the nAChR mutant $\alpha 5/h\beta 3^{V91S}$ subunits exert gain-of-function effects on $\alpha 6h\beta 4^*$ - or $\alpha 6h\beta 4^*$ -nAChR, they are unable to activate the function of $\alpha 6h\beta 2^*$ - or $\alpha 6h\beta 2^*$ -nAChR expressed in oocytes.

Studies of Human nAChR $\alpha 5$ Subunit Incorporation into Chimeric ($\alpha 6/h\alpha 3$)*-nAChR

Coexpression of $\alpha 5^{V91S}$ or $\alpha 5/h\beta 3^{V91S}$ Subunits with Chimeric $\alpha 6/h\alpha 3$ and $h\beta 2$ nAChR Subunits Produce Functional

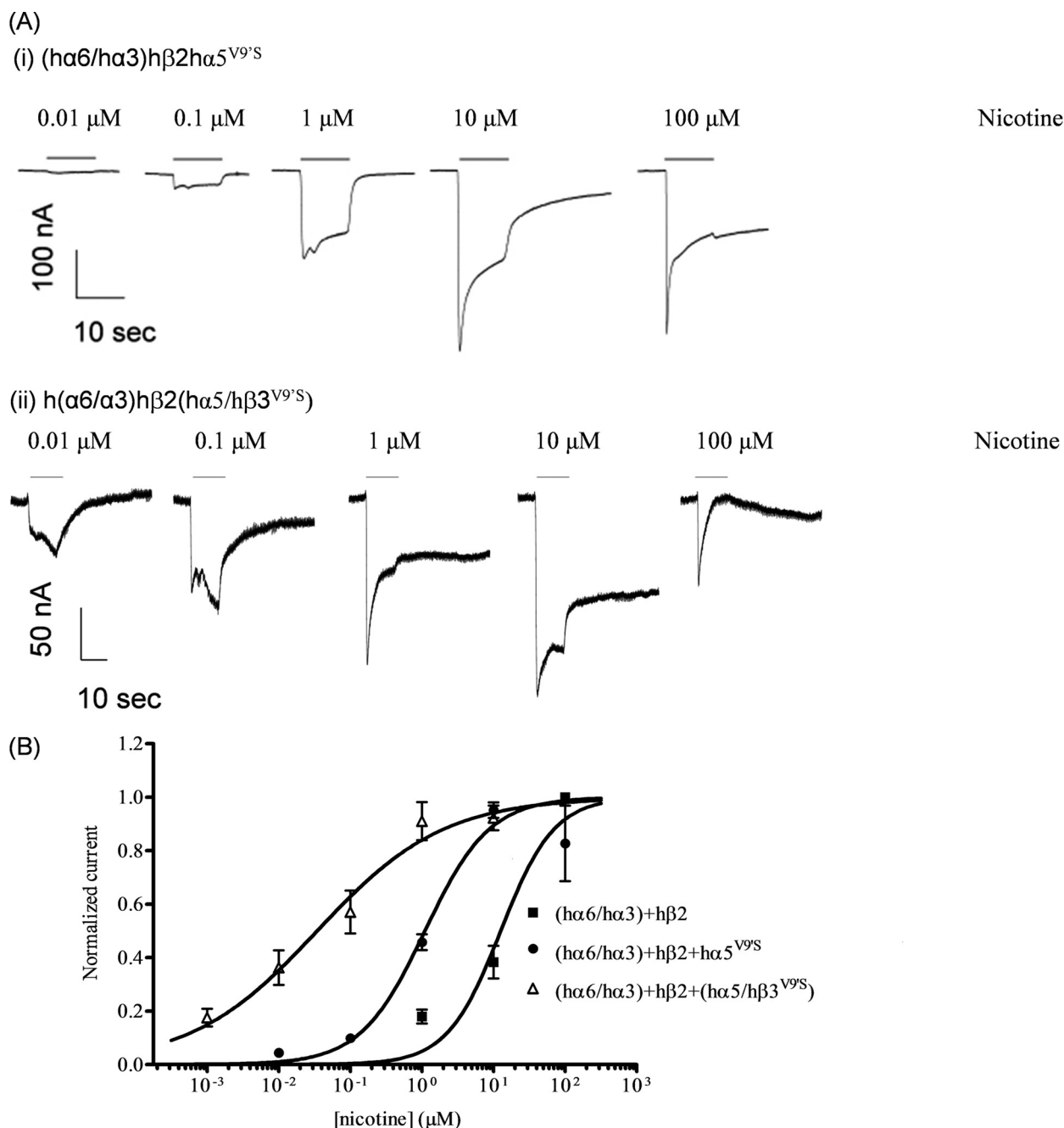


FIGURE 3. **Functional properties of $(\text{h}\alpha 6/\text{h}\alpha 3)\text{h}\beta 2^*$ -nAChR.** A, representative traces are shown for inward currents in oocytes held at -70 mV, responding to application at the indicated concentrations of nicotine (shown with the duration of drug exposure as *black bars* above the traces), and expressing nAChR $\text{h}\alpha 6/\text{h}\alpha 3$, $\text{h}\beta 2$, and either (i) $\text{h}\alpha 5^{\text{V9/S}}$ or (ii) $\text{h}\alpha 5/\text{h}\beta 3^{\text{V9/S}}$ 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 \pm S.E.; abscissa, ligand concentration in $\log \mu\text{M}$) for responses to nicotine as indicated for oocytes expressing nAChR $\text{h}\alpha 6/\text{h}\alpha 3$ and $\text{h}\beta 2$ subunits alone (■), with $\text{h}\alpha 5^{\text{V9/S}}$ subunits (●), or with $\text{h}\alpha 5/\text{h}\beta 3^{\text{V9/S}}$ 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 $\text{h}\alpha 5^{\text{V9/S}}$ or $\text{h}\alpha 5/\text{h}\beta 3^{\text{V9/S}}$ subunits ($p < 0.0001$; ~ 12 - and 433-fold lower EC_{50} values, respectively). See Table 2 for parameters.

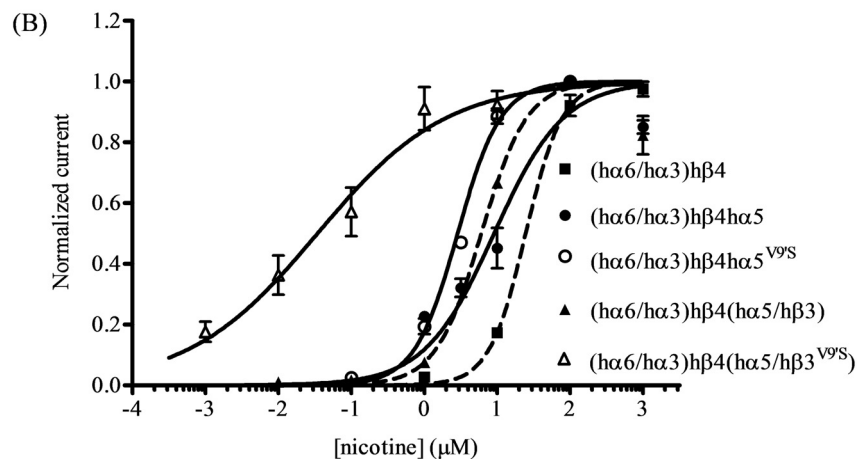
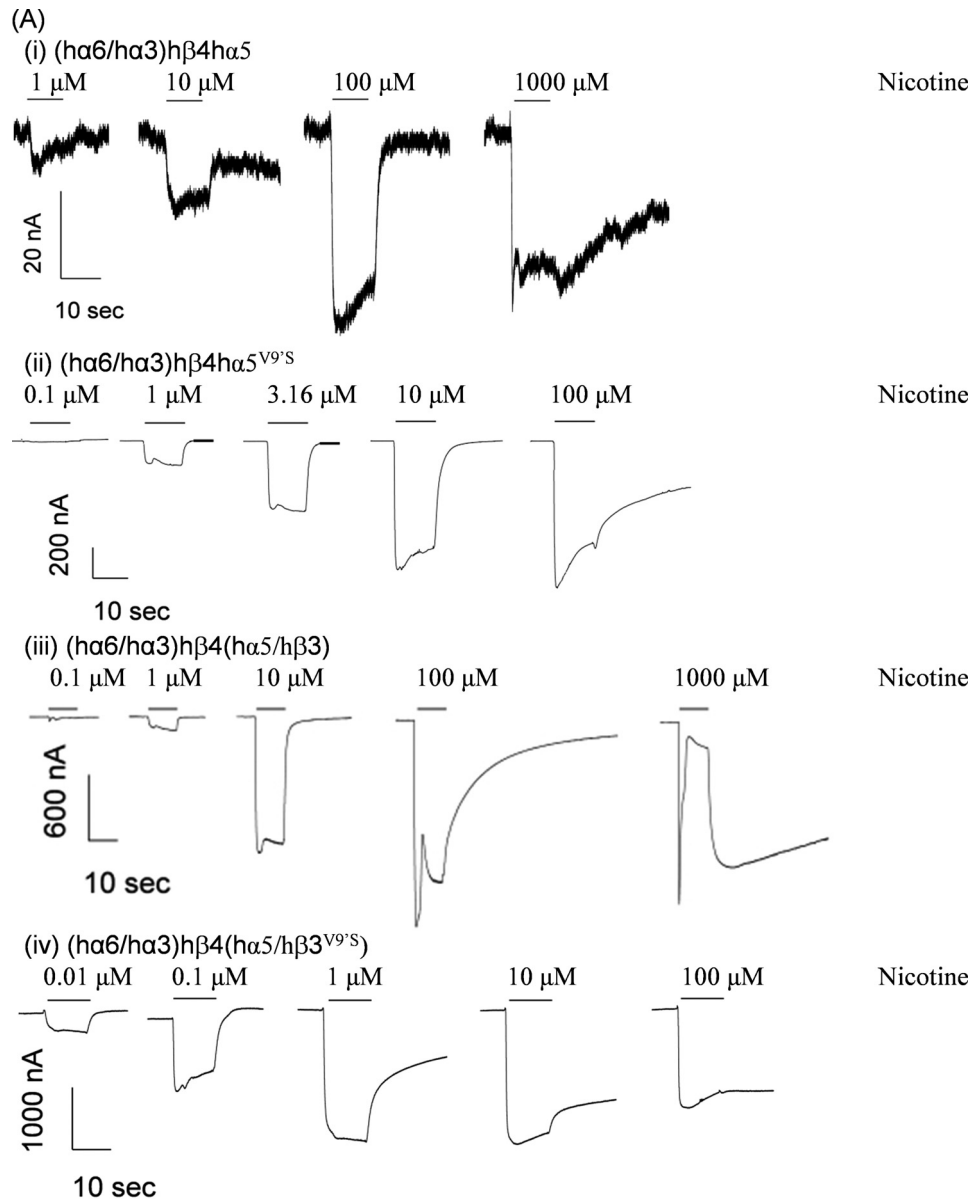
Receptors with Increased Sensitivity to Agonists—Oocytes expressing chimeric $\text{h}\alpha 6/\text{h}\alpha 3$ and $\text{h}\beta 2$ subunits occasionally displayed modest function that was reduced by coexpression with $\text{h}\alpha 5$ or $\text{h}\alpha 5/\text{h}\beta 3$ subunits (Table 2). By contrast, nearly all oocytes injected with $\text{h}\alpha 5^{\text{V9/S}}$ or $\text{h}\alpha 5/\text{h}\beta 3^{\text{V9/S}}$ subunit cRNAs along with nAChR $\text{h}\alpha 6/\text{h}\alpha 3$ and $\text{h}\beta 2$ subunit cRNAs yielded functional nicotinic responses (Fig. 3, Table 2). Oocyte-expressed $(\text{h}\alpha 6/\text{h}\alpha 3)\text{h}\beta 2(\text{h}\alpha 5/\text{h}\beta 3^{\text{V9/S}})$ -nAChR had

responses to nicotine at lower concentrations ($0.03 \mu\text{M}$ EC_{50}) than seen for receptors containing $\text{h}\alpha 5/\text{h}\beta 3$ subunits or no $\alpha 5$ subunit ($13 \mu\text{M}$ EC_{50}), and there also was higher sensitivity to nicotine for $(\text{h}\alpha 6/\text{h}\alpha 3)\text{h}\beta 2\text{h}\alpha 5^{\text{V9/S}}$ -nAChR ($1.1 \mu\text{M}$ EC_{50} ; Table 2). The findings for oocytes expressing $\text{h}\alpha 6/\text{h}\alpha 3$ and $\text{h}\beta 2$ subunits alone or with $\text{h}\alpha 5$ subunits are consistent with earlier observations by Kuryatov *et al.* (10). Interestingly, Hill coefficients for agonist responses of $(\text{h}\alpha 6/$

Modulation of $\alpha 6^*$ -Nicotinic Receptor Function: $\alpha 5$ Subunits

$h\alpha 3h\beta 2(h\alpha 5/h\beta 3^{V9/S})$ -nAChR are shallow ($n_H = 0.49$) and smaller than those for other functional $(h\alpha 6/h\alpha 3)\beta 2^*$ -nAChR, perhaps suggesting heterogeneity or negative coop-

erativity in the complexes formed. Moreover, agonist EC_{50} values are ~ 37 -fold lower for $(h\alpha 6/h\alpha 3)h\beta 2(h\alpha 5/h\beta 3^{V9/S})$ -nAChR than for $(h\alpha 6/h\alpha 3)h\beta 2(h\alpha 5^{V9/S})$ -nAChR.



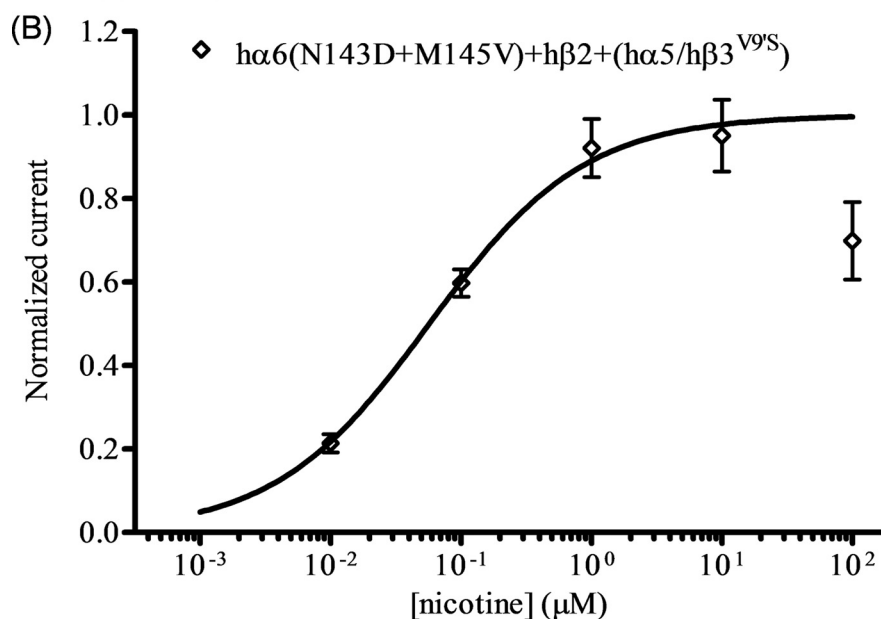
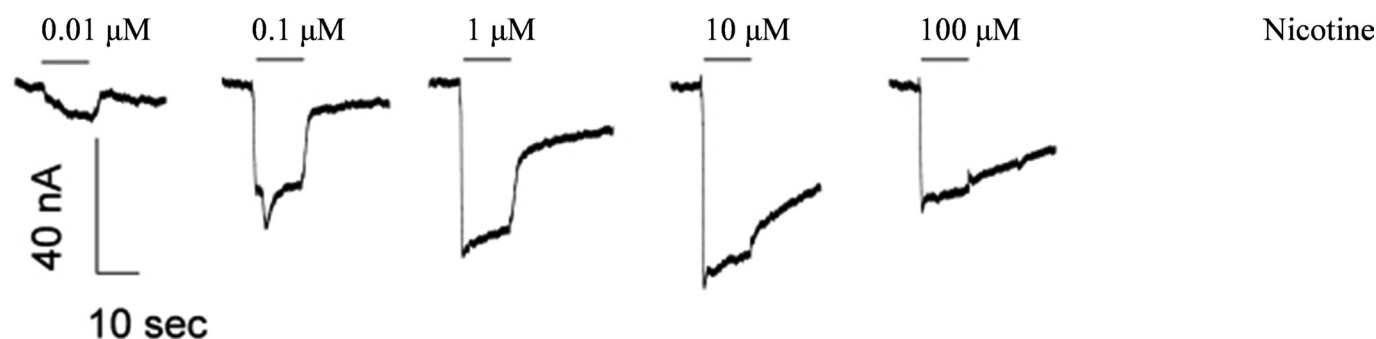
(A) $\alpha 6(N143D+M145V)h\beta 4(\alpha 5/h\beta 3^{V9/S})$


FIGURE 5. **Functional properties of $\alpha 6(N143D+M145V)h\beta 4^*$ -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 $\alpha 6(N143D+M145V)$, $h\beta 4$, and $(\alpha 5/h\beta 3^{V9/S})$ 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 \pm 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 $\alpha 5$, $\alpha 5^{V9/S}$, $\alpha 5/h\beta 3$, or $\alpha 5/h\beta 3^{V9/S}$ Subunits with Chimeric $\alpha 6/\alpha 3$ and $h\beta 4$ nAChR Subunits Produces Functional Receptors with Increased Sensitivity for Agonists—Nearly all oocytes injected with $\alpha 5$, $\alpha 5^{V9/S}$, $\alpha 5/h\beta 3$, or $\alpha 5/h\beta 3^{V9/S}$ subunit cRNAs along with nAChR ($\alpha 6/h\alpha 3$) and $h\beta 4$ subunit cRNAs yielded functional nicotinic responses (Table 2, Fig. 4). Oocytes expressing nAChR $\alpha 6/h\alpha 3$ and $h\beta 4$ subunits along with $\alpha 5$ subunits generally had 5-fold lower levels of function (although higher agonist sensitivity; $9.1 \mu M$ nicotine EC_{50} values) than oocytes expressing

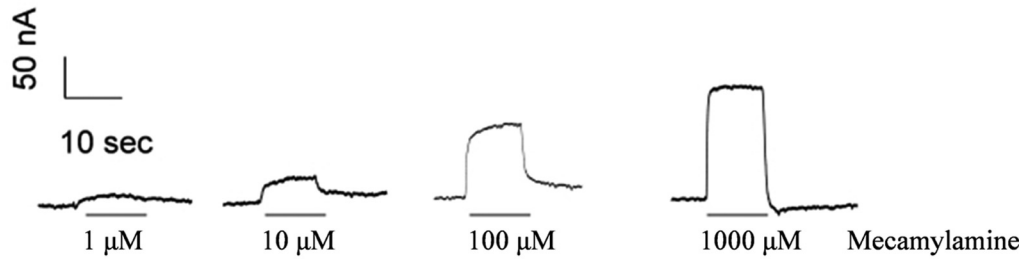
nAChR $\alpha 6/h\alpha 3$ and $h\beta 4$ subunits alone ($25 \mu M$ nicotine EC_{50} ; Table 2). However, oocytes expressing nAChR $\alpha 6/h\alpha 3$ and $h\beta 4$ subunits along with $\alpha 5/h\beta 3$ subunits had ~ 9 -fold higher levels of function (also higher agonist sensitivity; $6.1 \mu M$ nicotine EC_{50} values) than oocytes expressing nAChR $\alpha 6/h\alpha 3$ and $h\beta 4$ subunits alone. By contrast, both levels of functional receptor expression (3–13-fold higher than for $(\alpha 6/h\alpha 3)h\beta 4$ -nAChR) and agonist sensitivity (2.9 and $0.03 \mu M$ nicotine EC_{50} values, respectively; Fig. 4 and Table 2) are increased when $\alpha 6/h\alpha 3$ and $h\beta 4$ subunits are

FIGURE 4. **Functional properties of $(\alpha 6/h\alpha 3)h\beta 4^*$ -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 $\alpha 6/h\alpha 3$, $h\beta 4$ and $\alpha 5$ (i), $\alpha 5^{V9/S}$ (ii), $\alpha 5/h\beta 3$ (iii), or $\alpha 5/h\beta 3^{V9/S}$ (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 \pm S.E.; *abscissa*, ligand concentration in log μM) for responses to nicotine as indicated for oocytes expressing nAChR $\alpha 6/h\alpha 3$ and $h\beta 4$ subunits alone (\blacksquare), with wild-type $\alpha 5$ subunits (\blacklozenge), $\alpha 5^{V9/S}$ subunits (\circ), $\alpha 5/h\beta 3$ subunits (\blacktriangle), or $\alpha 5/h\beta 3^{V9/S}$ subunits (\triangle). 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 $\alpha 5$, $\alpha 5^{V9/S}$, $\alpha 5/h\beta 3$, or $\alpha 5/h\beta 3^{V9/S}$ subunits ($p < 0.0001$; ~ 3 -, ~ 9 -, ~ 3 -, and 833-fold, respectively). See Table 2 for parameters.

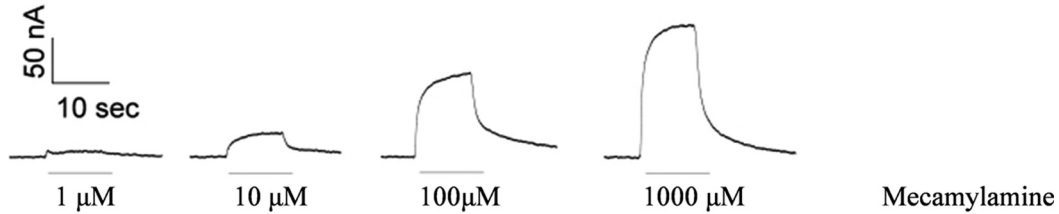
Modulation of $\alpha 6^*$ -Nicotinic Receptor Function: $\alpha 5$ Subunits

(A)

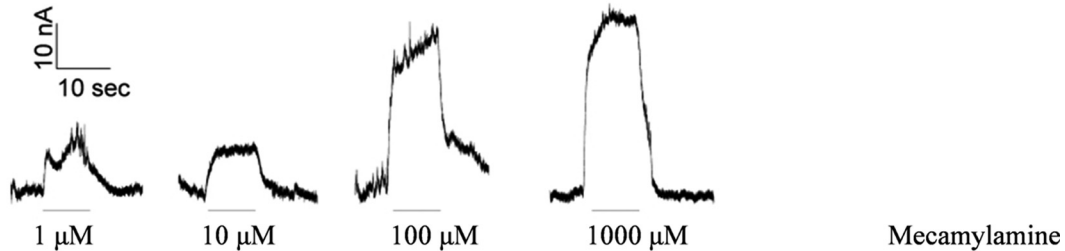
(i) $h\alpha 6h\beta 4(h\alpha 5/h\beta 3^{V9^*S})$ -nAChR



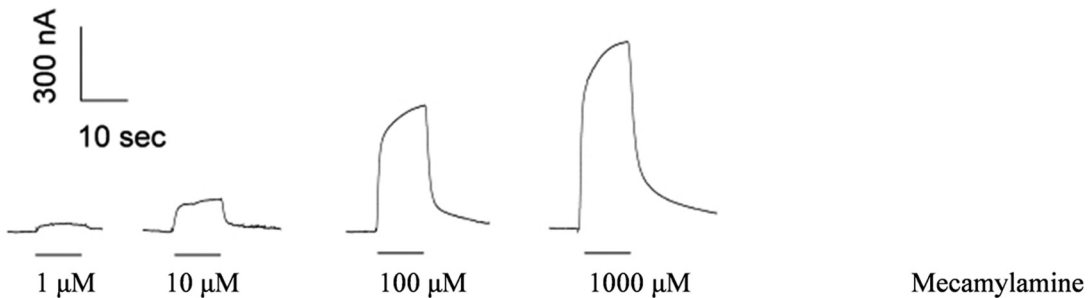
(ii) $m\alpha 6h\beta 4(h\alpha 5/h\beta 3^{V9^*S})$ -nAChR



(iii) $h(\alpha 6/\alpha 3)h\beta 2h(\alpha 5/\beta 3^{V9^*S})$ -nAChR



(iv) $(h\alpha 6/h\alpha 3)h\beta 4(h\alpha 5/h\beta 3^{V9^*S})$ -nAChR



(B)

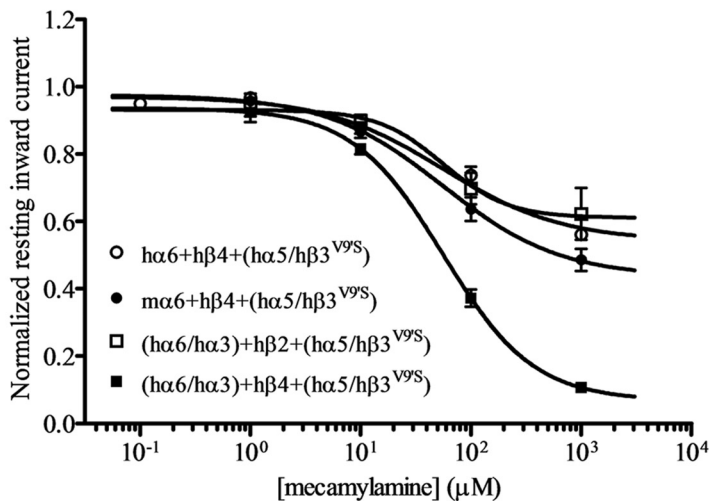


TABLE 3
Parameters for antagonist action at $\alpha 6^*$ -nAChR containing $\alpha 5/h\beta 3^{V91S}$ nAChR subunits

Inhibitor potencies (micromolar IC_{50} values with 95% confidence intervals), Hill coefficients ($n_H \pm$ S.E.), mean \pm S.E. peak responses (I_{max} in nanoamps), and concentrations where I_{max} is achieved (μM) are provided for mecamlamine 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 mecamlamine were seen for the following subunit combinations ($n = 9$ each): $\alpha 6 + h\beta 2$ with $\alpha 5^{V91S}$ or $\alpha 5/h\beta 3^{V91S}$; $\alpha 6 + h\beta 4$ with $\alpha 5^{V91S}$; $\alpha 6 + h\beta 2$ with $\alpha 5^{V91S}$ or $\alpha 5/h\beta 3^{V91S}$; $\alpha 6 + h\beta 4$ with $\alpha 5^{V91S}$; $\alpha 6/h\alpha 3 + h\beta 2$ with $\alpha 5^{V91S}$; and $\alpha 6/h\alpha 3 + h\beta 4$ with $\alpha 5^{V91S}$.

nAChR subunit combinations	Mecamlamine					
	n	Potency		$n_H \pm$ S.E.	Peak response	
		IC_{50} (95% CI)			Mean $I_{max} \pm$ S.E.	I_{max} concentration
		μM		nA	μM	
$\alpha 6 + h\beta 4 + \alpha 5/h\beta 3^{V91S}$	3	135 (23–779)	–0.65 \pm 0.23	3	150 \pm 21	1000
$\alpha 6 + h\beta 4 + \alpha 5/h\beta 3^{V91S}$	4	50 (20–125)	–0.84 \pm 0.29	4	125 \pm 35	1000
$\alpha 6/h\alpha 3 + h\beta 2 + \alpha 5/h\beta 3^{V91S}$	3	30 (4.6–196)	–0.86 \pm 0.45	3	67 \pm 22	1000
$\alpha 6/h\alpha 3 + h\beta 4 + \alpha 5/h\beta 3^{V91S}$	3	56 (40–78)	–1.1 \pm 0.16	3	695 \pm 95	1000

expressed with $\alpha 5^{V91S}$ or $\alpha 5/h\beta 3^{V91S}$ subunits. The findings for oocytes expressing $\alpha 6/h\alpha 3$ and $h\beta 4$ subunits alone or with $\alpha 5$ subunits are consistent with earlier observations by Kuryatov *et al.* (10). Once again, Hill coefficients for agonist responses of $(\alpha 6/h\alpha 3)h\beta 4^*$ -nAChR were lower ($n_H = 0.49$) in the presence of coexpressed $\alpha 5/h\beta 3^{V91S}$ subunits, even in the presence of $\alpha 5^{V91S}$ subunits, and smaller than those for other functional $(\alpha 6/h\alpha 3)\beta 2^*$ -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 $(\alpha 6/h\alpha 3)h\beta 4(\alpha 5/h\beta 3^{V91S})$ -nAChR than for $(\alpha 6/h\alpha 3)h\beta 4(\alpha 5^{V91S})$ -nAChR.

Coexpression of $\alpha 5/h\beta 3^{V91S}$ Subunits with $\alpha 6(N143D + M145V)$ and $h\beta 2$ nAChR Subunits Produces Functional Receptors—Earlier, we had shown (18) that mutations in the N-terminal domain of the nAChR $\alpha 6$ subunit enable nAChR $h\beta 3^{V91S}$ subunits to exert a gain-of-function effect at $\alpha 6(N143D + M145V)h\beta 2^*$ -nAChR (*i.e.* $\alpha 6(N143D + M145V)h\beta 2h\beta 3^{V91S}$ -nAChR are functional). This finding led us to explore effects of incorporation of $\alpha 5$ or $\alpha 5/h\beta 3$ subunits into $\alpha 6(N143D + M145V)h\beta 2^*$ -nAChR.

Oocytes injected with $\alpha 5$, $\alpha 5^{V91S}$, or $\alpha 5/h\beta 3$ subunit cRNAs along with nAChR $\alpha 6(N143D + M145V)$ and $h\beta 2$ subunit cRNAs did not yield functional nicotinic responses (Table 2). However, oocytes injected with $\alpha 5/h\beta 3^{V91S}$ subunit cRNAs along with nAChR $\alpha 6(N143D + M145V)$ and $h\beta 2$ 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 \pm S.E.) for 10 μM nicotine.

Exposure to Mecamlamine Reveals Spontaneous Channel Opening of nAChR Containing $\alpha 5/h\beta 3^{V91S}$ Subunits—Specificity of nicotine effects were routinely assessed based on the ability of the noncompetitive nAChR antagonist, mecamlamine, to block agonist-induced inward currents, but in doing so, we observed reversal of currents at very high concentrations of mecamlamine in the presence of nicotine. Effects of

mecamlamine alone were absent when assessed using oocytes expressing any combination of wild-type nAChR subunits (data not shown). However, exposure to mecamlamine alone produced concentration-dependent, reversible, outward currents in oocytes expressing $\alpha 6$ and $h\beta 4$ subunits in combination with $\alpha 5/h\beta 3^{V91S}$ subunits but not with $\alpha 5^{V91S}$ subunits (Fig. 6, Table 3). These results suggest that there is spontaneous opening of $\alpha 6^*$ -nAChR containing mutant $\alpha 5/h\beta 3^{V91S}$ subunits but not with $\alpha 5^{V91S}$ subunits, and that mecamlamine mediates the open channel block with an IC_{50} value of 135 μM at these spontaneously opening $\alpha 6h\beta 4(\alpha 5/h\beta 3^{V91S})$ -nAChR (Fig. 6, Table 3). Amplitudes of the mecamlamine-induced outward currents are about 66% of the amplitudes of agonist-induced inward currents, suggesting that a substantial percentage ($\sim 40\%$; 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 $\alpha 6h\beta 4^*$ -nAChR containing mutant $\alpha 5/h\beta 3^{V91S}$ subunits are spontaneously open at any time.

As was the case for oocytes expressing $\alpha 6$, $h\beta 4$, and mutant $\alpha 5/h\beta 3^{V91S}$ subunits or expressing $\alpha 6$, $h\beta 4$, and mutant $\alpha 5/h\beta 3^{V91S}$ subunits, oocytes co-injected with cRNAs for $\alpha 6$, $h\beta 4$, and $\alpha 5/h\beta 3^{V91S}$ subunits gave outward current responses to mecamlamine 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 mecamlamine. Estimates are that $\sim 40\%$ of these receptors are spontaneously open at any one time.

Similarly, oocytes expressing $(\alpha 6/h\alpha 3)h\beta 2(\alpha 5/h\beta 3^{V91S})$ - or $(\alpha 6/h\alpha 3)h\beta 4(\alpha 5/h\beta 3^{V91S})$ -nAChR gave outward current responses to mecamlamine in a concentration-dependent, reversible manner (Fig. 6, Table 3). This inhibition of spontaneously opening $(\alpha 6/h\alpha 3)h\beta 2(\alpha 5/h\beta 3^{V91S})$ - and $(\alpha 6/h\alpha 3)h\beta 4(\alpha 5/h\beta 3^{V91S})$ -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 $\alpha 6^*$ -nAChR containing $\alpha 5/h\beta 3^{V91S}$ subunits. A, representative traces are shown for outward currents in oocytes held at -70 mV, responding to application at the indicated concentrations of mecamlamine (shown with the duration of drug exposure as black bars above the traces), and expressing nAChR containing $\alpha 6$ and $h\beta 4$ (i), $\alpha 6$ and $h\beta 4$ (ii), $\alpha 6/h\alpha 3$ and $h\beta 2$ (iii), or $\alpha 6/h\alpha 3$ and $h\beta 4$ subunits (iv), all along with $\alpha 5/h\beta 3^{V91S}$ 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 \pm S.E.; abscissa, ligand concentration in log μM) for outward current responses to mecamlamine as indicated, for $\alpha 6h\beta 4(\alpha 5/h\beta 3^{V91S})$ - (○), $\alpha 6h\beta 4(\alpha 5/h\beta 3^{V91S})$ - (●), $(\alpha 6/h\alpha 3)h\beta 2(\alpha 5/h\beta 3^{V91S})$ - (□), or $(\alpha 6/h\alpha 3)h\beta 4(\alpha 5/h\beta 3^{V91S})$ -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 $\alpha 5$ subunit messages in the rodent brain, but left largely uninvestigated is how $\alpha 5$ subunits might incorporate as accessory partners into nAChR subtypes, specifically into $\alpha 6^*$ -nAChR. To understand how $\alpha 5$ subunits might incorporate into $\alpha 6^*$ -nAChR, we exploited the reporter or gain-of-function mutant strategy (20) to reveal whether $\alpha 5$ subunits or their variants integrate into $\alpha 6^*$ -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 $\beta 3$ subunits into $\alpha 6^*$ -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 $\beta 3$ (or other) subunit, (ii) incorporation of nAChR h $\beta 3$ subunits into $\alpha 6$ h $\beta 4^*$ -nAChR leads to formation of functional nAChR, and (iii) mutations in the E1 N-terminal domain of the nAChR $\alpha 6$ subunit are essential for successful assembly and formation of functional $\alpha 6$ (N143D+M145V)h $\beta 2$ h $\beta 3^{V9/S}$ -nAChR.

The principal observations of the current study are as follows. Coexpression in *Xenopus* oocytes of nAChR $\alpha 6$ subunits, h $\beta 4$ subunits, and wild-type $\alpha 5$ 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 $\alpha 6$ plus h $\beta 4$ subunits. No function is seen in the absence or presence of wild-type $\alpha 5$ subunits in oocytes expressing $\alpha 6$ and h $\beta 2$ subunits. Moreover, no function is seen for hybrid (a mixture of subunits from different species) $\alpha 6$ h $\beta 2^*$ - or $\alpha 6$ h $\beta 4^*$ -nAChR when those subunits are coexpressed with $\alpha 5^{V9/S}$ subunits or when $\alpha 6$ and either $\beta 2$ or $\beta 4$ subunits are coexpressed with chimeric $\alpha 5$ /h $\beta 3$ subunits. By contrast, there is very clear evidence for incorporation of chimeric, mutant $\alpha 5$ /h $\beta 3^{V9/S}$ subunits containing a valine 273 to serine mutation into $\alpha 6$ $\beta 4^*$ -nAChR, because oocytes expressing the indicated subunits have large, agonist-induced, peak inward current responses. Moreover, agonist sensitivity of $\alpha 6$ $\beta 4^*$ -nAChR containing mutant $\alpha 5$ /h $\beta 3^{V9/S}$ subunits is higher than for the equivalent receptors expressed in the absence of mutant subunits. Chimeric $\alpha 6$ /h $\alpha 3$ subunits (10) form functional nAChR when combined with either nAChR h $\beta 2$ or h $\beta 4$ subunits. Further incorporation of $\alpha 5$ or $\alpha 5$ /h $\beta 3$ subunits diminishes functional responsiveness, but levels of functional expression and sensitivity to nicotine are increased when $\alpha 6$ /h $\alpha 3$ and either h $\beta 2$ or h $\beta 4$ subunits are coexpressed with $\alpha 5^{V9/S}$ or $\alpha 5$ /h $\beta 3^{V9/S}$ subunits. Interestingly, (h $\alpha 6$ /h $\alpha 3$) $\beta 2^*$ - or (h $\alpha 6$ /h $\alpha 3$)h $\beta 4^*$ -nAChR complexes in oocytes coexpressing $\alpha 5$ /h $\beta 3^{V9/S}$ subunits have higher agonist sensitivity than in the presence of coexpressed $\alpha 5^{V9/S}$ subunits. When there is expression of functional receptors containing $\alpha 5$ /h $\beta 3^{V9/S}$ subunits, but not $\alpha 5^{V9/S}$ subunits,

mecamylamine exposure produces outward currents, indicative of spontaneous channel opening in the presence of $\alpha 5$ /h $\beta 3^{V9/S}$ subunits, and that mecamylamine blocks spontaneously open channels and agonist-induced open channels. Finally, there is expression of functional receptors when $\alpha 5$ /h $\beta 3^{V9/S}$ subunits (but not $\alpha 5$, $\alpha 5^{V9/S}$, or $\alpha 5$ /h $\beta 3$ subunits) are coexpressed with $\alpha 6$ (N143D+M145V) and h $\beta 2$ subunits.

We derive several conclusions from these findings. One is that nAChR $\alpha 5$ subunits or their variants can incorporate into heterologously expressed $\alpha 6$ $\beta 4^*$ -nAChR, where they exert dominant-negative (wild-type $\alpha 5$ or $\alpha 5$ /h $\beta 3$ subunits) or gain-of-function (mutant $\alpha 5$ /h $\beta 3^{V9/S}$ subunits) effects. The inability of wild-type, mutant, chimeric, or mutant chimeric $\alpha 5$ subunit variants to influence function of $\alpha 6$ $\beta 2^*$ -nAChR confounds the ability to make inferences about assembly of the indicated subunits. However, we also conclude from studies with $\alpha 6$ (N143D+M145V) subunits that the N-terminal domain of $\alpha 6$ subunits influences the ability to see a mutant $\alpha 5$ /h $\beta 3^{V9/S}$ subunit-mediated, gain-of-function effect at $\alpha 6$ $\beta 2^*$ -nAChR, consistent with earlier findings that h $\beta 3^{V9/S}$ subunits have a gain-of-function effect when expressed as part of $\alpha 6$ (N143D+M145V)h $\beta 2$ h $\beta 3^{V9/S}$ -nAChR. Effects of mecamylamine are interpreted as demonstrating that $\alpha 6^*$ -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 $\alpha 5$ /h $\beta 3^{V9/S}$ mutant chimeric subunit are largely similar to those found for $\beta 3^{V9/S}$ 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 $\alpha 6$ / $\alpha 3$ subunits provide a more reliable functional background when expressed as binary complexes with $\beta 2$ or $\beta 4$ subunits and underscore the interpretation that wild-type $\alpha 5$ or chimeric $\alpha 5$ /h $\beta 3$ subunits have a dominant-negative effect and that $\alpha 5^{V9/S}$, in addition to $\alpha 5$ /h $\beta 3^{V9/S}$ 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 $\alpha 5^{V9/S}$ or $\alpha 5$ /h $\beta 3^{V9/S}$ subunits, thus providing additional evidence that fine features of subunits influence their effects on $\alpha 6^*$ -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 $\alpha 6$ /h $\alpha 3$ subunits.

The current findings are in general agreement with and extend upon recent experimental successes demonstrating that nAChR $\alpha 6$ subunits, long refractory to heterologous expression (27, 28) in functional nAChR assemblies with other subunits, indeed can

interact with $\beta 2$ or $\beta 4$ subunits to form functional receptors in oocytes. The current studies have overcome difficulties in expressing and characterizing $\alpha 6\alpha 5^*$ -nAChR, in part by demonstrating that (i) a dominant-negative effect of wild-type $\alpha 5$ subunits on $\alpha 6^*$ -nAChR can be counteracted using a gain-of-function strategy and (ii) mutations in the N-terminal domain of $\alpha 6$ subunit are essential for a gain-of-function effect at $\alpha 6\beta 2^*$ -nAChR.

Recently, it was observed (17, 18) that incorporation of the human nAChR $\beta 3$ subunit has a dominant-negative effect on the function of specific nAChR subtypes including $\alpha 6\beta 4^*$ - and $\alpha 6\beta 2^*$ -nAChR heterologously expressed in oocytes. The current findings extend upon these previous findings and reinforce the perspective that $\beta 3$ and $\alpha 5$ subunits are highly homologous and serve as auxiliary subunits in formation of functional nAChR. Contrary to the gain-of-function observed for $\beta 3^{V91S}$ subunits, $\alpha 5^{V91S}$ subunits did not exert any gain-of-function in combination with wild-type $\alpha 6$ and $\beta 2$ or $\beta 4$ subunits. However, we have extended the work to show that gain-of-function occurs for $\alpha 5/h\beta 3^{V91S}$ subunits. Moreover, wild-type $\alpha 5$ subunits show a dominant-negative effect, and $\alpha 5^{V91S}$ subunits do exert a gain-of-function effect when coexpressed with chimeric $\alpha 6/h\alpha 3$ and $\beta 2$ or $\beta 4$ subunits, further reinforcing our interpretation.

Lack of or low level of function compromised our ability to definitively generate EC_{50} values for agonists at $\alpha 6\beta 2h\alpha 5^-$, $\alpha 6\beta 2h\alpha 5^-$, $\alpha 6\beta 2h\alpha 5^-$, $\alpha 6\beta 4h\alpha 5^-$, $\alpha 6\beta 4h\alpha 5^-$, $\alpha 6\beta 4h\alpha 5^-$, $(\alpha 6/h\alpha 3)h\beta 2h\alpha 5^-$ -nAChR, etc., but an EC_{50} value of $9.1 \mu M$ nicotine for $(\alpha 6/h\alpha 3)h\beta 4h\alpha 5^-$ -nAChR is from our studies. Nicotine EC_{50} values generated from the current study (0.03 – $2 \mu M$) for receptors containing $(\alpha 5/h\beta 3^{V91S})$ 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 $\alpha 6$ or even $\alpha 6(N143D+M145V)$ subunits plus $\beta 2$ or $\beta 4$ subunits in the presence of $\alpha 5^{V91S}$ subunits when previous studies (17, 18) demonstrated function of those combinations in the presence of $h\beta 3^{V91S}$ subunits, even though $\beta 3$ and $\alpha 5$ subunits are highly, but not perfectly, homologous. The fact that function is expressed in the presence of chimeric $\alpha 5/h\beta 3^{V91S}$ subunits suggests that features in the $\beta 3$ subunit as opposed to in the $\alpha 5$ 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 $\alpha 5/h\beta 3^{V91S}$ subunits. Studies of $(\alpha 6/h\alpha 3)h\beta 2^*$ - or $(\alpha 6/h\alpha 3)h\beta 4^*$ -nAChR showing more evident dominant-negative effects of $\alpha 5$ subunits and gain-of-function effects of $\alpha 5^{V91S}$ subunits also support these suggestions. This is because domains C-terminal to E1 from the $\alpha 3$ subunit might be more compatible than those from the $\alpha 6$ subunit with regard to productive assembly of functional nAChR in combination with $\alpha 5$ subunits, overcoming possible incompatibilities in those domains between $\alpha 6$ and $\alpha 5$ subunits. Moreover, the potentiation of chimeric $\alpha 5/\beta 3$ subunits rather than the dominant-negative influence of $\alpha 5$ subunits on function of $(\alpha 6/h\alpha 3)\beta 4^*$ -nAChR also is consistent with heretofore unrec-

ognized roles of $\alpha 5$ or $\beta 3$ domains C-terminal to E1 (and other than an M2 9' mutation itself) in formation of functional $\alpha 6^*$ -nAChR and agonist sensitivity.

Because there are indications that naturally expressed, rodent $\alpha 6\beta 2^*$ -nAChR seem to be functional, perhaps when combined with $\alpha 5$ subunits, and given the difficulties in heterologously expressing human $\alpha 6\beta 2^*$ -nAChR, we wondered whether there simply might be species-specific differences in the ability to heterologously express $\alpha 6\beta 2\alpha 5^*$ -nAChR, and so we chose to see if $\alpha 6$ and $h\beta 2$ nAChR subunits could be functionally expressed when combined with $\alpha 5$ subunits or their variants. However, we realized very similar outcomes in our studies of all human, all mouse, or hybrid $\alpha 6\beta 2\alpha 5^*$ -nAChR, even when $\alpha 5$ or $(\alpha 5/\beta 3)$ subunits had gain-of-function mutations and despite success of the $(\alpha 5/\beta 3)$ subunit gain-of-function strategy when applied to $\alpha 6\beta 4^*$ -nAChR. It is possible that a key assembly partner, such as $\alpha 4$ or $\alpha 3$ subunits, are required for formation of naturally expressed, functional $\alpha 6\beta 2\alpha 5^*$ -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 $\alpha 6\beta 2\alpha 5^*$ -nAChR.

Some of our other site-directed mutagenesis work implicated $\alpha 6$ residues 143 and 145 in the ability of $\beta 3$ subunits to affect $\alpha 6\beta 2^*$ -nAChR function (18). The $\alpha 6(N143D+M145V)$ mutations change the indicated residues to those that are in the $\alpha 6$ subunit and permits mutated $\alpha 6$ subunits to show function when coexpressed with $h\beta 2$ and either $h\beta 3^{V91S}$ or $\alpha 5/h\beta 3^{V91S}$ subunits when wild-type $\alpha 6$ 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 $\alpha 6(+)/(-)\beta 2$ or $\alpha 6(+)/(-)\beta 4$ subunit interfaces are where there would be productive and functionally relevant agonist binding. That is, they would be at the two $\alpha 6:\beta 2$ interfaces in a complex with the arrangement, counterclockwise when viewed from the extracellular space, of $\beta 3$ (or $\alpha 5$): $\alpha 6:\beta 2:\alpha 6:\beta 2$. However, $\alpha 6$ 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 $\alpha 6$ subunit interface thought not to engage in agonist binding influences formation of functional receptors with $\beta 2$ subunits and with subunits that contain either $\beta 3$ or $\alpha 5$ E1 domains coupled to $\beta 3$ subunit domains C-terminal to E1 (in addition to the reporter V293S mutation). This suggests that interactions between the $\alpha 6$ subunit (–) face with the (+) face from either $\beta 2$ subunits or from either $\beta 3$ or $\alpha 5$ subunits are important for functional $\alpha 6^*$ -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 $\alpha 6$ and $\beta 2$, $\beta 3$ or $\alpha 5$ subunits, and the ability of ligands to bind at that interface. Second, because the $\alpha 6$ subunit (–) face mutations affect functional nAChR forma-

Modulation of $\alpha 6^*$ -Nicotinic Receptor Function: $\alpha 5$ Subunits

tion with $\beta 2$ subunits and either $h\beta 3^{V9/S}$ or $h\alpha 5/h\beta 3^{V9/S}$ subunits, it does not seem that the $\alpha 5(+)/(-)\alpha 6$ or $\beta 3(+)/(-)\alpha 6$ E1 interfaces are critical, but rather that the $\beta 2(+)/(-)\alpha 6$ E1 interface is important. The analogous interface in $\alpha 4\beta 2$ -nAChR ($\beta 2(+)/(-)\alpha 4$) is an allosteric site of action of Zn^{2+} (30). $\beta 2:\alpha 6$ E1 subunit interfaces in the vicinity of loop E could play roles in subunit assembly leading to closure of functional, cell-surface $\alpha 6\beta 2\beta 3^{V9/S}$ -nAChR, although distal involvement of $\beta 2:\alpha 6$ subunit interfaces in ligand binding or transduction of ligand binding to channel gating cannot be discounted. The lack of function of $h\alpha 6(N143D+M145V)h\beta 2h\alpha 5^{V9/S}$ -nAChR again is consistent with limited compatibility between $\alpha 6$ and $\alpha 5$ subunit domains C-terminal to E1.

In conclusion, our results provide evidence that wild-type or mutant $\alpha 5$ subunits can incorporate into and either suppress/abolish or enhance, respectively, function of $\alpha 6\beta 4^*$ - (although low levels of function make this conclusion more tenuous), $(h\alpha 6/h\alpha 3)h\beta 2^*$ -, or $(h\alpha 6/h\alpha 3)h\beta 4^*$ -nAChR, but that effects are more evident in the presence of chimeric $\alpha 5/\beta 3$ subunits. These studies using the oocyte expression system have been useful in illuminating the $\alpha 6^*$ -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 $\alpha 6^*$ -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 $\alpha 6^*$ -nAChR to levels compatible with drug screening, perhaps using high throughput methods, could facilitate the development of new drugs selective for $\alpha 6^*$ -nAChR. This is of increasing importance given the potentially important roles for $\alpha 6^*$ -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)
 α 5 Subunits and/or Variants Modulate Function of α 6*-nAChR**

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