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Bhagirathi Dash

Minoti Bhakta

Yongchang Chang

Barrow Neurological Institute, yongchang.chang@dignityhealth.org

Ronald J. Lukas

Barrow Neurological Institute, ronald.lukas@dignityhealth.org

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Identification of N-terminal Extracellular Domain Determinants in Nicotinic Acetylcholine Receptor (nAChR) $\alpha 6$ Subunits That Influence Effects of Wild-type or Mutant $\beta 3$ Subunits on Function of $\alpha 6\beta 2^*$ - or $\alpha 6\beta 4^*$ -nAChR^{*S}

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Bhagirathi Dash¹, Minoti Bhakta, Yongchang Chang, and Ronald J. Lukas²

From the Division of Neurobiology, Barrow Neurological Institute, Phoenix, Arizona 85013

Background: $\alpha 6\beta 3^*$ -Nicotinic receptors (nAChRs) are physiologically important but difficult to express heterologously.

Results: Influences of $\beta 3$ subunits on $\alpha 6\beta 3^*$ -nAChR function are impacted by $\alpha 6$ subunit N-terminal domain loop E residues.

Conclusion: There are unexpected roles for the complementary face of the nAChR $\alpha 6$ subunit in receptor function.

Significance: Novel medicinals acting at new sites on $\alpha 6\beta 3^*$ -nAChRs could be useful antidepressants and/or smoking cessation aids.

Despite the apparent function of naturally expressed mammalian $\alpha 6^*$ -nicotinic acetylcholine receptors ($\alpha 6^*$ -nAChR; where * indicates the known or possible presence of additional subunits), their functional and heterologous expression has been difficult. Here, we report that coexpression with wild-type $\beta 3$ subunits abolishes the small amount of function typically seen for all-human or all-mouse $\alpha 6\beta 4^*$ -nAChR expressed in *Xenopus* oocytes. However, levels of function and agonist potencies are markedly increased, and there is atropine-sensitive blockade of spontaneous channel opening upon coexpression of $\alpha 6$ and $\beta 4$ subunits with mutant $\beta 3$ subunits harboring valine-to-serine mutations at 9'- or 13'-positions. There is no function when $\alpha 6$ and $\beta 2$ subunits are expressed alone or in the presence of wild-type or mutant $\beta 3$ subunits. Interestingly, hybrid nAChR containing mouse $\alpha 6$ and human (h) $\beta 4$ subunits have function potentiated rather than suppressed by coexpression with wild-type h $\beta 3$ subunits and potentiated further upon coexpression with h $\beta 3^{V9'S}$ subunits. Studies using nAChR chimeric mouse/human $\alpha 6$ subunits indicated that residues involved in effects seen with hybrid nAChR are located in the $\alpha 6$ subunit N-terminal domain. More specifically, nAChR h $\alpha 6$ subunit residues Asn-143 and Met-145 are important for dominant-negative effects of nAChR h $\beta 3$ subunits on h $\alpha 6\beta 4$ -nAChR function. Asn-143 and additional residues in the N-terminal domain of nAChR h $\alpha 6$ subunits are involved in the gain-of-function

effects of nAChR h $\beta 3^{V9'S}$ subunits on $\alpha 6\beta 2^*$ -nAChR function. These studies illuminate the structural bases for effects of $\beta 3$ subunits on $\alpha 6^*$ -nAChR function and suggest that unique subunit interfaces involving the complementary rather than the primary face of $\alpha 6$ subunits are involved.

There are at least six different nicotinic acetylcholine receptor (nAChR) α subunits ($\alpha 2$ – $\alpha 7$) and three nAChR β subunits ($\beta 2$ – $\beta 4$) expressed in the mammalian central nervous system (1). nAChR $\alpha 7$ subunits are thought principally to form homopentameric receptors when expressed in heterologous expression systems, whereas the other indicated subunits are thought to assemble into heteropentameric structures containing various combinations of α and β subunits. nAChR $\beta 3$ and $\alpha 5$ subunits are considered to be “wild cards,” as they do not form functional receptors when expressed alone or in binary complexes with any other single subunit. However, they seem capable of integrating as “accessory” subunits into complexes containing at least one other α and one other β subunit. $\alpha 6^*$ -nAChR (where the * indicates the known or possible presence of additional subunits in the complex) are expressed in the mammalian brain, predominantly in dopaminergic midbrain regions implicated in pleasure, reward, and drug (including nicotine) dependence; they modulate dopamine release and could be involved in schizophrenia and Parkinson disease (2–7). nAChR $\alpha 6$ and $\beta 3$ subunit messages share very similar expression patterns, and studies using knock-out animal and α -conotoxin sensitivity assessments suggest that $\beta 3$ subunit incorporation is important in the assembly and stability of mature $\alpha 6^*$ -nAChR, which also must have channel functions.

Mammalian $\alpha 6^*$ -nAChR are thought to naturally exist as combinations of $\alpha 6$ with $\beta 2$ alone or with addition of $\beta 3$ subunits and perhaps of $\alpha 6$ and $\beta 4$ subunits (1, 7). However, nAChR with these subunit compositions are not easily recreated in functional forms in artificial expression systems (8).

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[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental “Experimental Procedures,” Table S1, and Fig. S1.

¹ Present address: Dept. of Psychiatry and Neurobehavioral Sciences, School of Medicine, University of Virginia, Charlottesville, VA 22911.

² To whom correspondence should be addressed: Division of Neurobiology, Barrow Neurological Institute, 350 W. Thomas Rd., Phoenix, AZ 85013. Tel.: 602-406-3399; Fax: 602-406-4172; E-mail: rlukas@chw.edu.

³ The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine; h, human; m, mouse.

Coexpression of a human $\alpha 6/\alpha 4$ chimeric subunits with human $\beta 4$ subunits in *Xenopus* oocytes or HEK-293 human embryonic kidney cells results in functional receptors, although expression of wild-type $\alpha 6$ subunits with $\beta 4$ subunits did not produce ligand binding and functional $\alpha 6^*$ -nAChR (9). Chick or rat nAChR $\alpha 6$ subunits form functional channels when coexpressed with h $\beta 4$ subunits in *Xenopus* oocytes, but the $\alpha 6$ plus $\beta 2$ subunit combination fails to form functional channels (10). Chick $\alpha 6$ subunits also formed functional receptors when coexpressed with chick $\beta 2$ or $\beta 4$ subunits in BOSC 23 cells (11). More recently, Kuryatov *et al.* (8) reported functional expression of human $\alpha 6\beta 4$ -nAChR in *Xenopus* oocytes and formation of ligand-binding (nonfunctional) aggregates of $\alpha 6\beta 2$ -nAChR. Their study also suggested that $\beta 3$ subunits may facilitate $\alpha 6^*$ -nAChR trafficking to the cell membrane.

Recently (12), it was observed that coexpression with a large excess of human nAChR wild-type $\beta 3$ subunits has a dominant-negative effect on the function of human $\alpha 6^*$ -nAChR, whereas coexpression with a large excess of human mutant $\beta 3$ subunits (valine 273 to serine at position 9' in the putative second transmembrane domain; $\beta 3^{V273S} = \beta 3^{V9'S}$) potentiates the function of human $\alpha 6\beta 2^*$ - and $\alpha 6\beta 4^*$ -nAChR. This observation is surprising, because knock-out studies strongly suggest that the naturally expressed murine $\alpha 6^*$ -nAChR containing $\beta 2$ and $\beta 3$ subunits are functional and that $\alpha 6$ and $\beta 3$ subunits are needed to show sensitivity of these receptors to certain α -conotoxins (4–7).

To re-explore and expand on the prior findings, we further characterized human (h) or mouse (m) $\alpha 6\beta 3^*$ -nAChR heterologously expressed in *Xenopus* oocytes. Further study employing hybrid nAChR containing subunits from different species, and use of chimeric subunits having sequences for a given subunit from different species to guide finer site-directed mutagenesis studies, led us to find unexpectedly that amino acid residues in the N-terminal domain of $\alpha 6$ subunits influence sometimes the dominant-negative effects of wild-type $\beta 3$ subunits and are involved in gain-of-function effects of mutant $\beta 3^{V9'S}$ subunits on $\alpha 6^*$ -nAChR function. Hence, these results suggest that coassembly of $\beta 3$ with $\alpha 6$ and $\beta 2$ subunits to form functional nAChR is determined by the $\alpha 6$ subunit N-terminal extracellular region. These results also suggest that a novel interface between nAChR subunits exists and can influence subunit assembly and receptor function.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals for electrophysiology were obtained from Sigma. Fresh agonist (acetylcholine or nicotine) and antagonist (atropine or mecamylamine) stock solutions were made daily in Ringer's solution and diluted as needed.

Wild Type, Chimeric, or Point Mutation nAChR Subunits—cDNAs corresponding to human nAChR $\alpha 6$ (h $\alpha 6$), $\beta 2$ (h $\beta 2$), $\beta 3$ (h $\beta 3$), or $\beta 4$ (h $\beta 4$) subunits were excised from vectors containing them and subcloned into the oocyte expression vector pGEMHE. Similarly, cDNAs representing mouse nicotinic receptor $\alpha 6$ (m $\alpha 6$), $\beta 2$ (m $\beta 2$), $\beta 3$ (m $\beta 3$), or $\beta 4$ (m $\beta 4$) subunits (kind gifts from Dr. Jerry A. Stitzel, Department of Integrative Physiology, Institute for Behavioral Genetics, University of Colorado, Boulder) were subcloned into pGEMHE. Fully synthetic

nAChR h $\beta 2$ subunit with nucleotide sequences optimized for better heterologous expression (h $\beta 2_{opt}$ supplemental Fig. 1) was generated (GENEART, Burlingame, CA), subcloned into the pCI vector (Promega, San Luis Obispo, CA), and used in some studies, and although levels of function in complexes containing optimized $\beta 2$ subunits were generally slightly higher, the source of $\beta 2$ subunit did not materially affect outcomes.

Two chimeric nAChR subunits m $\alpha 6(1-350)/h\alpha 6(351-494)$ and m $\alpha 6(1-236)/h\alpha 6(237-494)$ were constructed as sketched in Fig. 1 and as described in detail in the supplemental material. Mutations in the m $\beta 3$ (V279S and V283S) or h $\beta 3$ (V273S and V277S) subunit transmembrane domain II 9'-(V9'S) or 13'-(V13'S)-position were introduced in 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 human nAChR $\alpha 6$ subunit (N91K, K94R, K114N, N143D, M145V, N91K/K94R, and N143D/M145V) were introduced using the QuikChange II site-directed mutagenesis kit. Primers used for mutagenesis are listed in supplemental Table S1. Construct integrity of all subunits was confirmed by sequencing.

In Vitro Transcription—All pGEMHE plasmids were linearized immediately downstream of the 3'-polyadenylation sequence. NheI was used to linearize m $\alpha 6$, m $\beta 2$, m $\beta 3$, m $\beta 3^{V9'S}$, m $\beta 3^{V13'S}$, m $\beta 4$, h $\alpha 6$, h $\beta 3$, h $\beta 3^{V9'S}$, h $\beta 3^{V13'S}$, h $\beta 4$, h $\alpha 6^{N91K}$, h $\alpha 6^{K94R}$, h $\alpha 6^{K114N}$, h $\alpha 6^{N143D}$, h $\alpha 6^{M145V}$, h $\alpha 6^{N91K+K94R}$, h $\alpha 6^{N143D+M145V}$, m $\alpha 6(1-350)/h\alpha 6(351-494)$ and m $\alpha 6(1-236)/h\alpha 6(237-494)$ subunit containing plasmids. SbfI was used to linearize h $\beta 2$ subunits. Capped mRNA was transcribed from linearized plasmids in a reaction mixture (25 μ l) containing 1 \times transcription buffer, 1.6 mM rNTPs (Promega, San Luis Obispo, CA), 0.5 mM 7m-CAP (New England Biolabs, Ipswich, MA), 1 μ l of RNasin Plus (New England Biolabs), and 1 μ l of T7 RNA polymerase (New England Biolabs) following standard protocols. Integrity and quality of the cRNA were 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, 5 HEPES, 50 mg/ml gentamycin, 50 units/ml penicillin, and 50 μ g/ml 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 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 μ m 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

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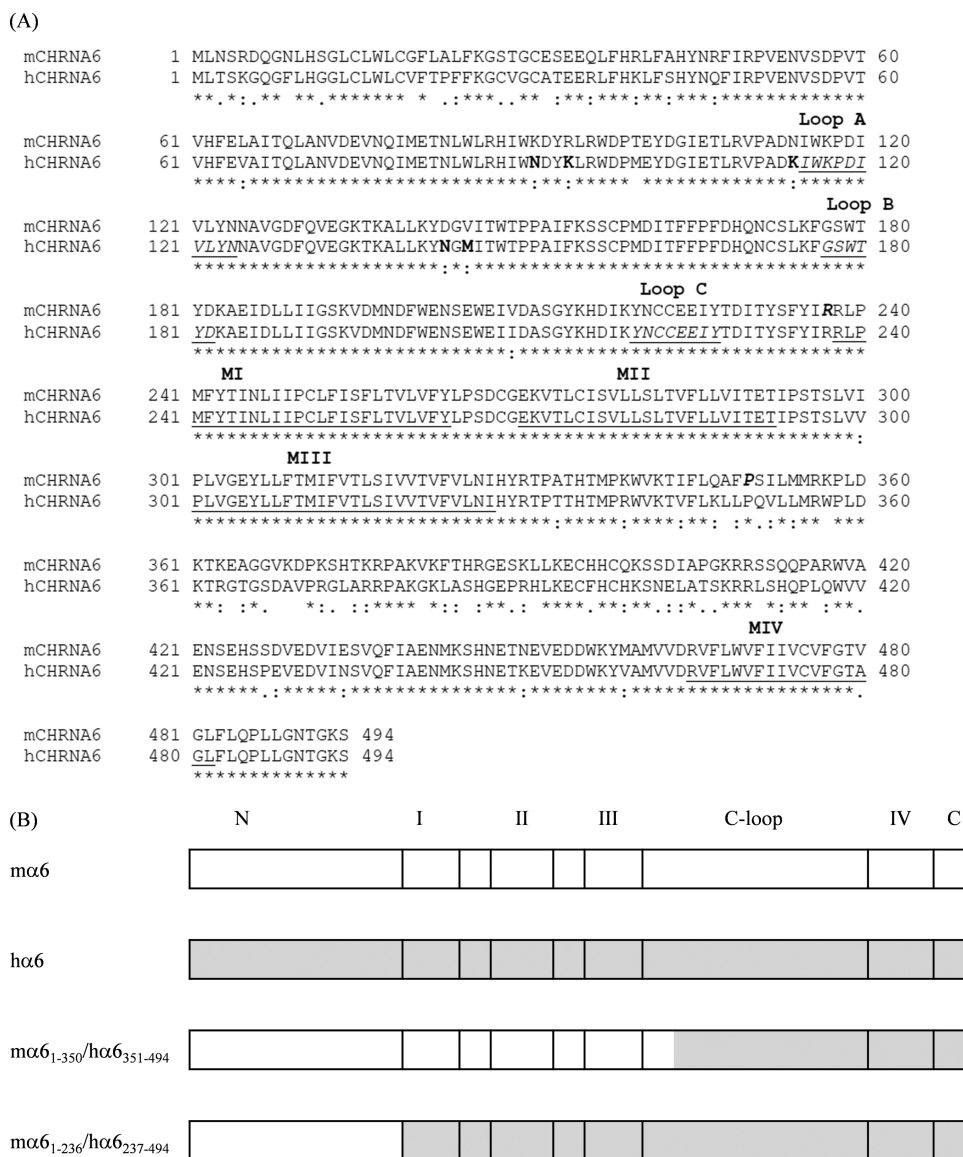


FIGURE 1. A, sequence alignment for mouse (*m*) or human (*h*) nAChR $\alpha 6$ subunits (GenBank™ NP_004189.1 (*Homo sapiens*) and NP_067344.2 (*Mus musculus*); single letter code, numbering begins at translation start methionine). Symbols below sequences indicate fully (*) or weakly (:) conserved residues, and the lack of a symbol indicates amino acid divergence, and **boldface type** in $h\alpha 6$ subunit indicates residues given prime attention in mutagenesis studies. Underlining in the $h\alpha 6$ subunit sequence indicates putative transmembrane domains. Underlined and italicized type in the $h\alpha 6$ subunit indicates putative domains involved in ligand binding (loops A–C), and **boldface and italicized type** in the $m\alpha 6$ subunit indicates junctions for chimeric subunits. B, schematic diagrams of wild-type, human, or mouse $\alpha 6$ subunits or chimeric subunits. Notations are: N = N-terminal domain; I, II, III, or IV = respective transmembrane domains; C-loop = cytoplasmic loop; C = C terminus.

nAChR in oocytes, about 4 ng of cRNA corresponding to each nAChR subunit was injected.

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, Sunnyvale, CA). 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 pClamp10.2. Electrodes contained 3 M KCl and had a resistance of 1–2 megohms. Drugs (agonists and antagonists) were prepared daily in

bath solution. Drug was applied using a ValveLink 8.2 perfusion system (Automate scientific, Berkeley, CA). 1 μ M atropine was always coapplied for acetylcholine (ACh)-based recordings to eliminate muscarinic AChR (mAChR) responses. All electrophysiological measurements were conducted or checked in at least two batches of oocytes.

Experimental Controls—Injection of cRNA corresponding to one subunit alone or pairwise combinations of $\beta 3$ or $\beta 3^{V9^5S}$ or $\beta 3^{V13^5S}$ subunits with either a α subunit or $\beta 2$ or $\beta 4$ or chimeric subunits (10–12 ng of total cRNA) did not result in the expression of functional nAChR. Current responses to 100 μ M nicotine were less than 5–20 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 as measures of functional nAChR expression and results pooled across experiments (mean \pm S.E. for data from at least three oocytes). Assessment of true I_{max} values for different nAChR subunit combinations was made based on 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). *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 (13, 14). We made no attempt to measure or control for subunit combination-specific effects, but whenever preliminary studies revealed possible differences in peak current amplitudes, the findings were further confirmed across different subunit combinations using the same batch of oocytes and the same time between cRNA injection and recording. Whenever we make statements about results comparing ligand potencies and efficacies across subunit combinations, the observations are clear and significant (one-way analyses of variance followed by Tukey's multiple comparison tests).

RESULTS

Wild-type or Mutant nAChR $\beta 3$ Subunits from Either Humans or Mice Incorporate into $\alpha 6\beta 4^*$ -nAChR

In initial studies (Fig. 2A), coexpression of nAChR wild-type $\alpha 6$ and $\beta 4$ subunits produced nicotinic responses in only about 3–5% of injected oocytes, and functional responses to 100 μM ACh when present were very modest (22 ± 3 nA; Table 1). Responses to nicotine were less reliable (Table 1). Coexpression with wild-type $\beta 3$ subunits suppressed the modest responses to 100 μM ACh (8.4 ± 1.0 nA; Fig. 2A). By contrast, coexpression with mutant $\beta 3^{V9'S}$ subunits significantly increased nicotinic responses to 100 μM ACh (290 ± 19 nA), and nearly every oocyte injected with nAChR $\alpha 6$, $\beta 4$, and mutant $\beta 3^{V9'S}$ subunits expressed functional nAChR (Fig. 2A and Table 1).

Although the small amplitudes of current in the few oocytes that yielded functional receptors when injected with cRNA encoding nAChR $\alpha 6$ and $\beta 4$ subunits plus wild-type $\beta 3$ subunits confounded detailed analyses, studies done comparing nicotine and ACh efficacies and apparent potencies done on the same day after injection of the same batch of oocytes indicated that these agonists were equally efficacious ($p > 0.05$) (Table 1) at $\alpha 6\beta 4\beta 3^{V9'S}$ -nAChR. Also, ACh and nicotine were equally potent at $\alpha 6\beta 4\beta 3^{V9'S}$ -nAChR, yielding EC_{50} values of 0.43 and 0.42 μM , respectively (Fig. 2B and Table 1). Our studies also demonstrated that there also was emergence of receptor function when oocytes were injected with cRNA for nAChR $\alpha 6$ and $\beta 4$ subunits along with $\beta 3^{V13'S}$ subunits (Fig. 2C and Table 1). Apparent potency and efficacy for nicotine did not differ across 9' and 13' $\beta 3$ subunit mutations (EC_{50} values of 0.42 and 0.30 μM , respectively). However, the EC_{50} value for ACh of 1.2 μM at $\alpha 6\beta 4\beta 3^{V13'S}$ -nAChR differs significantly ($p = 0.0011$) from that of 0.43 μM at $\alpha 6\beta 4\beta 3^{V9'S}$ -

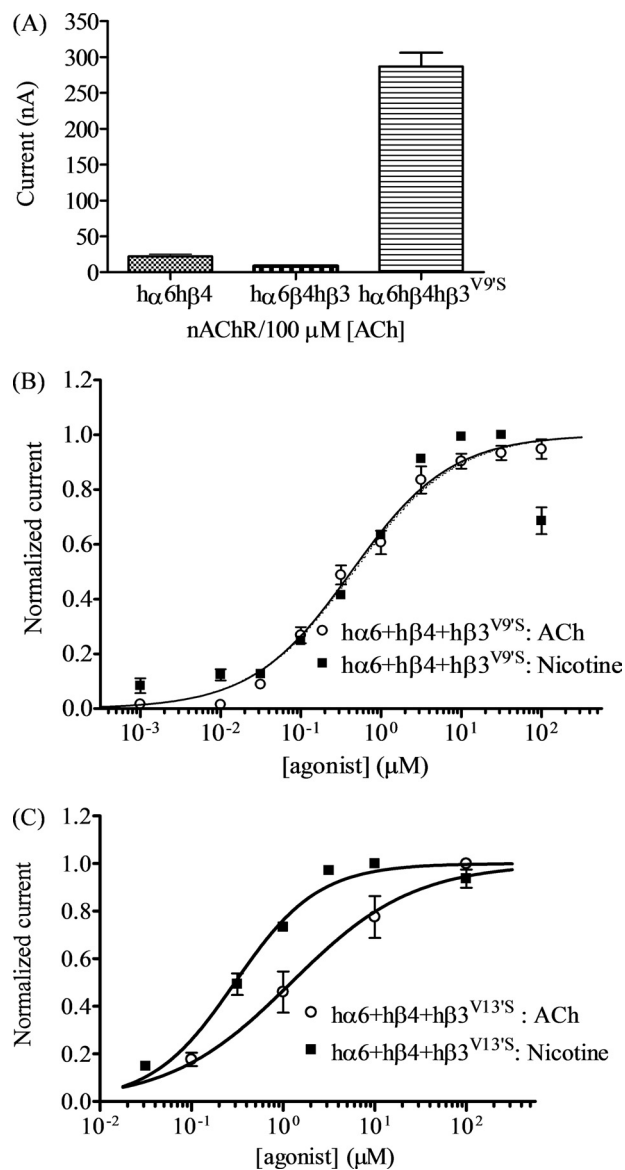


FIGURE 2. Functional properties of $\alpha 6\beta 4^*$ -nAChR. A, mean peak inward current amplitude (\pm S.E.; abscissa; nA) elicited by oocytes expressing the indicated human nAChR subunit combinations in response to application of 100 μM ACh. The level of nAChR function in oocytes expressing $\alpha 6$ and $\beta 4$ subunits is reduced by addition of wild-type $\beta 3$ subunits but increased in the presence of $\beta 3^{V9'S}$ subunits ($p < 0.05$). B and C, results 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 ACh (○) or nicotine (■) as indicated for oocytes expressing nAChR $\alpha 6$ and $\beta 4$ subunits with either $\beta 3^{V9'S}$ (B) or $\beta 3^{V13'S}$ (C) subunits. Concentration-response curves for ACh and nicotine are almost superimposable for oocytes expressing $\alpha 6$, $\beta 4$, and $\beta 3^{V9'S}$ subunits, but EC_{50} values for ACh and nicotine are different ($p < 0.0001$) for oocytes expressing $\alpha 6$, $\beta 4$, and $\beta 3^{V13'S}$ subunits (see Table 1 for parameters).

nAChR, and average ACh efficacy also was lower for the former set of receptors (Table 1).

Difficulties in expressing functional $\alpha 6^*$ -nAChR from human subunits gave us pause, and so we undertook studies of $\alpha 6^*$ -nAChR heterologously expressed from mouse subunits, because the literature strongly suggests that naturally expressed mouse $\alpha 6^*$ -nAChR are functional (2–7). Many oocytes injected with cRNA encoding nAChR wild-type $\alpha 6$

Effects of $\beta 3$ Subunits on $\alpha 6^*$ -Nicotinic Receptor Function

TABLE 1

Parameters for agonist action at nAChR containing human or mouse $\alpha 6$ subunits

Potencies (micromolar EC_{50} values with 95% confidence intervals), Hill coefficients ($n_H \pm$ S.E.), mean \pm S.E. efficacies (I_{max} in nA), and concentrations where maximal peak current amplitudes (I_{max}) are achieved (in μM) are provided for the indicated agonist (ACh or 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 the figures. \uparrow or \downarrow indicates a significant ($p < 0.05$) increase or decrease, respectively, in potency or efficacy of the indicated agonist at the indicated nAChR subtype relative to nAChR containing the same subunits but in the absence of the indicated $\beta 3$ subunit; \blacktriangle or \blacktriangledown indicates a significant increase or decrease, respectively, in indicated agonist potency or efficacy at the indicated nAChR subtype relative to nAChR containing the same subunits in the presence wild-type $\beta 3$ subunits, and Δ or ∇ indicates a significant increase or decrease, respectively, in potency or efficacy of the indicated agonist at the indicated nAChR containing $\beta 3^{V9^S}$ subunits relative to the same complex containing $\beta 3^{V13^S}$ subunits. Note that no responses or very rare and then small responses were seen for the following subunit combinations ($n = 9$ each) to ACh or nicotine: $\alpha 6 + \beta 2$ alone or with $\beta 3$ or $\beta 3^{V9^S}$ or $\beta 3^{V13^S}$; and $\alpha 6 + \beta 2$ alone or with $\beta 3$ or $\beta 3^{V9^S}$ or $\beta 3^{V13^S}$. – indicates that absent or inconsistent functional responses in two-electrode voltage clamp studies precluded determination of the parameter of interest.

Drug	nAChR subunit combinations	n	Potency			Peak response	
			EC_{50} (95% CI)	$n_H \pm$ S.E.	n	Mean $I_{max} \pm$ S.E.	I_{max} concentration
			μM			nA	μM
ACh	$\alpha 6 + \beta 4$	9	–	–	3	22 ± 3	100
	$\alpha 6 + \beta 4 + \beta 3$	9	–	–	9	–	–
	$\alpha 6 + \beta 4 + \beta 3^{V9^S}$	9	0.43 (0.34–0.54) $\uparrow \blacktriangle$	0.69 ± 0.04	11	$2100 \pm 220 \uparrow \blacktriangle$	$10 \uparrow \blacktriangle$
	$\alpha 6 + \beta 4 + \beta 3^{V13^S}$	3	1.2 (0.66–2.3) $\uparrow \blacktriangledown$	0.65 ± 0.10	3	$910 \pm 150 \uparrow \blacktriangle \nabla$	$10 \uparrow \blacktriangle$
	$\alpha 6 + \beta 4$	3	38 (25–58)	0.72 ± 0.09	3	65 ± 25	1000
	$\alpha 6 + \beta 4 + \beta 3$	9	–	–	9	–	–
	$\alpha 6 + \beta 4 + \beta 3^{V9^S}$	4	0.42 (0.25–0.71) $\uparrow \blacktriangle$	0.57 ± 0.08	3	$2100 \pm 190 \uparrow \blacktriangle$	$100 \uparrow \blacktriangle$
	$\alpha 6 + \beta 4 + \beta 3^{V13^S}$	4	0.30 (0.22–0.41) $\uparrow \blacktriangle$	0.71 ± 0.07	4	$2400 \pm 420 \uparrow \blacktriangle$	$100 \uparrow \blacktriangle$
Nicotine	$\alpha 6 + \beta 4$	9	–	–	9	–	–
	$\alpha 6 + \beta 4 + \beta 3$	9	–	–	9	–	–
	$\alpha 6 + \beta 4 + \beta 3^{V9^S}$	5	0.42 (0.26–0.68) $\uparrow \blacktriangle$	0.70 ± 0.19	8	$1900 \pm 340 \uparrow \blacktriangle$	$10 \uparrow \blacktriangle$
	$\alpha 6 + \beta 4 + \beta 3^{V13^S}$	4	0.30 (0.24–0.37) $\uparrow \blacktriangle$	0.96 ± 0.08	4	$1600 \pm 54 \uparrow \blacktriangle$	$10 \uparrow \blacktriangle$
	$\alpha 6 + \beta 4$	3	26 (18–38)	0.65 ± 0.13	5	27 ± 7	1000
	$\alpha 6 + \beta 4 + \beta 3$	9	–	–	9	–	–
	$\alpha 6 + \beta 4 + \beta 3^{V9^S}$	6	0.07 (0.04–0.10) $\uparrow \blacktriangle$	0.89 ± 0.14	7	$2500 \pm 250 \uparrow \blacktriangle$	$10 \uparrow \blacktriangle$
	$\alpha 6 + \beta 4 + \beta 3^{V13^S}$	4	0.26 (0.19–0.36) $\uparrow \blacktriangle \nabla$	0.79 ± 0.08	4	$3000 \pm 360 \uparrow \blacktriangle$	$10 \uparrow \blacktriangle$

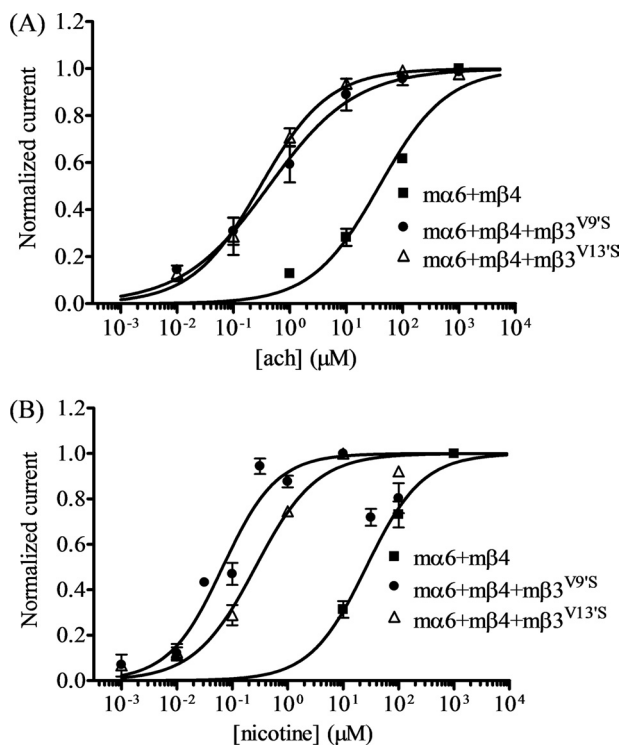


FIGURE 3. Functional properties of $\alpha 6\beta 4^*$ -nAChR. Results averaged across experiments were used to produce concentration-response curves (ordinate – mean normalized current \pm S.E.; abscissa – ligand concentration in log μM) for responses to ACh (A) or nicotine (B) as indicated for oocytes expressing nAChR $\alpha 6$ and $\beta 4$ subunits alone (\blacksquare) or with either $\beta 3^{V9^S}$ (\bullet) or $\beta 3^{V13^S}$ (Δ) subunits. Leftward shifts in agonist concentration-response curves are evident for functional nAChR containing $\beta 3^{V9^S}$ or $\beta 3^{V13^S}$ subunits ($p < 0.0001$; ~ 91 - and ~ 130 -fold, respectively, for ACh and ~ 370 - and 100 -fold, respectively for nicotine). See Table 1 for parameters.

and $\beta 4$ subunits yielded functional nicotinic responses (Fig. 3), but coinjection of wild-type $\beta 3$ subunits failed to yield oocytes that responded to nicotinic agonists (Table 1).

As with human subunits, oocytes expressing nAChR $\alpha 6$ and $\beta 4$ with $\beta 3^{V9^S}$ or $\beta 3^{V13^S}$ subunits yielded relatively large responses to nicotinic agonists (Fig. 3 and Table 1). Agonist concentration-response curves (Fig. 3) reveal that ACh and nicotine sensitivities are 100–400-fold higher for $\alpha 6\beta 4\beta 3^{V9^S}$ - or $\alpha 6\beta 4\beta 3^{V13^S}$ -nAChR than for $\alpha 6\beta 4$ -nAChR (Fig. 3).

These results indicated that both wild-type $\beta 3$ and mutant $\beta 3^{V9^S}$ or $\beta 3^{V13^S}$ subunits incorporate into at least some complexes containing $\alpha 6$ and $\beta 4$ subunits. Incorporation of $\beta 3$ subunits has a dominant-negative effect, reflected by lowering of levels 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 the nAChR $\beta 3^{V9^S}$ or $\beta 3^{V13^S}$ subunit produces a gain-of-function effect reflected by an increase in agonist sensitivity and in absolute levels of functional receptor expression.

There was no functional expression represented by nicotinic agonist-induced current responses in oocytes expressing nAChR $\alpha 6$ and $\beta 2$ subunits alone, in combination with wild-type $\beta 3$ subunits, or in combination with either mutant $\beta 3^{V9^S}$ or $\beta 3^{V13^S}$ subunits from either human or mouse (Table 1). This confounded the ability to interpret results, but they do indicate that coexpression with mutant $\beta 3^{V9^S}$ or $\beta 3^{V13^S}$ subunits does not have a gain-of-function effect on $\alpha 6\beta 2^*$ -nAChR from either species, contrary to effects on $\alpha 6\beta 4^*$ -nAChR.

Studies Using Hybrid nAChR Containing Subunits from Different Species Indicate Forms of These $\alpha 6^*$ -nAChR into Which nAChR Wild-type or Mutant $\beta 3$ Subunits Can Incorporate

For a given nAChR subunit ($\alpha 6$, $\beta 2$, $\beta 4$, and $\beta 3$) across species, amino acid residues are nearly perfectly matched in

TABLE 2

Parameters for drug action at hybrid $\alpha 6^*$ nAChR

Potencies (micromolar EC_{50} values with 95% confidence intervals for ACh or nicotine effects), Hill coefficients ($n_H \pm S.E.$), mean \pm S.E. peak response (I_{max} in nA), and concentrations where maximal peak current amplitudes (I_{max}) are achieved (in μM) are provided for the indicated agonist (ACh or 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 the figures. \uparrow or \downarrow indicates a significant ($p < 0.05$) increase or decrease, respectively, in potency or peak response elicited by the indicated agonist at the indicated nAChR subtype relative to nAChR containing the same subunits but in the absence of the indicated $\beta 3$ subunit. \blacktriangle or \blacktriangledown indicates a significant increase or decrease, respectively, in indicated agonist potency or peak response at the indicated nAChR subtype relative to nAChR containing the same subunits in the presence wild-type $\beta 3$ subunits. Note that no or very rare and then small responses were seen for the following subunit combinations ($n = 9$ each) to nicotine: $\alpha 6 + m\beta 2$ alone or with $m\beta 3$, $h\beta 3$, $h\beta 3^{V97S}$, or $m\beta 3^{V97S}$; $\alpha 6 + m\beta 4$ alone or with $h\beta 3$ or $m\beta 3$; $\alpha 6 + h\beta 2$ alone or with $m\beta 3$, $h\beta 3$, or $m\beta 3^{V97S}$; $\alpha 6 + h\beta 4$ alone or with $m\beta 3$. — indicates that absent or inconsistent functional responses in two-electrode voltage clamp studies precluded determination of the parameter of interest.

Drug	nAChR subunit combinations	Potency			Peak response		
		n	EC_{50} (95% CI) μM	$n_H \pm S.E. n_H$	n	Mean $I_{max} \pm S.E.$ nA	I_{max} concentration μM
Nicotine	$\alpha 6 + h\beta 2 + h\beta 3^{V97S}$	4	0.03 (0.02–0.05) $\uparrow \blacktriangle$	0.67 ± 0.07	4	$280 \pm 50 \uparrow \blacktriangle$	$1 \uparrow \blacktriangle$
	$\alpha 6 + m\beta 4 + h\beta 3^{V97S}$	3	0.27 (0.1–0.73) $\uparrow \blacktriangle$	0.46 ± 0.09	3	$40 \pm 2 \uparrow \blacktriangle$	$10 \uparrow \blacktriangle$
	$\alpha 6 + m\beta 4 + m\beta 3^{V97S}$	5	0.06 (0.03–0.15) $\uparrow \blacktriangle$	0.48 ± 0.08	5	$600 \pm 120 \uparrow \blacktriangle$	$10 \uparrow \blacktriangle$
	$\alpha 6 + h\beta 4 + h\beta 3$	7	14 (9.6–21) \uparrow	0.7 ± 0.08	8	$57 \pm 7 \uparrow$	$1000 \uparrow$
	$\alpha 6 + h\beta 4 + h\beta 3^{V97S}$	3	0.08 (0.03–0.02) $\uparrow \blacktriangle$	0.53 ± 0.14	3	$1600 \pm 98 \uparrow \blacktriangle$	$10 \uparrow \blacktriangle$
	$\alpha 6 + h\beta 4 + m\beta 3^{V97S}$	3	0.42 (0.28–0.64) $\uparrow \blacktriangle$	0.69 ± 0.08	3	$1000 \pm 12 \uparrow \blacktriangle$	$10 \uparrow \blacktriangle$
ACh	$\alpha 6 + h\beta 2 + h\beta 3^{V97S}$	4	0.34 (0.18–0.62) $\uparrow \blacktriangle$	0.52 ± 0.07	4	$300 \pm 90 \uparrow \blacktriangle$	$100 \uparrow \blacktriangle$

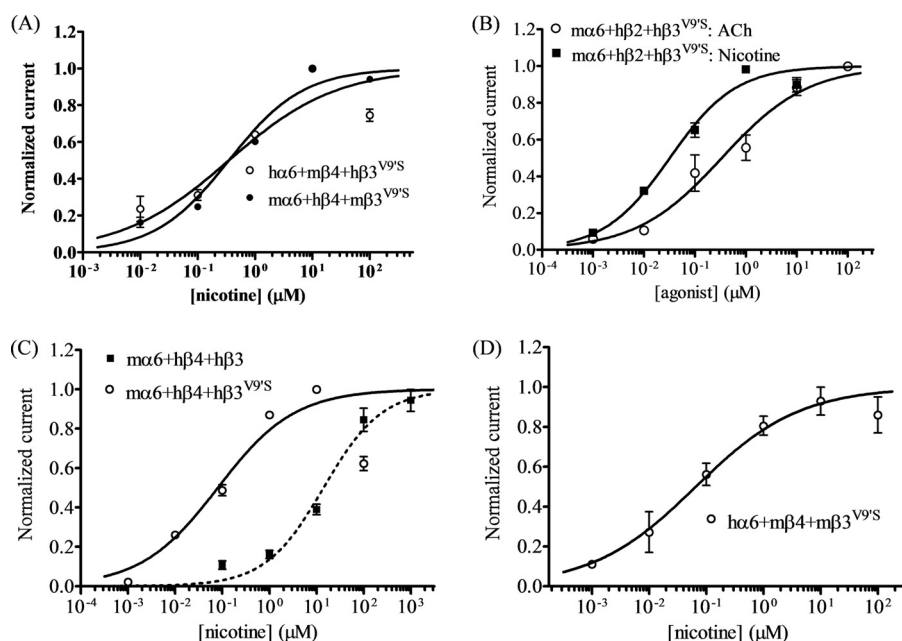


FIGURE 4. **Functional properties of hybrid $\alpha 6^*$ -nAChR.** Concentration (abscissa; log μM)-response (ordinate – mean normalized current \pm S.E.) curves are shown for responses as follows. A, to nicotine for oocytes expressing $\alpha 6m\beta 4h\beta 3^{V97S}$ (\circ) or $\alpha 6h\beta 4m\beta 3^{V97S}$ (\bullet) nAChR; B, to ACh (\circ) or nicotine (\blacksquare) for oocytes expressing $\alpha 6h\beta 2h\beta 3^{V97S}$ -nAChR; C, to nicotine for oocytes expressing nAChR $\alpha 6$ and $h\beta 4$ subunits with either $h\beta 3$ (\blacksquare) or $h\beta 3^{V97S}$ (\circ) subunits; or D, to nicotine for oocytes expressing nAChR $\alpha 6$, $m\beta 4$, and $m\beta 3^{V97S}$ subunits. A leftward shift in the nicotine concentration-response curve is evident for functional nAChR containing $h\beta 3^{V97S}$ subunits relative to nAChR containing wild-type $h\beta 3$ subunits ($p < 0.0001$; ~ 173 -fold). See Table 2 for parameters.

transmembrane domains, but there are some differences in other regions of the N terminus, first extracellular domain, and in the second, large cytoplasmic loop. We hypothesized that switching between mouse and human $\alpha 6$ plus $\beta 2$ or $\beta 4$ subunits would lead to formation of functional $\alpha 6^*$ -nAChR and/or $\alpha 6^*$ -nAChR with function influenced by $\beta 3$ subunits.

Human nAChR $\alpha 6$ Subunits Coexpressed With $m\beta 4$ (but Not With $m\beta 2$) and $h\beta 3^{V97S}$ Subunits Produce Functional Receptors—Oocytes expressing $\alpha 6$ plus either $m\beta 2$ or $m\beta 4$ subunits alone or additionally coexpressing $h\beta 3$ subunits did not respond appreciably to nicotinic agonists nor were there responses in oocytes coexpressing $\alpha 6$, $m\beta 2$, and $h\beta 3^{V97S}$ subunits (Table 2). However, coexpression of $\alpha 6$, $m\beta 4$, and mutant $h\beta 3^{V97S}$ subunits yielded oocytes with functional nicotinic responses (~ 40 -nA peak response, see Fig. 4A and Table 2).

Mouse nAChR $\alpha 6$ Subunits Coexpressed with $h\beta 4$ (but Not with $h\beta 2$) and $m\beta 3^{V97S}$ Subunits Produce Functional Receptors—Coexpression with $m\beta 3^{V97S}$ subunits significantly increased nicotinic responses in oocytes also expressing $\alpha 6$ and $h\beta 4$ subunits (~ 1022 -nA peak response, Fig. 4A, Table 2), although there was not formation of functional $\alpha 6h\beta 4$ - or $\alpha 6h\beta 4m\beta 3$ -nAChR. Also, there was no appreciable function in oocytes expressing $\alpha 6$ plus $h\beta 2$ subunits alone or in the additional presence of wild-type $m\beta 3$ or $m\beta 3^{V97S}$ subunits (Table 2).

Mouse nAChR $\alpha 6$ Subunits Coexpressed with $h\beta 2$ and $h\beta 3^{V97S}$ (but Not with $h\beta 3$) Subunits Produce Functional Receptors—There is functional nAChR expression in oocytes expressing nAChR $\alpha 6$, $h\beta 2$, and mutant $h\beta 3^{V97S}$ subunits (Fig. 4B and Table 2) but not for hybrid $\alpha 6h\beta 2h\beta 3$ -

Effects of $\beta 3$ Subunits on $\alpha 6^*$ -Nicotinic Receptor Function

TABLE 3

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

Potencies (micromolar IC_{50} values with 95% confidence intervals), Hill coefficients ($n_H \pm$ S.E.), mean \pm S.E. efficacies (I_{max} in nA), and concentrations where maximal peak current amplitudes (I_{max}) are achieved (in μM) are provided for the indicated antagonist (atropine or 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 figures. ∇ indicates a significant decrease in potency or efficacy of the indicated antagonist at the indicated nAChR containing $\beta 3^{V9^9S}$ subunits relative to the same complex containing $\beta 3^{V13^9S}$ subunits. — indicates that absent or inconsistent functional responses in two-electrode voltage clamp studies precluded determination of the parameter of interest.

Drug	nAChR subunit combinations	Potency			Peak response			Spontaneous opening
		n	IC_{50} (95% CI)	$n_H \pm$ S.E.	n	Mean $I_{max} \pm$ S.E.	I_{max} concentration	
Atropine	$\alpha 6 + \beta 4 + \beta 3^{V9^9S}$	5	11 (5.7–21)	-1.2 ± 0.62	5	500 ± 76	1000	19
	$\alpha 6 + \beta 4 + \beta 3^{V13^9S}$	4	35 (30–41) ∇	-1.1 ± 0.06	3	630 ± 81	1000	41
	$\alpha 6 + \beta 4 + \beta 3^{V9^9S}$	3	6.1 (1–36)	-1.1 ± 0.91	3	450 ± 210	1000	15
	$\alpha 6 + \beta 4 + \beta 3^{V13^9S}$	3	7.8 (1.3–48)	-1.6 ± 2.8	3	$120 \pm 56\bar{\nabla}$	1000	4
	$\alpha 6 + \beta 2 + \beta 3^{V9^9S}$	3	—	—	3	42 ± 6	1000	13
	$\alpha 6 + \beta 4 + \beta 3^{V9^9S}$	3	11 (5.7–21)	-1.20 ± 0.13	3	230 ± 7.9	1000	13
Mecamylamine	$\alpha 6 + \beta 4 + \beta 3^{V9^9S}$	4	18 (12.6–25)	-1.07 ± 0.17	4	300 ± 110	1000	33
	$\alpha 6(1-350)/\alpha 6(351-494) + \beta 4 + \beta 3^{V9^9S}$	3	—	—	3	16 ± 1.6	100	10
	$\alpha 6(1-236)/\alpha 6(237-494) + \beta 4 + \beta 3^{V9^9S}$	3	4.4 (0.06–309)	-1.8 ± 3.62	3	46 ± 14	100	8

nAChR. $\alpha 6\beta 2\beta 3^{V9^9S}$ -nAChR have maximal responses of ~ 300 nA at $100 \mu M$ ACh and at $1 \mu M$ nicotine, and EC_{50} values are $0.34 \mu M$ for ACh and $0.03 \mu M$ for nicotine (Fig. 4B and Table 2). Concentration-response curves show little to no evidence of what would be expected to be low efficacy, low agonist sensitivity responses that could be attributed to $\alpha 6\beta 2$ -nAChR (Fig. 4B). These studies suggest that the presence of $\alpha 6$ instead of $\alpha 6$ subunits is key to the function of $\alpha 6\beta 2\beta 3^{V9^9S}$ -nAChR.

Mouse nAChR $\alpha 6$ Subunits Coexpressed with nAChR $\beta 4$ and $\beta 3$ Subunits Produce Functional Receptors with Increased Agonist Sensitivity and Responsiveness—Hybrid nAChR produced in oocytes expressing $\alpha 6$, $\beta 4$, and $\beta 3$ subunits have functional responses to nicotinic agonists that are elevated relative to the negligible to no levels of function observed for $\alpha 6\beta 4$ -nAChR (Table 2 and Fig. 4C). Furthermore, coexpression with mutant $\beta 3^{V9^9S}$ subunits significantly increases nicotinic responses of oocytes also expressing $\alpha 6$ and $\beta 4$ subunits (Fig. 4C and Table 2), as is the case for oocytes expressing $\alpha 6$, $\beta 4$, and $\beta 3^{V9^9S}$ subunits or $\alpha 6$, $\beta 4$, and $\beta 3^{V9^9S}$ subunits. Pairwise comparisons show that differences in amplitudes of responses to nicotine are statistically significant ($p < 0.001$) between $\alpha 6\beta 4\beta 3$ - and $\alpha 6\beta 4\beta 3^{V9^9S}$ -nAChR (~ 60 and ~ 1600 nA, respectively; Table 2). Moreover, nicotine potency is increased for $\alpha 6\beta 4\beta 3^{V9^9S}$ -nAChR ($EC_{50} = 0.08 \mu M$) relative to that for $\alpha 6\beta 4\beta 3$ -nAChR ($EC_{50} = 14 \mu M$; Table 2). These results indicate that both wild-type $\beta 3$ and mutant $\beta 3^{V9^9S}$ subunits incorporate into at least some complexes containing $\alpha 6$ and $\beta 4$ subunits. However, although mutant $\beta 3^{V9^9S}$ subunits have the reasonably expected gain-of-function effect, wild-type $\beta 3$ subunits have potentiation rather than an expected, dominant-negative effect. These studies again suggest importance of $\alpha 6$ instead of $\alpha 6$ subunits in these effects.

Human nAChR $\alpha 6$ Subunits Coexpressed with nAChR $\beta 4$ (but Not with $\beta 2$) and $\beta 3^{V9^9S}$ Subunits Produce Functional Receptors—There is no appreciable function of nAChR in oocytes expressing $\alpha 6$ plus either $\beta 2$ or $\beta 4$ subunits alone or in the additional presence of $\beta 3$ subunits nor does coexpression with $\beta 3^{V9^9S}$ subunits produce functional nAChR in oocytes also expressing $\alpha 6$ and $\beta 2$ subunits (Table 2). However, coexpression with mutant $\beta 3^{V9^9S}$ subunits significantly

increases nicotinic responses in oocytes also expressing $\alpha 6$ and $\beta 4$ subunits (~ 600 -nA peak response, nicotine $EC_{50} = 0.06 \mu M$; Fig. 4D and Table 2).

Evidence for Spontaneous Opening of $\alpha 6^*$ -nAChR Containing Mutant $\beta 3^{V9^9S}$ or $\beta 3^{V13^9S}$ Subunits

To eliminate possible contributions of muscarinic AChRs to responses in oocytes expressing nAChR $\alpha 6$ and other subunits, ACh always was applied in the presence of $1 \mu M$ atropine, a muscarinic receptor antagonist that at higher concentrations also can noncompetitively block nAChR function. To help define specificity of agonist action, additional studies involved regular assessment of effects on nAChR function of the nAChR antagonist, mecamylamine, which also acts noncompetitively through the open channel block. Effects of atropine or mecamylamine alone were absent when assessed using oocytes expressing any combination of wild-type nAChR subunits from any species (data not shown). However, exposure to atropine or mecamylamine alone resulted in what seemed to be outward currents in oocytes expressing $\alpha 6^*$ -nAChR containing $\beta 3^{V9^9S}$ or $\beta 3^{V13^9S}$ subunits (Table 3). These effects were reversible, in that effects on currents ceased when atropine or mecamylamine were removed. They also were typically concentration-dependent, in that the magnitudes of the apparent outward currents were largest at the highest concentrations of atropine or mecamylamine (Fig. 5).

Inward currents elicited by nicotinic agonists from oocytes expressing nAChR $\alpha 6$ and $\beta 2$ or $\beta 4$ subunits in the presence of mutant $\beta 3$ subunits were actually reversed to apparent outward currents (relative to base-line levels) in the presence of added atropine or mecamylamine, even when agonist was first applied alone prior to application of agonist in the presence of antagonist. These phenomena made it evident that atropine and mecamylamine were in fact blocking not just inward current responses to agonists but that they also were blocking resting inward currents rather than inducing outward currents. Our interpretation of these results is that $\alpha 6^*$ -nAChR also containing mutant $\beta 3$ subunits and that had large functional responses to nicotinic agonists has a finite level of spontaneous channel opening that contributes to a resting inward current. This inward current can be ceased in the presence of adequately high concentrations of atropine or mecamylamine thanks to their

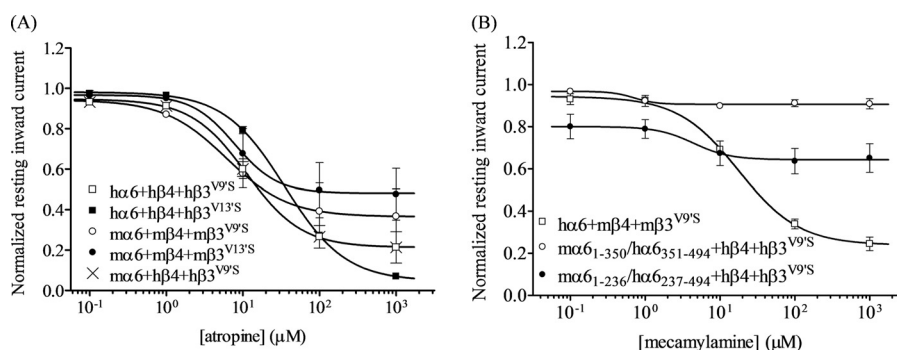


FIGURE 5. Evidence for spontaneous opening of $\alpha 6\beta 4^*$ -nAChR containing mutant $\beta 3^{V9/S}$ or $\beta 3^{V13/S}$ subunits. A, concentration-response curves (ordinate – mean normalized current \pm S.E.; abscissa – ligand concentration in log μM) are shown for apparent outward current responses as follows: A, to atropine for oocytes expressing $\alpha 6\text{h}\beta 4\text{h}\beta 3^{V9/S}$ - (○); $\alpha 6\text{h}\beta 4\text{h}\beta 3^{V13/S}$ - (●); $\alpha 6\text{m}\beta 4\text{m}\beta 3^{V9/S}$ - (○); $\alpha 6\text{m}\beta 4\text{m}\beta 3^{V13/S}$ - (●); or $\alpha 6\text{h}\beta 4\text{h}\beta 3^{V9/S}$ - (×) nAChR (note that curves are superimposable for oocytes expressing $\alpha 6\text{h}\beta 4\text{h}\beta 3^{V9/S}$ - or $\alpha 6\text{h}\beta 4\text{h}\beta 3^{V13/S}$ -nAChR), or B, to mecamlamine for oocytes expressing $\alpha 6\text{m}\beta 4\text{m}\beta 3^{V9/S}$ - (□); ($\alpha 6(1-350)/\alpha 6(351-494)\text{h}\beta 4\text{h}\beta 3^{V9/S}$ - (○) or ($\alpha 6(1-236)/\alpha 6(237-494)\text{h}\beta 4\text{h}\beta 3^{V9/S}$ - (●) nAChR. The interpretation is that coexpression with mutant $\beta 3$ subunits produces nAChR that open spontaneously, producing a stable inward current that is progressively blocked in the presence of increasing concentrations of antagonists acting as open channel blockers. See Table 3 for parameters.

open channel blocking abilities. It is not uncommon for gain-of-function mutations in second transmembrane domains of nAChR subunits to confer spontaneous channel opening to nAChR that contain those subunits (1). This means that atropine or mecamlamine potencies when acting alone (Table 3) actually are measures of ligand antagonist IC_{50} values, and additional studies assessing effects of these agents in block of agonist-induced responses yield similar IC_{50} values (data not shown). Antagonist potency determinations (IC_{50} values; typically $\sim 10 \mu\text{M}$ for both ligands) for atropine or mecamlamine are summarized in Table 3.

The results also can be used to estimate the number of nAChR that are spontaneously open. For example, at maximally efficacious concentrations, the atropine-alone sensitive component of its effect plus the inward current response to a full agonist ($42 \text{ nA}/(42 + 280 \text{ nA})$; Tables 2 and 3) indicate that about 13% of $\alpha 6\text{h}\beta 2\text{h}\beta 3^{V9/S}$ -nAChR is spontaneously open even in the absence of agonist. Similar calculations indicate that 4–41% of $\alpha 6^*$ -nAChR containing $\beta 3^{V9/S}$ or $\beta 3^{V13/S}$ subunits are spontaneously open (Tables 1–4).

Contributions of nAChR $\alpha 6$ Subunit N-terminal Domain to Effects of nAChR Wild-type or Mutant $\beta 3$ Subunits on Function of $\alpha 6^*$ -nAChR

Deviating from the predominant, dominant-negative effect of nAChR $\beta 3$ subunits on any function of $\alpha 6\beta 4$ -nAChR, there is facilitation of function by nAChR $\text{h}\beta 3$ subunits in $\alpha 6\text{h}\beta 4\text{h}\beta 3$ -nAChR (Table 2 and Fig. 4). Moreover, deviating from the predominant absence of function for $\alpha 6\beta 2$ -, $\alpha 6\beta 2\beta 3$ -, and $\alpha 6\beta 2\beta 3^{V9/S}$ - or $\alpha 6\beta 2\beta 3^{V13/S}$ -nAChR, nAChR $\text{h}\beta 3^{V9/S}$ subunits exert a gain-of-function effect in $\alpha 6\text{h}\beta 2\text{h}\beta 3^{V9/S}$ -nAChR (Table 2 and Fig. 4). These effects could only be attributed to the presence of nAChR $\alpha 6$ instead of $\text{h}\alpha 6$ subunits in these receptors. Furthermore, the strongest indication of a gain-of-function effect of mutant $\beta 3$ subunits on $\alpha 6\beta 2^*$ -nAChR function comes from studies of hybrid receptors containing $\alpha 6$, $\text{h}\beta 2$, and $\text{h}\beta 3^{V9/S}$ subunits and are not seen for complexes instead containing $\text{h}\alpha 6$ subunits. To understand the bases for these effects, we extended our studies to work using chimeric mouse/human $\alpha 6$ nAChR subunits.

Coexpression of Chimeric $\alpha 6(1-350)/\alpha 6(351-494)$ nAChR Subunits in Oocytes with nAChR $\text{h}\beta 4$ and Either Wild-type $\text{h}\beta 3$ or Mutant $\text{h}\beta 3^{V9/S}$ Subunits Produces Functional nAChR—There is functional expression represented by nicotinic agonist-induced current responses in oocytes expressing nAChR chimeric $\alpha 6(1-350)/\alpha 6(351-494)$ subunits in combination with $\text{h}\beta 4$ and $\text{h}\beta 3$ or $\text{h}\beta 3^{V9/S}$ subunits (Fig. 6 and Table 4). These results indicate that the nAChR $\alpha 6$ subunit region from cytoplasmic loop residue Pro-350 through to the C terminus does not strongly influence effects of wild-type $\beta 3$ subunits and has a limited influence on the effects of mutant $\beta 3$ subunits on function of $\alpha 6\beta 4^*$ -nAChR. Reciprocally, the results suggested that the region N-terminal to Pro-350 is likely to account for differences in effects of wild-type or mutant $\beta 3$ subunits on function of $\alpha 6\text{h}\beta 4^*$ -nAChR relative to effects on function of $\alpha 6\text{h}\beta 4^*$ -nAChR.

Coexpression of nAChR Chimeric $\alpha 6(1-236)/\alpha 6(237-494)$ Subunits in Oocytes with $\text{h}\beta 4$ and Either nAChR Wild-type $\text{h}\beta 3$ or Mutant $\text{h}\beta 3^{V9/S}$ Subunits Produces Functional Receptors—To further narrow the search for the region of the nAChR $\alpha 6$ subunit important for interactions with wild-type or mutant $\beta 3$ subunits, we assessed function of nAChR-containing chimeric $\alpha 6(1-236)/\alpha 6(237-494)$ subunits that link the N-terminal domain of the $\alpha 6$ subunit to the transmembrane domains, cytoplasmic loops, and C terminus of the $\text{h}\alpha 6$ subunit. There is functional expression represented by nicotinic agonist-induced current responses in oocytes expressing nAChR $\alpha 6(1-236)/\alpha 6(237-494)$ and $\text{h}\beta 4$ subunits in combination with wild-type nAChR $\text{h}\beta 3$ subunits (Fig. 6 and Table 4). The potentiation rather than the dominant-negative suppression of $\alpha 6\beta 4^*$ -nAChR function in the presence of wild-type $\beta 3$ subunits thus seems to be influenced by $\alpha 6$ subunit residues in the N-terminal extracellular domain. Although not as strong from the perspectives of nicotine potency and levels of functional expression, the N-terminal extracellular domain of $\alpha 6$ subunits also influences gain-of-function effects of mutant $\beta 3^{V9/S}$ subunits on $\alpha 6\beta 4^*$ -nAChR, including susceptibility to spontaneous opening sensitive to open channel block (Table 3).

Region(s) Apart from the N-terminal Domain and First Three Transmembrane Domains of nAChR $\alpha 6$ Subunits Seem to Be

Effects of $\beta 3$ Subunits on $\alpha 6^*$ -Nicotinic Receptor Function

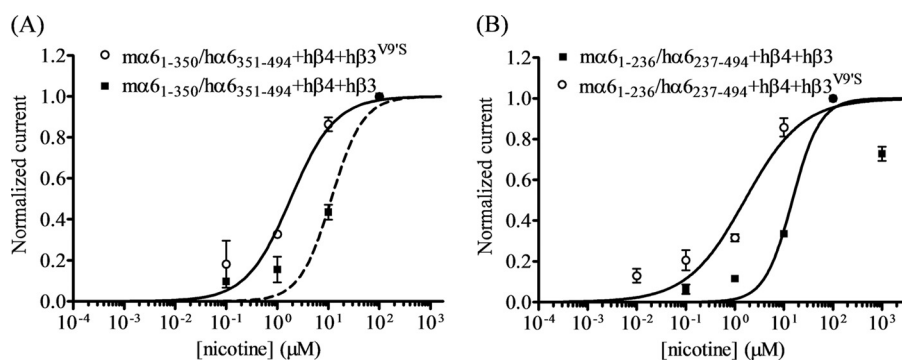


FIGURE 6. **Functional properties of nAChR containing chimeric $\alpha 6$ subunits.** A, concentration-response curves (ordinate — mean normalized current \pm S.E.; abscissa — ligand concentration in $\log(\mu\text{M})$) are shown for responses to nicotine for oocytes expressing nAChR chimeric $\alpha 6(1-350)/\text{ha}6(351-494)$ subunits (A) or $\alpha 6(1-236)/\text{ha}6(237-494)$ subunits (B) with $\text{h}\beta 4$ subunits along with either $\text{h}\beta 3$ (■) or $\text{h}\beta 3^{\text{V99S}}$ (○) subunits. A leftward shift in the nicotine concentration-response curve is evident for functional nAChR containing $\text{h}\beta 3^{\text{V99S}}$ subunits relative to nAChR containing wild-type $\text{h}\beta 3$ subunits ($p < 0.0001$; $\sim 7-9$ -fold). See Table 4 for parameters.

TABLE 4

Parameters for drug action at nAChR containing chimeric mouse/human $\alpha 6$ subunits and human $\beta 2$ or $\beta 4$ subunits alone or in combination with wild-type or mutant human $\beta 3$ subunits

Potencies (micromolar EC_{50} values with 95% confidence intervals for nicotine effects or micromolar IC_{50} values with 95% confidence intervals for mecamylamine effects), Hill coefficients ($n_{\text{H}} \pm \text{S.E.}$), mean \pm S.E. peak response (I_{max} in nA) and concentrations where maximal peak current amplitudes (I_{max}) are achieved (in μM) are provided for the indicated agonist (nicotine) or antagonist (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 figures. \uparrow or \downarrow indicates a significant ($p < 0.05$) increase or decrease, respectively, in potency or peak response elicited by the indicated agonist at the indicated nAChR subtype relative to nAChR containing the same subunits but in the absence of the indicated $\beta 3$ subunit. \blacktriangle or \blacktriangledown indicates a significant increase or decrease, respectively, in indicated agonist potency or peak response at the indicated nAChR subtype relative to nAChR containing the same subunits in the presence wild-type $\beta 3$ subunits. Note that no response or very rare and then small responses were seen for the following subunit combinations ($n = 4-9$ each) to nicotine: $\alpha 6(1-350)/\text{ha}6(351-494) + \text{h}\beta 2$ alone or with $\text{h}\beta 3$ or $\text{h}\beta 3^{\text{V99S}}$; $\alpha 6(1-236)/\text{ha}6(237-494) + \text{h}\beta 2$ alone or with $\text{h}\beta 3$ or $\text{h}\beta 3^{\text{V99S}}$ subunits. — indicates that absent or inconsistent functional responses in two-electrode voltage clamp studies precluded determination of the parameter of interest.

Drug	nAChR subunit combinations	n	Potency		Peak response		
			EC_{50} or IC_{50} (95% CI)	$n_{\text{H}} \pm \text{S.E.}$	n	Mean $I_{\text{max}} \pm \text{S.E.}$	I_{max} concentration
			μM		nA	μM	
Nicotine	$\alpha 6(1-350)/\text{ha}6(351-494) + \text{h}\beta 4$	9	—	—	9	—	—
	$\alpha 6(1-350)/\text{ha}6(351-494) + \text{h}\beta 4 + \text{h}\beta 3$	4	12 (8.6–15) \uparrow	1 ± 0.4	4	$33 \pm 14 \uparrow$	100
	$\alpha 6(1-350)/\text{ha}6(351-494) + \text{h}\beta 4 + \text{h}\beta 3^{\text{V99S}}$	4	1.8 (1.3–2.6) $\uparrow \blacktriangle$	1.4 ± 0.14	4	$140 \pm 48 \uparrow \blacktriangle$	100
	$\alpha 6(1-236)/\text{ha}6(237-494) + \text{h}\beta 4$	3	—	—	3	—	—
	$\alpha 6(1-236)/\text{ha}6(237-494) + \text{h}\beta 4 + \text{h}\beta 3$	5	15 (9.5–22) \uparrow	1.5 ± 0.48	5	$120 \pm 26 \uparrow$	100
	$\alpha 6(1-236)/\text{ha}6(237-494) + \text{h}\beta 4 + \text{h}\beta 3^{\text{V99S}}$	4	1.6 (0.97–2.6) $\uparrow \blacktriangle$	0.74 ± 0.11	4	$500 \pm 160 \uparrow \blacktriangle$	100

Required for the nAChR $\text{h}\beta 3^{\text{V99S}}$ Subunit to Exert Gain-of-Function Effects on $\alpha 6\text{h}\beta 2^*$ -nAChR—Oocytes coexpressing nAChR chimeric $\alpha 6(1-236)/\text{ha}6(237-494)$ or $\alpha 6(1-350)/\text{ha}6(351-494)$ subunits with $\text{h}\beta 2$ subunits alone or in the presence of wild-type $\text{h}\beta 3$ or mutant $\text{h}\beta 3^{\text{V99S}}$ subunits do not respond to nicotinic agonists (Table 4). These results indicate that the ability of nAChR $\text{h}\beta 3^{\text{V99S}}$ subunits to exert gain-of-function effects on $\alpha 6\text{h}\beta 2^*$ -nAChR (Table 2), unlike effects on $\alpha 6\beta 4^*$ -nAChR, must be influenced by regions aside from those in the N-terminal domains and first three transmembrane domains of nAChR $\alpha 6$ subunits.

Residues in the nAChR $\alpha 6$ Subunit That Influence Function of $\alpha 6^*$ -nAChR

Studies described above using hybrid $\alpha 6^*$ -nAChR and chimeric $\alpha 6$ subunits suggested that residues in the highly conserved N-terminal domain of nAChR $\alpha 6$ subunits influence effects of $\beta 3$ subunits on $\alpha 6\beta 4^*$ -nAChR function. It is thought that productive agonist binding occurs at selected interfaces between specific subunits in nAChR assemblies, more specifically in a pocket formed by loops A, B, and C on the + or primary face of α subunits except for $\alpha 5$ and loops D, E, and F on the — or complementary face of neighboring subunits ($\beta 2$ or $\beta 4$ in the central or autonomic nervous systems as partners to

$\alpha 2$, $\alpha 3$, $\alpha 4$, or $\alpha 6$ subunits, $\alpha 7$ in $\alpha 7$ -nAChR homomers, and γ or δ subunits as partners to $\alpha 1$ subunits in muscle-type nAChR). Consideration of the alignment of mouse and human $\alpha 6$ subunit amino acid sequences (Fig. 1) pointed us to residues in loop A ($\text{ha}6$ Lys-114) but also in loops D ($\text{ha}6$ Asn-91 and Lys-94) and E ($\text{ha}6$ Asn-143 and Met-145). Each of these residues in the nAChR $\text{ha}6$ subunit was mutated to their counterpart in the nAChR $\alpha 6$ subunit individually or in specific combinations (*i.e.* $\text{ha}6^{\text{N91K}}$, $\text{ha}6^{\text{K94R}}$, $\text{ha}6^{\text{K114N}}$, $\text{ha}6^{\text{N143D}}$, $\text{ha}6^{\text{M145V}}$, $\text{ha}6^{\text{N91K+K94R}}$, and $\text{ha}6^{\text{N143D+M145V}}$; see Fig. 1).

Residues at Positions 143 and 145 in the N-terminal Domain of nAChR $\alpha 6$ Subunits Influence Effects of nAChR $\beta 3$ Subunits on $\alpha 6\beta 4^*$ -nAChR Function—Coexpression of the double mutant $\text{ha}6^{\text{N143D/M145V}}$ subunit (but not any other single point mutations or the $\text{ha}6^{\text{N91K/K94R}}$ double mutation) with $\text{h}\beta 4$ subunits yields oocytes responding to 100 μM nicotine (mean \pm S.E. peak response = 35 ± 12 nA; Fig. 7A). When the same set of mutant $\text{ha}6$ subunits are coexpressed with nAChR $\text{h}\beta 4$ and $\text{h}\beta 3$ subunits, significant and reproducible peak current responses (\pm S.E.) to 100 μM nicotine are obtained only for $\text{ha}6^{\text{N91K}}\text{h}\beta 4\text{h}\beta 3$ -nAChR (28 ± 4 nA), $\text{ha}6^{\text{N143D}}\text{h}\beta 4\text{h}\beta 3$ -nAChR (31 ± 5 nA), and double mutant $\text{ha}6^{\text{N143D/M145V}}\text{h}\beta 4\text{h}\beta 3$ -nAChR (74 ± 11 nA; Fig. 7A). However, only nAChR

Effects of $\beta 3$ Subunits on $\alpha 6^*$ -Nicotinic Receptor Function

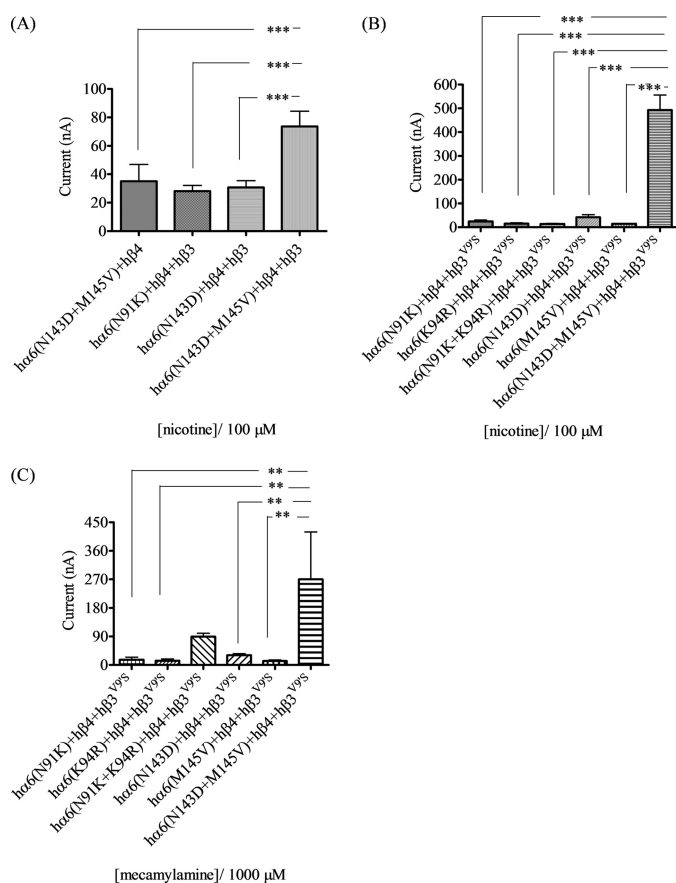


FIGURE 7. Effects of nAChR $\alpha 6$ subunit N-terminal domain amino acid substitutions on functional responsiveness of $\alpha 6\beta 4\beta 3$ -nAChR to nicotinic ligands. A and B, mean (\pm S.E.) peak inward current responses upon exposure (10 s) to 100 μ M nicotine (10 s exposure; *ordinate*) from oocytes ($n = 4$) voltage-clamped at -70 mV and heterologously expressing the indicated nAChR subunits. C, mean (\pm S.E.) apparent peak outward current responses upon exposure (10 s) to 1000 μ M mecamylamine (10 s exposure; *ordinate*) from oocytes ($n = 4$) voltage-clamped at -70 mV and heterologously expressing the indicated nAChR subunits. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. $\alpha 6(N143D)$ subunits are present in $\alpha 6h\beta 4^*$ -nAChR that have the largest amplitude responses to nicotine and the largest amplitude for mecamylamine-sensitive spontaneous openings, whether in the presence of wild-type or mutant $h\beta 3$ subunits.

containing the double mutant $\alpha 6^{N143D/M145V}$ subunit show the peak current response potentiation by coexpression with $\beta 3$ subunits seen for $\alpha 6h\beta 4h\beta 3$ -nAChR.

There is some level of functional response (peak \pm S.E.) to 100 μ M nicotine in oocytes coexpressing nAChR $h\beta 4$ and $h\beta 3^{V9'S}$ subunits in combination with $\alpha 6^{N91K}$ (25 ± 6 nA), $\alpha 6(K94R)$ (16 ± 3 nA), $\alpha 6^{N143D}$ (42 ± 12 nA), $\alpha 6^{M145V}$ (15 ± 1 nA), or $\alpha 6^{N91K+K94R}$ (15 ± 1 nA) subunits (Fig. 7B), but none of these combinations yields responses like those seen for $\alpha 6h\beta 4h\beta 3^{V9'S}$ -nAChR (~ 1600 nA) or even ($\alpha 6(1-236)/\alpha 6(237-494)h\beta 4h\beta 3^{V9'S}$ -nAChR (~ 140 nA) or ($\alpha 6(1-350)/\alpha 6(351-494)h\beta 4h\beta 3^{V9'S}$ -nAChR (~ 500 nA). Curiously, coexpression of nAChR $h\beta 4$ and $h\beta 3^{V9'S}$ with nAChR $\alpha 6^{K114N}$ subunits yields oocytes giving outward current responses to 100 μ M nicotine (data not shown). Significantly, only coexpression of double mutant $\alpha 6^{N143D/M145V}$ subunits in combination with $h\beta 4$ and $h\beta 3^{V9'S}$ subunits yields oocytes giving peak current responses (490 ± 63 nA) to 100

μ M nicotine that rival those of $\alpha 6h\beta 4h\beta 3^{V9'S}$ - or ($\alpha 6(1-236)/\alpha 6(237-494)h\beta 4h\beta 3^{V9'S}$ -nAChR.

Upon coexpression with $h\beta 4$ and $h\beta 3^{V9'S}$ subunits, exposure to 1 mM mecamylamine elicits apparently outward currents in oocytes also injected with $\alpha 6^{N91K}$ (17 ± 8 nA), $\alpha 6^{K94R}$ (14 ± 5 nA), $\alpha 6^{N143D}$ (90 ± 11 nA), $\alpha 6^{M145V}$ (31 ± 5 nA), or $\alpha 6^{N91K/K94R}$ (13 ± 3 nA) subunit cRNA (Fig. 7C). However, the largest apparently outward current is mediated by $\alpha 6^{N143D/M145V}h\beta 4h\beta 3^{V9'S}$ -nAChR ($270 + 150$ nA; Fig. 7C), with an amplitude comparable with that seen for atropine action on $\alpha 6h\beta 4h\beta 3^{V9'S}$ -nAChR and ~ 5 -fold higher than that seen for mecamylamine action at ($\alpha 6(1-236)/\alpha 6(237-494)h\beta 4h\beta 3^{V9'S}$ -nAChR. This indicates that there is some spontaneous opening of receptors containing the indicated subunits.

These findings indicate that nAChR $\alpha 6$ subunit residues at positions 143 and 145 influence the ability to form functional $\alpha 6\beta 4$ -, $\alpha 6\beta 4\beta 3$ -, and $\alpha 6\beta 4\beta 3^{V9'S}$ -nAChR, dictate whether $\beta 3$ subunits have dominant-negative suppression or gain-of-function effects, and are involved in gain-of-function effects of mutant $\beta 3^{V9'S}$ subunits on $\alpha 6\beta 4^*$ -nAChR. Clearly, wild-type or mutant $\beta 3$ subunits are incorporated into functional $\alpha 6\beta 4^*$ -nAChR complexes when residues at positions 143 and 145 are the same as those in the nAChR $\alpha 6$ subunit.

Residues at Positions 91 and 143 Are among Those in the N-terminal Domain of nAChR $\alpha 6$ Subunits That Influence Effects of nAChR $\beta 3^{V9'S}$ Subunits on $\alpha 6\beta 2^$ -nAChR Function—* Although studies with chimeric nAChR $\alpha 6$ subunits suggested that residues C-terminal to the third transmembrane domain influenced assembly of functional $\alpha 6\beta 2\beta 3^{V9'S}$ -nAChR, we also examined the effects of $\alpha 6$ subunit-like mutations in the N-terminal extracellular domain of the $\alpha 6$ subunit on the function of nAChR produced in oocytes upon coexpression with $h\beta 2$ subunits alone or in addition to $h\beta 3$ or $h\beta 3^{V9'S}$ subunits. Coexpression of $\alpha 6^{N91K}$, $\alpha 6^{K94R}$, $\alpha 6^{N143D}$, $\alpha 6^{M145V}$, $\alpha 6^{N91K+K94R}$, or $\alpha 6^{N143D+M145V}$ subunits with nAChR $h\beta 2$ subunits alone or in combination with wild-type $h\beta 3$ subunits does not result in consistent production of function upon exposure to 100 μ M nicotine (data not shown). However, and surprisingly, coexpression of $\alpha 6^{N91K}$, $\alpha 6^{K94R}$, $\alpha 6^{N143D}$, $\alpha 6^{M145V}$, or $\alpha 6^{N143D/M145V}$ subunits with $h\beta 2$ and $h\beta 3^{V9'S}$ subunits resulted in production of oocytes giving inward current responses to 100 μ M nicotine or apparently outward current responses to 1 mM mecamylamine (Fig. 8). Oocytes coexpressing $\alpha 6^{N91K+K94R}$ subunits with $h\beta 2$ and $h\beta 3^{V9'S}$ subunits failed to produce consistent response to 100 μ M nicotine or outward current responses to 1 mM mecamylamine (data not shown). These results suggest that the indicated trinary complexes are formed and have some function, although levels of functional expression and spontaneous channel opening are less than 1/3rd of that seen for $\alpha 6h\beta 2h\beta 3^{V9'S}$ -nAChR.

DISCUSSION

Patterns of nAChR $\alpha 6$ and $\beta 3$ subunit coexpression in primates or rodents *in vivo* (15–17) and clear evidence of the functional importance of native $\alpha 6^*$ -nAChR in the same species (2–7, 18, 19) suggest that $\alpha 6\beta 3^*$ -nAChR exist in functional

Effects of $\beta 3$ Subunits on $\alpha 6^*$ -Nicotinic Receptor Function

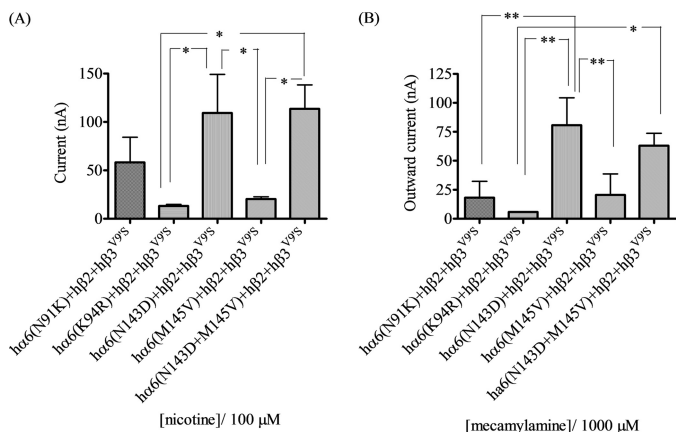


FIGURE 8. Effects of nAChR $\alpha 6$ subunit N-terminal domain amino acid substitutions on functional responsiveness of $\alpha 6\beta 3$ -nAChR to nicotinic ligands. A, mean (\pm S.E.) peak inward current responses upon exposure (10 s) to 100 μ M nicotine (10 s exposure; *ordinate*) from oocytes ($n = 4$) voltage-clamped at -70 mV and heterologously expressing the indicated nAChR subunits. B, mean (\pm S.E.) apparent peak outward current responses upon exposure (10 s) to 1000 μ M mecamylamine (10 s exposure; *ordinate*) from oocytes ($n = 4$) voltage-clamped at -70 mV and heterologously expressing the indicated nAChR subunits. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. h $\alpha 6$ (N143D) subunits are present in h $\alpha 6$ h $\beta 2^*$ -nAChR that have the largest amplitude responses to nicotine and the largest amplitude for mecamylamine-sensitive spontaneous openings, whether in the presence of wild-type or mutant h $\beta 3$ subunits.

forms that should be evident upon heterologous expression. However, some of the prior studies of heterologous expression of $\alpha 6^*$ -nAChR indicated that hybrid receptors containing combinations of chicken and human nAChR subunits exhibit some level of function, although all-human or all-chicken $\alpha 6^*$ -nAChR did not (10). Moreover, and although these studies were done using oocytes manipulated to express disproportionate ratios of subunits, incorporation of nAChR h $\beta 3$ subunits present in presumed excess have a dominant-negative effect on the function of all-human $\alpha 2\beta 2^*$ -, $\alpha 2\beta 4^*$ -, $\alpha 3\beta 2^*$ -, $\alpha 3\beta 4^*$ -, $\alpha 4\beta 2^*$ -, or $\alpha 4\beta 4^*$ -nAChR that is reversed upon substitution of mutant, h $\beta 3^{V9'S}$ for wild-type $\beta 3$ subunits (12). Similar effects are mentioned (but not described in detail) of effects of wild-type or mutant $\beta 3$ subunits on function of $\alpha 6\beta 2^*$ - and $\alpha 6\beta 4^*$ -nAChR (11). Interestingly, other studies using chimeric ($\alpha 6/\alpha 3$) subunits (containing the N-terminal domain of the nAChR $\alpha 6$ subunit substituting for that of the otherwise $\alpha 3$ subunit) instead of wild-type $\alpha 6$ subunits showed potentiating effects of wild-type $\beta 3$ subunit coexpression on $\alpha 6^*$ -nAChR (8). Here, we have extended these lines of studies to make novel and sometimes surprising findings that help to crystallize our cumulative understanding of $\alpha 6\beta 3^*$ -nAChR function.

One of the conclusions from this work is that nAChR wild-type or mutant $\beta 3$ subunits can incorporate into heterologously expressed $\alpha 6\beta 4^*$ -nAChR, where they predominantly exert dominant-negative (wild-type $\beta 3$ subunits) or gain-of-function (mutant $\beta 3^{V9'S}$ or $\beta 3^{V13'S}$ subunits) effects, respectively. The abilities of wild-type m $\beta 3$ subunits to mimic dominant-negative effects of wild-type h $\beta 3$ subunits suggests that $\beta 3$ subunits from either species have the same features needed for negative dominance or gain-of-function. These findings are of interest for nAChR structure-function relationships (see below), but their physiological significance is tempered because there are

few brain regions in rodents where all three subunits are expressed, although the circumstance may be different in primates, which might express higher levels of $\beta 4$ subunits and do so more broadly (15, 17–19).

It is clear from a variety of studies, including those done using knock-out animals and nicotinic agonist-activated neurotransmitter release assays (2, 4), that naturally expressed mouse nAChR containing $\alpha 6$, $\beta 3$, and $\beta 2$ subunits seem to be functional and sensitive to blockade by specific α -conotoxins (7), but there are fewer indications that human $\alpha 6\beta 2\beta 3^*$ -nAChR are functional when naturally or heterologously expressed. Thus, we wondered whether there simply might be species-specific differences in the ability to heterologously express $\alpha 6\beta 2\beta 3^*$ -nAChR, and so we chose to see if murine $\alpha 6\beta 2\beta 3^*$ -nAChR could be functionally expressed when equivalent human receptors could not. However, we realized very similar outcomes in our studies of all-human or all-mouse $\alpha 6\beta 2\beta 3^*$ -nAChR, even when $\beta 3$ subunits had gain-of-function mutations and despite success of the gain-of-function strategy when applied to $\alpha 6\beta 4\beta 3^*$ -nAChR. The inability of wild-type or mutant $\beta 3$ subunits to influence function of $\alpha 6\beta 2^*$ -nAChR confounds the ability to make inferences about assembly of the indicated subunits. Also, the lack of function of h $\alpha 6$ h $\beta 2$ h $\beta 3^{V9'S}$ - or h $\alpha 6$ h $\beta 2$ h $\beta 3^{V13'S}$ -nAChR observed in this work is in contrast to the observation by Broadbent *et al.* (12) that human $\alpha 6\beta 2\beta 3^{V9'S}$ -nAChR are functional and to our findings that h $\alpha 6$ h $\beta 4$ h $\beta 3^{V9'S}$ - or h $\alpha 6$ h $\beta 4$ h $\beta 3^{V13'S}$ -nAChR are functional.

We tried to solve this problem by using two different approaches. One approach involved switching sequences between m $\beta 2$ and h $\beta 2$ subunits, but this did not influence the ability of wild-type or mutant $\beta 3$ subunits to affect function of $\alpha 6\beta 2^*$ -nAChR, although h $\alpha 6$ m $\beta 4$ h $\beta 3^{V9'S}$ - and m $\alpha 6$ h $\beta 4$ m $\beta 3^{V9'S}$ -nAChR were functional. The other approach entailed switching sequences between m $\alpha 6$ and h $\alpha 6$ subunits and surprisingly showed that m $\alpha 6$ h $\beta 2$ h $\beta 3^{V9'S}$ - and m $\alpha 6$ h $\beta 4$ h $\beta 3$ -nAChR are functional.

The persisting lack of function seen for all-human or all-mouse $\alpha 6\beta 2$ - or $\alpha 6\beta 2\beta 3$ -nAChR suggests that native and physiologically relevant receptors likely contain an additional assembly partner, perhaps $\alpha 4$ or $\alpha 3$ subunits. 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 $\alpha 6\beta 2\beta 3^*$ -nAChR.

However, success in formation of functional, hybrid m $\alpha 6$ h $\beta 2$ h $\beta 3^{V9'S}$ - and m $\alpha 6$ h $\beta 4$ h $\beta 3$ -nAChR indicated that features of $\alpha 6$ subunits influence the function or functional assembly of $\alpha 6\beta 3^*$ -nAChR. Chimeric $\alpha 6$ subunits containing different length segments of the m $\alpha 6$ subunit fused to otherwise h $\alpha 6$ subunits localized key determinants involved in effects of $\beta 3$ subunits on $\alpha 6\beta 4^*$ -nAChR function to the N-terminal extracellular domain of the nAChR $\alpha 6$ subunit but gave little initial indication that the same region influenced effects of $\beta 3$ subunits on $\alpha 6\beta 2^*$ -nAChR function. Our studies of $\alpha 6\beta 2^*$ -nAChR also suggest that the cytoplasmic loop (and perhaps, although less likely, the fourth transmembrane domain and the C-terminal tail) of the $\alpha 6$ subunit can influence functional expression of receptors and their interactions with $\beta 3$ subunits, perhaps con-

	91 94	114	143 145
Human	HIW NDY KL R WDPMEYDGIETLRVPAD K IWK P DIVLYNNAVGDFQVEG K T K ALLK Y NG M ITW		
Chimpanzee	HIW NDY KL R WDPMEYDGIETLRIPAD K IWK P DIVLYNNAVGDFQVEG K T K ALLK Y NG M ITW		
Human	HIW NDY KL R WDPMEYDGIETLRVPAD K IWK P DIVLYNNAVGDFQVEG K T K ALLK Y NG M ITW		
Monkey	HIW NDY KL R WDPMEYDGIETLRVPAD K IWK P DIVLYNNAVGDFQVEG K T K ALLK Y NG M ITW		
Rat	HWKDYRLCWDPT EY DGIETLRVPAD N IWK P DIVLYNNAVGDFQVEG K T K ALLK Y DG V ITW		
Mouse	HIWKDYRLRWDPT EY DGIETLRVPAD N IWK P DIVLYNNAVGDFQVEG K T K ALLK Y DG V ITW		
Cow	HIW NDY KL R WDPTEYDGIETLRVPA E M I WK P DIVLYNNAVGDFQ V KG K T K ALLK Y DG M ITW		
Chicken	HIW NDY KL R WDPREYDGI E FV R VPAD K IWK P DIVLYNNAVGDFQVEG K T K ALLR Y DG M ITW		
Zebrafish	HIW NDY KL K WSPAEFDG I E F IRV P SN K IWR P DIVLYNNAVGDFL V ED K T K ALL K F D GT I W		
	::*:*:* *.* *:*:*:* :*:*:* :*:*:*:*:*:*:*:*:*:* *.:*****::: * * *		

FIGURE 9. Multiple sequence alignment of nAChR $\alpha 6$ subunit proteins from several species (GenBank™ accession number NP_004189.1 (human, *Homo sapiens*), NP_001029266.1 (chimpanzee, *Pan troglodytes*), XP_001099152.1 (monkey, *Macaca mulatta*), NP_476532.1 (rat, *Rattus norvegicus*), NP_067344.2 (mouse, *Mus musculus*), XP_584902.3 (cow, *Bos taurus*), NP_990695.1 (chicken, *Gallus gallus*), and NP_001036149.1 (zebrafish, *Danio rerio*)). Numbering begins at translation start methionine of human nAChR $\alpha 6$ subunit protein and is shown in the N-terminal domain region of interest. Symbols below sequences indicate fully (*) or weakly (.) conserved residues, and underlining in boldface indicates numbered residues given prime attention in human nAChR $\alpha 6$ subunit mutagenesis studies.

sistent with the observations by Kuryatov *et al.* (8) who used chimeric subunits possessing intracellular domains of the $\alpha 3$ instead of $\alpha 6$ subunit.

Site-directed mutagenesis work, simplified to some extent by the remarkable homology between human and mouse nAChR $\alpha 6$ subunits, indicated that the function like that seen in hybrid receptors occurring for $\text{h}\alpha 6^{\text{N143D/M145V}}$ $\text{h}\beta 4$ -nAChR is potentiated in the presence of wild-type $\text{h}\beta 3$ subunits and is further potentiated upon substitution of mutant $\text{h}\beta 3^{\text{V97S}}$ for wild-type $\text{h}\beta 3$ subunits. These findings indicated that amino acid residues 143 and 145 in the $\alpha 6$ subunit are key determinants influencing effects of $\beta 3$ subunits on $\alpha 6\beta 4^*$ -nAChR function. $\text{m}\alpha 6$ differs from $\text{h}\alpha 6$ by having a negatively charged rather than a polar side chain at position 143 and a residue with a slightly higher hydrophobicity and smaller side chain volume at position 145. These differences might enhance interactions between $\alpha 6$ and $\beta 3$ and/or $\beta 4$ subunits. These residues are in loop E on the – or complementary face of the $\alpha 6$ subunit that would be involved in presumed interactions with residues on the + or primary faces of the neighboring $\beta 3$ subunit and/or of the neighboring $\beta 4$ subunit in a complex that has the presumed (counterclockwise when viewed from the extracellular space) arrangement as follows: $-\beta 3_+:-\alpha 6_+:-\beta 4_+:-\alpha 6_+:-\beta 4_+$. Neighboring (–) face residues in loop E at consensus positions 139, 141, 147, and 149 (Leu, Lys, Thr, and Thr, respectively, preserved across mammalian $\alpha 6$ subunits, although chickens have Pro-141; see Fig. 9) have been implicated in agonist binding based largely on mutagenesis or structural studies of muscle-type or $\alpha 7$ -nAChR (20). It is unexpected that agonist binding would occur at $\beta 3_+:-\alpha 6$ or $\beta 4_+:-\alpha 6$ subunit interfaces, as it would be expected to be confined to $\alpha 6_+:-\beta 4$ (or $\alpha 6_+:-\beta 2$) interfaces (but see Moroni *et al.* (21) for evidence that nAChR $\beta 2_+:-\alpha 4$ interfaces are engaged in allosteric effects of Zn^{2+}). It is possible that $\beta 3_-:+\alpha 6$ and/or $\beta 4_-:+\alpha 6$ subunit interfaces in the vicinity of loop E are important for subunit assembly leading to closure of functional cell-surface expression of $\alpha 6\beta 4\beta 3$ -nAChR complexes, and distal involvement of $\beta 3_-:+\alpha 6$ and $\beta 4_-:+\alpha 6$ subunit interfaces in ligand binding or transduction of ligand binding to channel gating cannot be discounted. It is notable that mouse and chicken $\alpha 6$ subunits share the presence of aspartate at position 143 as opposed to the human asparagine, perhaps more deeply implicating that residue in the function of hybrid $\alpha 6^*$ -nAChR.

Although studies using chimeric mouse/human $\alpha 6$ subunits did not initially implicate the N-terminal region in interactions with $\beta 3$ subunits, consistent with the site-directed mutagenesis work at some of the N-terminal domain residues investigated, coexpression of nAChR $\text{h}\alpha 6^{\text{N91K}}$ or $\text{h}\alpha 6^{\text{N143D}}$ subunits together with nAChR $\text{h}\beta 2$ and $\text{h}\beta 3^{\text{V97S}}$ subunits resulted in production of functional receptors as evident by inward current responses to nicotine and apparently outward current responses to mecamylamine. The $\text{h}\alpha 6^{\text{N143D}}$ single mutation also accounted for effects when it was coupled with the more conservative, $\text{h}\alpha 6^{\text{M145V}}$ mutation. Although absolute levels of function were not particularly high and were just 1/3rd of those for hybrid $\text{m}\alpha 6\text{h}\beta 2\text{h}\beta 3^{\text{V97S}}$ -nAChR, again suggesting as did chimeric subunit studies that residues C-terminal to the third transmembrane domain play a role in influencing effects of $\beta 3$ subunits on $\alpha 6\beta 2^*$ -nAChR function, these studies implicate sites involved in formation of functional $\alpha 6\beta 2\beta 3$ -nAChR. Aside from the considerations already described above about residue 143 in the E loop of the $\alpha 6$ subunit engaging in interactions with neighboring $\beta 3$ or, in this case, $\beta 2$ subunits, there are potential influences of the introduction of a positively charged instead of a polar side chain at position 91 that could influence effects of $\beta 3$ subunits on $\alpha 6\beta 2^*$ -nAChR. Interestingly, residue 91 is in loop D and on the – face of the $\alpha 6$ subunit, slightly C-terminal to residues at positions 85 and 87 (Trp and Arg, respectively, preserved across mammalian, chicken, and fish $\alpha 6$ subunits). Residue 91 is an asparagine in primates, cows, and chickens but is a lysine in rats and mice (Fig. 9). It, as opposed to residue 143, might not account for differences between chicken and human $\alpha 6$ subunits in hybrid receptors, but it seems to contribute to differences between mouse and human $\alpha 6$ subunits. Perhaps the incomplete (relative to hybrid $\alpha 6\beta 2\beta 3^{\text{V97S}}$ -nAChR) potentiation of $\alpha 6\beta 2^*$ -nAChR function is because the changes in position 143 reflect influences on $\beta 3_+:-\alpha 6$ and $\beta 4_+:-\alpha 6$ subunit interactions but not $\beta 2_+:-\alpha 6$ subunit interactions. Reciprocally, changes in position 91 might have effects only because they influence $\beta 2_+:-\alpha 6$ and not $\beta 4_+:-\alpha 6$ subunit interactions. Again, given that it would be unexpected for agonist binding to occur at $\beta 3_+:-\alpha 6$ or $\beta 2_+:-\alpha 6$ subunit interfaces, as opposed to at $\alpha 6_+:-\beta 2$ interfaces, changes in residues in loops D and E probably affect assembly and closure of functional $\alpha 6\beta 2\beta 3$ -nAChR pentamers, although they could have allosteric effects on ligand binding and/or coupling to channel

opening (e.g. see Ref. 21), maybe even serving as coagonist-binding sites.

Finally, effects of atropine or mecamylamine interpreted as that of $\alpha 6^*$ -nAChR showing gain-of-function have a significant probability of spontaneous channel opening that is abated by atropine- or mecamylamine-mediated open channel block. Gain-of-function effects due to mutations occurring at the 9'- or 13'-positions in the $\beta 3$ subunit second transmembrane domain have largely similar effects but in some cases yield functional receptors with different agonist sensitivities, suggesting subtleties in coupling between ligand binding and channel opening.

In conclusion, our results provide evidence that wild-type $\beta 3$ or mutant $\beta 3^{V9'S}$ or $\beta 3^{V13'S}$ subunits can incorporate into and either suppress/abolish or enhance function of $\alpha 6\beta 4^*$ -nAChR but not $\alpha 6\beta 2^*$ -nAChR. These observations, along with the demonstration that $\beta 3$ subunits can form functional receptors with $\alpha 7$ subunits (22) and that $\beta 3^{V273T}$ subunits participate in formation of $\alpha 3\beta 4^*$ -nAChR (23, 24), help to define nAChR subtypes capable of containing $\beta 3$ subunits, thus providing insights into roles of $\beta 3^*$ -nAChR in nicotinic signaling. However, there remain puzzles about the makeup of functional, all-human or all-mouse $\alpha 6^*$ -nAChR, especially $\alpha 6\beta 3^*$ -nAChR. Nevertheless, our results provide further evidence that wild-type and/or mutant $\beta 3$ subunits not only form functional receptors in combination with $\alpha 6$ subunits but also influence $\alpha 6^*$ -nAChR function. We also show for the first time that dominant-negative suppression or potentiation of $\alpha 6\beta 4^*$ -nAChR upon heterologous coexpression with wild-type $\beta 3$ subunits is influenced in hybrid receptors and in the presence of chimeric $\alpha 6$ subunits in ways affected by selected residues that unexpectedly are found on the N-terminal extracellular domain – face of $\alpha 6$ subunits. These and additional residues influence effects of mutant $\beta 3$ subunits on function of $\alpha 6\beta 2^*$ -nAChR. The current findings suggest that the molecular description of functional nAChR is incomplete and that novel interfaces (i.e. other than the consensus interface thought to be involved in productive agonist binding, $\alpha 6_+ : \beta 2$ or $\alpha 6_+ : \beta 4$) between nAChR subunits play heretofore unappreciated roles in receptor assembly, ligand recognition, and/or function. Finally, the development of oocytes that express $\alpha 6\beta 3^*$ -nAChR and the prospect that cell lines also containing the same assemblies in functional forms provide potentially useful tools for development of $\alpha 6^*$ -nAChR-selective or -specific ligands, with the caveat that any $\alpha 6^*$ -nAChR-containing subunits with gain-of-function properties or chimeric subunits may have different sensitivities for agonists or perhaps other types of ligands than $\alpha 6^*$ -nAChR composed of fully wild-type subunits. This is important due to the growing interest in functional $\alpha 6\beta 3^*$ -nAChR based on their demonstrated or perceived importance in locomotion, reward and reinforcement behavior, schizophrenia, and Parkinson disease (2, 15, 25). Perhaps of high significance is the association of single nucleotide polymorphisms in the genes (*CHRNA6* and *CHRNB3*) encoding $\alpha 6$ and $\beta 3$ subunits with nicotine dependence, number of quit attempts, and subjective responses to nicotine (26–30). Studies as described here are essential for

an improved understanding of structure and function and ultimately of biological roles of $\alpha 6\beta 3^*$ -nAChR.

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Bhagirathi Dash, Minoti Bhakta, Yongchang Chang and Ronald J. Lukas

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