

Modulation of Gain-of-function $\alpha 6^*$ -Nicotinic Acetylcholine Receptor by $\beta 3$ Subunits*

Received for publication, November 9, 2011, and in revised form, January 27, 2012. Published, JBC Papers in Press, February 7, 2012, DOI 10.1074/jbc.M111.322610

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Background: Function of physiologically important $\alpha 6\beta 3^*$ -nicotinic receptor (nAChR) is differentially impacted by $\beta 3$ subunits.

Results: nAChR expressed in several novel ways indicates that $\beta 3$ subunits mostly potentiate gain-of-function $\alpha 6^*$ -nAChR.

Conclusion: Extracellular domain loop E region in $\alpha 6$ subunits governs effect of $\beta 3$ subunit on gain-of-function $\alpha 6^*$ -nAChR.

Significance: Novel $\alpha 6\beta 3^*$ -nAChR reported could be used to assess and/or develop smoking cessation aids.

We previously have shown that $\beta 3$ subunits either eliminate (e.g. for all-human (h) or all-mouse (m) $\alpha 6\beta 4\beta 3$ -nAChR) or potentiate (e.g. for hybrid $m\alpha 6h\beta 4h\beta 3$ - or $m\alpha 6m\beta 4h\beta 3$ -nAChR containing subunits from different species) function of $\alpha 6^*$ -nAChR expressed in *Xenopus* oocytes, and that nAChR $\alpha 6$ subunit residues Asn-143 and Met-145 in N-terminal domain loop E are important for dominant-negative effects of nAChR $\beta 3$ subunits on $\alpha 6^*$ -nAChR function. Here, we tested the hypothesis that these effects of $\beta 3$ subunits would be preserved even if nAChR $\alpha 6$ subunits harbored gain-of-function, leucine- or valine-to-serine mutations at 9' or 13' positions (L9'S or V13'S) in their second transmembrane domains, yielding receptors with heightened functional activity and more amenable to assessment of effects of $\beta 3$ subunit incorporation. However, coexpression with $\beta 3$ subunits potentiates rather than suppresses function of all-human, all-mouse, or hybrid $\alpha 6^{(L9'S \text{ or } V13'S)}\beta 4^*$ - or $\alpha 6(N143D+M145V)^{L9'S}\beta 2^*$ -nAChR. This contrasts with the lack of consistent function when $\alpha 6^{(L9'S \text{ or } V13'S)}$ and $\beta 2$ subunits are expressed alone or in the presence of wild-type $\beta 3$ subunits. These results provide evidence that gain-of-function $\alpha 6h\beta 2^*$ -nAChR (i.e. $\alpha 6(N143D+M145V)^{L9'S}h\beta 2h\beta 3$ nAChR) could be produced *in vitro*. These studies also indicate that nAChR $\beta 3$ subunits can be assembly partners in functional $\alpha 6^*$ -nAChR and that 9' or 13' mutations in the nAChR $\alpha 6$ subunit second transmembrane domain can act as gain-of-function and/or reporter mutations. Moreover, our findings suggest that $\beta 3$ subunit coexpression promotes function of $\alpha 6^*$ -nAChR.

Nicotinic acetylcholine receptors (nAChR)³ are pentameric ligand-gated ion channels expressed throughout the nervous system. Those other than the muscle-type (embryonic $\alpha 1\beta 1\gamma\delta$ - or adult $\alpha 1\beta 1\gamma\epsilon$ -) nAChR are thought to be composed of different permutations of eight α subunits ($\alpha 2$ - $\alpha 7$, $\alpha 9$ - $\alpha 10$) and three β subunits ($\beta 2$ - $\beta 4$) in humans (1). Of specific interest to us in this study are $\alpha 6\beta 3^*$ -nAChR (where * indicates the known or possible presence of nAChR subunits other than those specified) (2–4). $\alpha 6\beta 3^*$ -nAChR have been implicated in dopaminergic neurotransmission, nicotine dependence, anxiety, and other important neurophysiological processes (5–13).

In vitro expression of functional, all-mouse (m) or all-human (h), wild-type $\alpha 6\beta 3^*$ -nAChR has been difficult to achieve despite strong evidence for expression of $\alpha 6\beta 3^*$ -nAChR in rodent brain (3, 4, 6, 7, 10, 12–15). Functional expression of $\alpha 6^*$ -nAChR only has been achieved in *Xenopus* oocytes when using specific forms of mutant or chimeric subunits or in hybrid $\alpha 6^*$ -nAChR composed of subunits from different species (16–20). For example, function is achieved when chimeric, $h\alpha 6/h\alpha 3$ subunits (composed of the N-terminal, first extracellular domain of the $h\alpha 6$ subunit fused to the first transmembrane domain through to the C terminus of the $h\alpha 3$ subunit) are coexpressed with $h\beta 2$ or $h\beta 4$ subunits alone or in the presence of $h\beta 3$ subunits (19). $\alpha 6^*$ -nAChR are functional when expressed as hybrids of mouse and human $\alpha 6$ and other subunits, and there is function of some complexes containing $\beta 3$ subunits mutated at specific residues in their second transmembrane domains (leucine- or valine-to-serine mutations at 9' or 13' positions; L9'S or V13'S) to confer gain-of-function effects (4, 15, 21). Potentiation of function is sometimes seen when wild-type $\beta 3$ subunits are incorporated into hybrid complexes, but this is in contrast to dominant-negative effects of coexpression with wild-type $\beta 3$ subunits on function of $\alpha 6\beta 4^*$ -nAChR when all subunits are from the same species (4, 21). There may be host cell specificity in some of these effects because nAChR $h\beta 3$ subunits promote expression and nicotine-induced up-regulation of $h\alpha 6^*$ -nAChR in transfected cell lines (22).

* This work was supported, in whole or in part, by National Institutes of Health Grant DA015389 (to R. J. L.), by Philip Morris USA Inc. and Philip Morris International through their External Research Program, and by endowment and capitalization funds from the Men's and Women's Boards of the Barrow Neurological Foundation. Portions of this work have been presented in abstract form (Dash, B., Chang, Y., and Lukas, R. J. (2008) Molecular and pharmacological characterization of recombinant $\alpha 6\beta 4^*$ -nAChR. *Soc. Neurosci. Abst.* 34, 233.13).

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³ The abbreviations used are: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor(s); I_{max} , peak current response; m, mouse; h, human.

Gain-of-function $\alpha 6^*$ -Nicotinic Receptor and $\beta 3$ Subunits

We and others have taken advantage of gain-of-function mutations in the nAChR $\beta 3$ subunit to produce functional nAChR, including those containing $\alpha 6$ subunits, in part to assess capabilities of subunits to coassemble, but also as a strategy to increase functional gain (signal:noise) to facilitate such assessments (4, 15, 21). For example, coexpression with $\beta 3^{V9'S}$ subunits increases agonist sensitivity and efficacy for $\alpha 6^*$ -nAChR. We hypothesized that similar mutations in nAChR $\alpha 6$ subunits would increase agonist sensitivity and efficacy of $\alpha 6^{(L9'S \text{ or } V13'S)}$ ($\beta 4$ or $\beta 2$)*-nAChR to provide enough functional gain to facilitate evaluation of effects of wild-type $\beta 3$ subunits on complexes and even to ensure that we can detect incorporation of wild-type $\beta 3$ subunits into $\alpha 6^{(L9'S \text{ or } V13'S)}$ *-nAChR. We also hypothesized that wild-type $\beta 3$ subunits would have the same effects, dominant-negative or potentiating, depending on the subunit combination investigated, on gain-of-function $\alpha 6^{(L9'S \text{ or } V13'S)}$ *-nAChR as they did on wild-type $\alpha 6^*$ -nAChR. This would help us assess whether any reduction or abolishment of function is due to altered open channel probability (21) or due to reduced surface expression of nAChR because $\beta 3$ subunit incorporation facilitates formation of dead end intermediates (23). Our results indicated that whenever nAChR $\beta 3$ subunits are incorporated into ($\alpha 6$ or $\alpha 6(N143D+M145V)$)($L9'S$ or $V13'S$)*-nAChR, function is potentiated (*i.e.* there is higher agonist potency and larger magnitude responses) irrespective of whether there are dominant-negative or potentiating effects of $\beta 3$ subunits on wild-type $\alpha 6^*$ -nAChR.

EXPERIMENTAL PROCEDURES

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

Subcloning, Mutagenesis, and in Vitro Transcription of nAChR Subunits—Human or mouse nAChR $\alpha 6$, $\beta 2$, $\beta 3$, or $\beta 4$ subunits were subcloned into the oocyte expression vector, pGEMHE, as earlier (4, 15). Fully synthetic, nAChR h $\beta 2$ subunit GenBank JN565027 with nucleotide sequence optimized for better heterologous expression (h $\beta 2_{opt}$) was generated (GENEART, Burlingame, CA) and subcloned into the pCI vector (Promega, San Luis Obispo, CA) as earlier (4). Mutations in the nAChR subunits were introduced in the pGEMHE background using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotides used for a creation of the 9' mutant in the $\alpha 6$ subunit (L9'S; L280S) were 5'-cgc-ttgatttcagtcctgtctctctgactgtgttttgc-3' (forward) and 5'-gca-aaaacacagtcagagaagacagactgaaatacaagcg-3' (reverse). Similarly, oligonucleotides used for creation of the 9' mutant in the $\alpha 6$ subunit (L9'S; L280S) were 5'-ctcttgcatctcgttctgattctctcactgtcttttgc-3' (forward) and 5'-gcaaaaagacagtgagagaactcagaacggagatgcaaagag-3' (reverse). Also, a 13' mutation in the $\alpha 6$ subunit (V13'S; V284S) was created by using 5'-cgttctgcttctctcactagcttttctgctggtgattacag-3' (forward) and 5'-ctg-taactcaccagcaaaagcctagtgagagaagcagaacg-3' (reverse) oligonucleotides. Mutations in the N-terminal domain of the nAChR $\alpha 6$ subunit (*i.e.* N143D+M145V) were introduced as earlier (4, 15). This $\alpha 6$ subunit mutant (*i.e.* N143D+M145V) was

further subjected to a 9' mutation using the primers stated earlier. Identities of all wild-type or mutant subunits were confirmed by sequencing referenced to nucleotide/protein sequences available in GenBank.

All pGEMHE plasmids were linearized immediately downstream of the 3'-polyadenylation sequence. NheI was used to linearize nAChR $\alpha 6$, $\alpha 6^{L9'S}$, $\alpha 6(N143D+M145V)$, $\alpha 6(N143D+M145V)^{L9'S}$, h $\beta 3$, h $\beta 4$, m $\alpha 6$, m $\alpha 6^{L9'S}$, m $\alpha 6^{L13'S}$, m $\beta 2$, m $\beta 3$, and m $\beta 4$ subunit-containing plasmids, and SbfI was used for linearizing the h $\beta 2$ subunit-containing plasmid. SwaI was used to linearize h $\beta 2_{opt}$. Capped mRNA was transcribed from linearized plasmids in a reaction mixture (25 μ l) containing 1 \times transcription buffer, 1.6 mM rNTPs (Promega, WI), 0.5 mM m⁷G(5')ppp(5')G RNA Cap Structure Analog (New England Biolabs), 1 μ l of RNasin plus (New England Biolabs) and 1 μ l T7 RNA polymerase (New England Biolabs) following standard protocols or using mMESSAGE mMACHINE[®] T7 kit (Ambion/Invitrogen) and following the manufacturer's instructions. The 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). The 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 supplemented with 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. Ovarian lobes were cut into small pieces and digested with 0.08 Wünsch 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 \sim 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 \sim 60 nl. To express nAChR in oocytes, about 4 ng of cRNA corresponding to each subunit was injected; *i.e.* at ratios of 1:1 or 1:1:1 for binary or trinary receptors, respectively, with the exception that for coexpression of $\alpha 6(N143D+M145V)$ +h $\beta 2^*$ -nAChR in the presence or absence nAChR h $\beta 3$ subunit, about 10 ng of cRNA corresponding to each subunit including nAChR h $\beta 2_{opt}$ subunit was injected.

Oocyte Electrophysiology—Two to seven days after injection, oocytes were placed in a small-volume chamber and continuously perfused with oocyte Ringer's 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 an AxoClamp 900A and the pClamp 10.2 software (Axon Instruments, 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 an Axon Digidata1440A and the pClamp10.2 software. Electrodes contained 3 M KCl and had a resistance of 1–2 megaohms. Drugs (agonists and antagonists) were prepared daily in bath solution. Drug was 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 $\beta 3$ subunits with either an $\alpha 6$ or a mutant $\alpha 6$ subunit or $\beta 2$ or $\beta 4$ subunits (8–20 ng total of 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 as measures of functional nAChR expression and results 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 assessment 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, although 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 variation in oocytes, time between cRNA injection and recording, and subunit combination-specific parameters, such as open probability (influenced by gating rate constants, rates, and extents of desensitization), single channel conductance, assembly efficiency, and efficiency of receptor trafficking to the cell surface (24). 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 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 given in tables sometimes differ. However, when we make statements about results comparing ligand potencies and peak current amplitudes across subunit combinations, we do so for studies done under the same or very similar conditions, and the observations are clear, statistically significant, and in agreement whether for pooled data or for results from smaller sets of studies (one-way anal-

yses of variance followed by Tukey's multiple comparison tests).

RESULTS

Human nAChR $\alpha 6^{\text{L9'S}}$ Subunits Form Functional Receptors in Association with nAChR $h\beta 4$ and $h\beta 3$ Subunits with Increased Receptor Agonist Sensitivity and Efficacy—Earlier, we observed that oocytes coinjected with nAChR $h\alpha 6$ and $h\beta 4$ subunit cRNAs produce functional $h\alpha 6h\beta 4$ -nAChR in only a few out of many injected oocytes and then only have minimal responses to nicotinic agonists (4). Although we could measure a peak current of 22 ± 3 nA for $h\alpha 6h\beta 4$ -nAChR in response to 100 μM acetylcholine, we were unable to measure reliable and reproducible functional responses to nicotine. Also, oocytes injected with nAChR $h\alpha 6$, $h\beta 4$, and $h\beta 3$ subunit cRNAs do not produce reliable and reproducible functional $h\alpha 6h\beta 4h\beta 3$ -nAChR, suggesting that the small amount of function seen for $h\alpha 6h\beta 4$ -nAChR is either reduced or completely eliminated, probably due to $\beta 3$ subunits exerting a negative effect on function of $h\alpha 6h\beta 4$ -nAChR. We replicated those findings in the current work, and we also found that oocytes coexpressing nAChR $h\alpha 6^{\text{L9'S}}$ and $h\beta 4$ subunit cRNAs have marginally increased, but more reproducible, responses to nicotine (peak current of 32 ± 7 nA for $h\alpha 6^{\text{L9'S}}h\beta 4$ -nAChR in response to 100 μM nicotine; Fig. 1; Table 1). Thus, replacement of $h\alpha 6^{\text{L9'S}}$ for $h\alpha 6$ subunits does not have as great of a gain-of-function effect on $h\alpha 6h\beta 4$ -nAChR as does introduction of $h\beta 3^{\text{V9'S}}$ subunits (4) into otherwise wild-type $h\alpha 6h\beta 4$ -nAChR.

Consistent with our previous observations regarding introduction of gain-of-function $\beta 3$ subunits into $\alpha 6^*$ -nAChR (4), oocytes coexpressing nAChR $h\alpha 6^{\text{L9'S}}$ and $h\beta 4$ subunits and exposed to the nAChR noncompetitive antagonist and open channel blocker, mecamylamine, respond with an apparent outward peak current of 12 ± 5 nA (Table 1). Because mecamylamine coexposure more than blocks inward currents produced by nicotinic agonists, also leading under those conditions to production of apparent outward current responses, and does so in a concentration-dependent manner, we again interpret these effects as showing the ability of mecamylamine to block spontaneous opening of $h\alpha 6^{\text{L9'S}}h\beta 4$ -nAChR channels (Table 1). Given the magnitudes of peak current responses to nicotine alone and to mecamylamine alone, about 27% of $h\alpha 6^{\text{L9'S}}h\beta 4$ -nAChR appear to be spontaneously open at any given time ($(12 + 32) = 0.27$).

When nAChR $h\alpha 6^{\text{L9'S}}$ and $h\beta 4$ were coexpressed with $h\beta 3$ subunits instead of alone, oocyte responsiveness to nicotine (EC_{50} value of 0.9 μM) increases over 10-fold (to a peak current response of 350 ± 52 nA; Fig. 1, Table 1). This suggests that wild-type $\beta 3$ subunits incorporate into $h\alpha 6^{\text{L9'S}}h\beta 4$ -nAChR and strongly potentiate receptor function. However, this does not occur with a change in agonist potency upon $h\beta 3$ subunit incorporation into $h\alpha 6^{\text{L9'S}}h\beta 4$ -nAChR because there is not a significant change in nicotine EC_{50} values (Table 1). Outward current production in the same oocytes (9.5 ± 1.5 nA) in response to 1000 μM mecamylamine indicates that there is spontaneous opening of $h\alpha 6^{\text{L9'S}}h\beta 4h\beta 3$ -nAChR, but levels of spontaneous opening are comparable with those for $h\alpha 6^{\text{L9'S}}h\beta 4$ -nAChR in the absence of $h\beta 3$ subunits, indicating

Gain-of-function $\alpha 6^{L9'S}$ -Nicotinic Receptor and $\beta 3$ Subunits

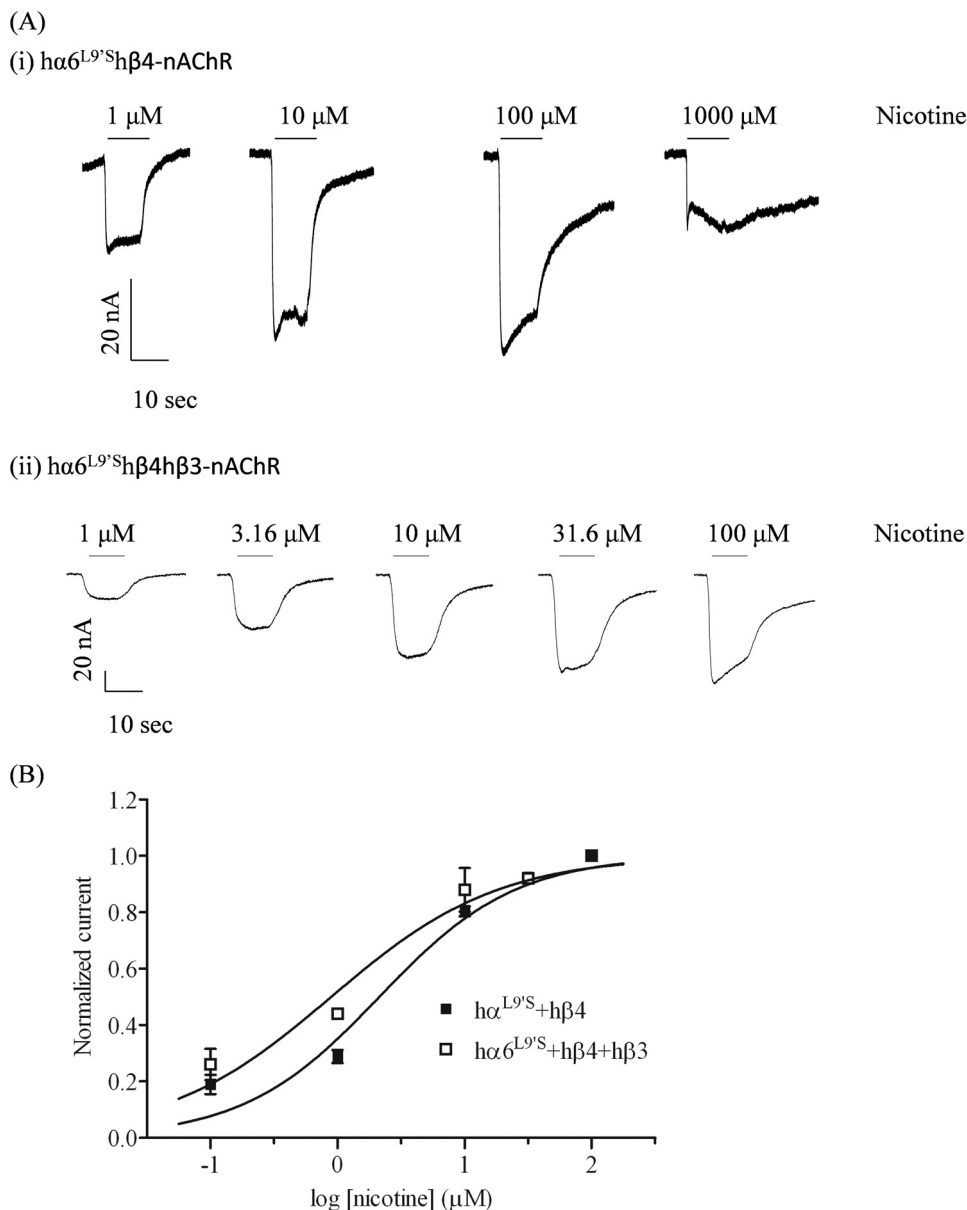


FIGURE 1. **Functional properties of $\alpha 6^{L9'S}$ -nAChR.** *A*, representative traces are shown for inward currents in oocytes held at -70 mV, responding to application at the indicated concentrations of nicotine (shown with the duration of drug exposure as *black bars* above the traces), and expressing nAChR $h\alpha 6^{L9'S}$ and $h\beta 4$ subunits (*i*) or nAChR $h\alpha 6^{L9'S}$, $h\beta 4$, and $h\beta 3$ subunits (*ii*). *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 μ M) for inward current responses to nicotine as indicated for oocytes expressing nAChR $h\alpha 6^{L9'S}$ and $h\beta 4$ subunits alone (\blacksquare) or with $h\beta 3$ subunits (\square), where current amplitudes are represented as a fraction of the peak inward current amplitude in response to the most efficacious concentration of nicotine. Much higher levels of evoked currents are evident for functional nAChR containing $h\alpha 6^{L9'S}$, $h\beta 4$, and $h\beta 3$ subunits when compared with receptors lacking $h\beta 3$ subunits. See Table 1 for parameters.

that a smaller proportion of $h\alpha 6^{L9'S}h\beta 4h\beta 3$ -nAChR is spontaneously open at any time ($9.5/(9.5 + 350) = 0.026$; Table 1) than for $h\alpha 6^{L9'S}h\beta 4$ -nAChR. No function was observed in response to nicotine or mecamylamine in oocytes coexpressing nAChR $h\alpha 6$ or $h\alpha 6^{L9'S}$ subunits plus $h\beta 2$ subunits with or without $h\beta 3$ subunits.

Mouse nAChR $\alpha 6^{V13'S}$ Subunits Form Functional Receptors in Association with nAChR $m\beta 4$ and $m\beta 3$ Subunits with Increased Receptor Agonist Sensitivity and Efficacy—We have shown earlier that oocytes coinjected with $m\alpha 6$ and $m\beta 4$ nAChR subunit cRNAs form functional nAChR, but with minimal responses to nicotinic agonists, and function is further reduced in the presence of nAChR $m\beta 3$ subunits, indicating

that nAChR $m\beta 3$ subunits exert dominant-negative effects on the function of $m\alpha 6m\beta 4^*$ -nAChR (4). Here, we observed that oocytes coexpressing either wild-type $m\alpha 6$ or mutant $m\alpha 6^{L9'S}$ along with $m\beta 4$ subunits give comparably modest peak current responses to 100μ M nicotine ($I_{max} = 27 \pm 7$ or 29 ± 1 nA, respectively; Table 1). Thus, although oocytes expressing $m\alpha 6^{L9'S}$ and $m\beta 4$ subunits give outward current responses to mecamylamine, consistent with spontaneous channel opening, the 9' mutation in the nAChR $m\alpha 6$ subunit does not significantly increase the magnitude of functional responsiveness (Table 1). Similarly, there is no increase in functional responsiveness to nicotine for oocytes coexpressing nAChR $m\alpha 6^{L9'S}$, $m\beta 4$, and $m\beta 3$ subunits (peak current = 26 ± 4 nA), although

TABLE 1

Parameters for agonist or antagonist action at nAChR containing gain-of-function $\alpha 6$ mutant subunits

Potencies (micromolar EC_{50} or IC_{50} values with 95% confidence intervals), Hill coefficients ($n_H \pm$ S.E.), mean \pm S.E. efficacies (two-electrode voltage-clamp peak responses, I_{max} , in nanoamperes), and concentrations where maximal peak current amplitudes (I_{max} concentration in micromolar) are achieved (μM) are provided for nicotine as an agonist or mecamylamine as an antagonist 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 the figures. *Closed up arrows* or *closed down arrows* indicate a significant ($p < 0.05$) increase or decrease, respectively, in potency or efficacy of the indicated agent at the indicated nAChR subtype relative to nAChR containing the wild type $\alpha 6$ subunit. *Filled triangle* indicates a significant increase in indicated agonist or antagonist potency or efficacy at the indicated nAChR containing $\alpha 6^{L9'S}$ or $\alpha 6^{V13'S}$ subunits relative to the same complex but lacking $\beta 3$ subunits. *Open up arrows* or *open down arrows* indicate a significant increase or decrease, respectively, in potency or efficacy of the indicated agonist at the indicated nAChR containing $\alpha 6^{V13'S}$ subunits relative to nAChR containing $\alpha 6^{L9'S}$ subunits. Note that no or very rare and then small responses to nicotine were seen for the following subunit combinations ($n = 6-9$ each): $\alpha 6$ or $\alpha 6^{L9'S}$ plus $\beta 2$ alone or with $\beta 3$; $\alpha 6$ or $\alpha 6^{L9'S}$ or $\alpha 6^{V13'S}$ plus $\beta 2$ alone or with $\beta 3$; and $\alpha 6$ or $\alpha 6^{L9'S}$ plus $\beta 2$ alone or with $\beta 3$. - indicates that absent or inconsistent functional responses in two-electrode voltage-clamp studies precluded determination of the parameter of interest; # indicates data from (4).

Drug	nAChR subunit combinations	Potency			Peak response			
		n	EC_{50} or IC_{50} (95% CI) (μM)	$n_H \pm$ SE	n	Mean $I_{max} \pm$ SE (nA)	I_{max} Conc. (μM)	
Nicotine	$\alpha 6 + \beta 4$	3	-	-	-	-	100	
	$\alpha 6^{L9'S} + \beta 4$	5	2.1 (1.5-3.1)	0.81 \pm 0.1	5	32 \pm 7 \uparrow	100	
	$\alpha 6 + \beta 4 + \beta 3$ #	9	-	-	9	-	-	
	$\alpha 6^{L9'S} + \beta 4 + \beta 3$	3	0.89 (0.53-1.5)	0.66 \pm 0.09	3	350 \pm 52 $\uparrow\blacktriangle$	100	
	$\alpha 6 + \beta 4$	3	26 (14-50)	0.65 \pm 0.13	3	27 \pm 7	1000	
	$\alpha 6^{L9'S} + \beta 4$	3	-	-	3	29 \pm 1	100 \uparrow	
	$\alpha 6^{V13'S} + \beta 4$	9	-	-	9	- $\downarrow\downarrow$	100 \uparrow	
	$\alpha 6 + \beta 4 + \beta 3$ #	9	-	-	9	-	-	
	$\alpha 6^{L9'S} + \beta 4 + \beta 3$	3	-	-	3	26 \pm 4 \uparrow	100 \uparrow	
	$\alpha 6^{V13'S} + \beta 4 + \beta 3$	3	1.2 (1-1.4) \uparrow	0.97 \pm 0.07	3	800 \pm 190 $\uparrow\blacktriangle\uparrow$	100 \uparrow	
	$\alpha 6 + \beta 4 + \beta 3$	4	18 (12-28)	0.62 \pm 0.07	4	360 \pm 130	1000	
	$\alpha 6^{L9'S} + \beta 4 + \beta 3$	4	0.48 (0.33-0.7) \uparrow	1.1 \pm 0.17	3	680 \pm 32 $\uparrow\blacktriangle$	100 \uparrow	
	$\alpha 6 + \beta 4$	9	-	-	9	-	1000	
	$\alpha 6^{L9'S} + \beta 4$	6	3.1 (2.1-4.6)	0.75 \pm 0.09	6	80 \pm 18 \uparrow	100 \uparrow	
	$\alpha 6 + \beta 4 + \beta 3$	7	14 (9.6-21)	0.70 \pm 0.08	8	57 \pm 7	1000	
	$\alpha 6^{L9'S} + \beta 4 + \beta 3$	4	2.3 (1.7-2.9) \uparrow	0.93 \pm 0.09	4	870 \pm 270 $\uparrow\blacktriangle$	100 \uparrow	
	$\alpha 6(N143D+M145V)^{L9'S} + \beta 2$	3	-	-	3	-	100	
	$\alpha 6(N143D+M145V)^{L9'S} + \beta 2 + \beta 3$	3	0.02 (0.01-0.03) \uparrow	1.1 \pm 0.12	5	98 \pm 21 \uparrow	1 \uparrow	
	Mecamylamine	$\alpha 6^{V9'S} + \beta 4$	5	-	-	5	12 \pm 5	1000
		$\alpha 6^{V9'S} + \beta 4 + \beta 3$	5	-	-	5	9.5 \pm 1.5	1000
$\alpha 6^{L9'S} + \beta 4$		9	-	-	3	7.8 \pm 2.3	1000	
$\alpha 6^{V13'S} + \beta 4$		9	-	-	9	-	1000	
$\alpha 6^{L9'S} + \beta 4 + \beta 3$		9	-	-	3	12 \pm 3	1000	
$\alpha 6^{V13'S} + \beta 4 + \beta 3$		3	-	-	3	9.0 \pm 0.5	1000	
$\alpha 6^{L9'S} + \beta 4 + \beta 3$		4	-	-	4	8.5 \pm 3.3	1000	
$\alpha 6^{L9'S} + \beta 4$		3	-	-	4	12 \pm 9	1000	
$\alpha 6^{L9'S} + \beta 4 + \beta 3$		3	-	-	3	41 \pm 15 \blacktriangle	100 \blacktriangle	

mecamylamine-induced outward current indicated that there is spontaneous opening of $\alpha 6^{L9'S}\beta 4\beta 3$ -nAChR (Table 1).

By contrast, unexpectedly, and interestingly, oocytes expressing nAChR $\alpha 6^{V13'S}$, $\beta 4$, and $\beta 3$ subunits exhibit a >25 -fold increase in peak current responses to nicotine (800 ± 190 nA) relative to responses of $\alpha 6^{L9'S}\beta 4$ - or $\alpha 6^{L9'S}\beta 4\beta 3$ -nAChR. Also, we were able to define an increase in nicotine potency when acting at $\alpha 6^{V13'S}\beta 4\beta 3$ -nAChR ($EC_{50} = 1.2 \mu M$) relative to nicotine potency at $\alpha 6\beta 4$ -nAChR (EC_{50} value of $26 \mu M$; Table 1, Fig. 2). In addition, there also is spontaneous opening of $\alpha 6^{V13'S}\beta 4\beta 3$ -nAChR, although responses to nicotine or mecamylamine are absent for $\alpha 6^{V13'S}\beta 4$ -nAChR (Table 1). No function was observed in response to nicotine or mecamylamine in oocytes coexpressing nAChR $\alpha 6$ or $\alpha 6^{L9'S}$ or $\alpha 6^{V13'S}$ subunits plus $\beta 2$ subunits with or without $\beta 3$ subunits.

Mouse nAChR $\alpha 6^{L9'S}$ Subunits Form Functional Receptors in Association with nAChR $\beta 4$ and $\beta 3$ Subunits with Increased Receptor Agonist Sensitivity and Efficacy—We reported earlier that oocytes coinjected with nAChR $\alpha 6$, $\beta 4$, and $\beta 3$ subunit cRNAs tend to form functional $\alpha 6\beta 4\beta 3$ -nAChR, whereas oocytes coexpressing nAChR $\alpha 6$ and $\beta 4$ subunit cRNAs do not respond to nicotinic agonists (4). Here, we show that oocytes coexpressing nAChR $\alpha 6^{L9'S}$ and $\beta 4$ subunits yield peak function of 80 ± 18 nA in response to $100 \mu M$ nicotine (Fig. 3, Table 1) and outward current responses (12 ± 9 nA) to mecamylamine, consistent with spontaneous channel opening of $\alpha 6^{L9'S}\beta 4$ -nAChR (Table 1). Moreover, oocytes coexpressing nAChR $\alpha 6^{L9'S}$, $\beta 4$, and $\beta 3$ subunits respond to nicotine with an EC_{50} value of $2.3 \mu M$ and give an even larger peak current (870 ± 270 nA; Fig. 3; Table 1). Also, oocytes coexpressing $\alpha 6^{L9'S}$, $\beta 4$, and $\beta 3$ subunits give relatively large, outward current responses (peak current of 41 ± 15 nA)

Gain-of-function $\alpha 6^*$ -Nicotinic Receptor and $\beta 3$ Subunits

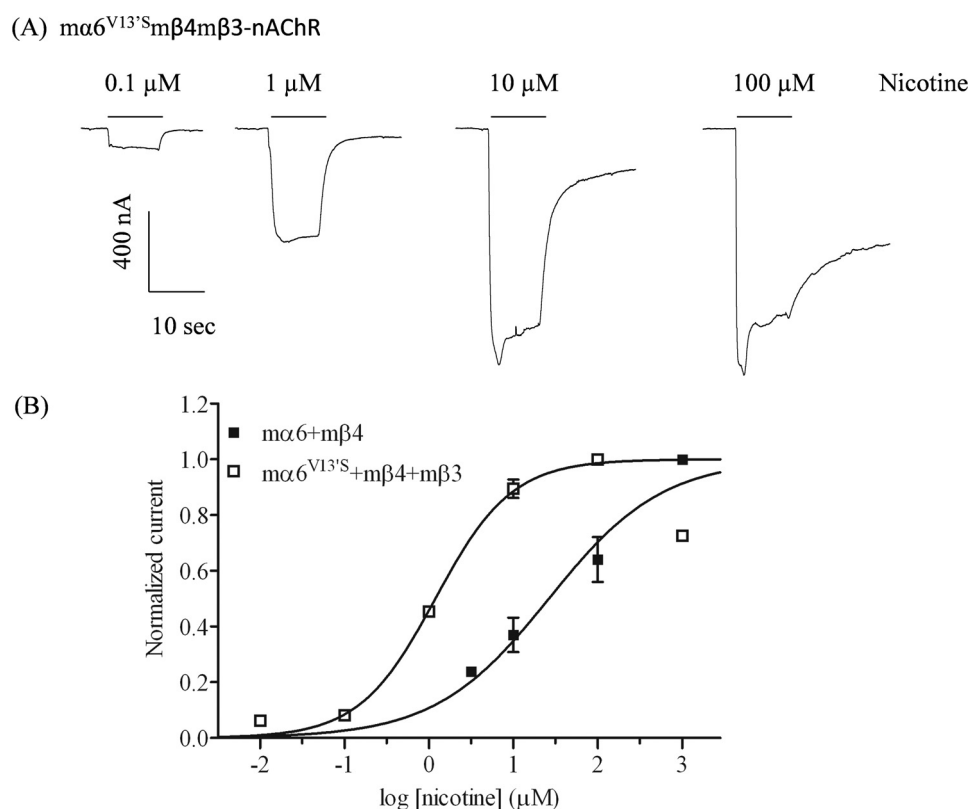


FIGURE 2. **Functional properties of $\alpha 6^*$ -nAChR.** A, representative traces are shown for inward currents in oocytes held at -70 mV, responding to application at the indicated concentrations of nicotine (shown with the duration of drug exposure as *black bars* above the traces), and expressing nAChR $\alpha 6^{V13'S}$, $m\beta 4$, and $m\beta 3$ subunits. 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 inward current responses to nicotine as indicated for oocytes expressing nAChR $\alpha 6$ and $m\beta 4$ subunits (■) or $\alpha 6^{V13'S}$ and $m\beta 4$ and $m\beta 3$ subunits (□), where current amplitudes are represented as a fraction of the peak inward current amplitude in response to the most efficacious concentration of nicotine. Much higher levels of evoked currents are evident for functional nAChR containing $\alpha 6^{V13'S}$, $m\beta 4$, and $m\beta 3$ subunits when compared with receptors lacking $\alpha 6^{V13'S}$ subunits. See Table 1 for parameters.

to mecamylamine, again an indication that functional and spontaneously opening $\alpha 6^{L9'S}h\beta 4h\beta 3$ -nAChR are formed (Table 1). The kinetics of traces generated in response to application of nicotine differs between $\alpha 6^{L9'S}h\beta 4$ - and $\alpha 6^{L9'S}h\beta 4h\beta 3$ -nAChR. $\alpha 6^{L9'S}h\beta 4h\beta 3$ -nAChR in response to activation by $1000 \mu\text{M}$ nicotine exhibit a tail current that is significantly reduced or absent in $\alpha 6^{L9'S}h\beta 4$ -nAChR when activated by the same concentration of nicotine. Precisely, in the presence of $h\beta 3$ subunits, there is a pronounced functional block of the receptor at higher concentration of nicotine. Removal of the functional block imposed by nicotine (probably acting as an open channel blocker), as a result of switching out to buffer application, leads to activation of the receptor by the residual nicotine that results in formation of a tail current. This is not unusual given that incorporation of accessory subunits into nAChR differentially affects various functional characteristics of the receptor (25).

Minimal and inconsistent nAChR function was observed when $\alpha 6^{L9'S}$ subunits were coexpressed with $h\beta 2$ and $h\beta 3$ subunits. This is in contrast to our earlier observation that nAChR $\alpha 6$ subunits along with $h\beta 2$ subunits and gain-of-function $h\beta 3$ subunits ($h\beta 3^{V9'S}$; $h\beta 3^{V273S}$) form functional $\alpha 6h\beta 2h\beta 3^{V9'S}$ -nAChR (4). This suggests that the gain-of-function mutation in the $\alpha 6^{L9'S}$ subunit is inadequate to overcome what seems to be a strong, dominant-negative effect of nAChR $\beta 3$ subunits in the presence of $\beta 2$ subunits.

Mouse nAChR $\alpha 6^{L9'S}$ Subunits Form Functional Receptors in Association with nAChR $m\beta 4$ and $h\beta 3$ Subunits with Increased Receptor Agonist Sensitivity and Efficacy—Oocytes coinjected with nAChR $\alpha 6$, $m\beta 4$, and $h\beta 3$ subunit cRNAs give >10 -fold larger and ~ 2 -fold more sensitive responses to nicotinic agonists than do oocytes coinjected $\alpha 6$ and $m\beta 4$ subunit cRNAs and in stark contrast to the elimination of functional responses in oocytes coexpressing nAChR $\alpha 6$, $m\beta 4$, and $m\beta 3$ subunits (Table 1). Interestingly, here we found that oocytes coexpressing nAChR $\alpha 6^{L9'S}$, $m\beta 4$, and $h\beta 3$ subunits responded to nicotine with an EC_{50} value of $0.48 \mu\text{M}$ and with large peak currents 680 ± 32 nA; Fig. 4; Table 1) and gave outward current responses (8.5 ± 3.3 nA) when exposed to $1000 \mu\text{M}$ mecamylamine (Table 1). No function was observed when $\alpha 6^{L9'S}$ subunits were coexpressed with $m\beta 2$ and $h\beta 3$ subunits.

Human nAChR $\alpha 6(N143D+M145V)^{L9'S}$ Subunits Form Functional Receptors in Association with nAChR $h\beta 2$ and $h\beta 3$ Subunits with Increased Receptor Agonist Sensitivity and Efficacy—Earlier (4), we had shown that mutations in the N-terminal domain of the nAChR $\alpha 6$ subunit enable nAChR $h\beta 3^{V9'S}$ subunits to exert a gain-of-function effect at $\alpha 6(N143D+M145V)h\beta 2^*$ -nAChR (*i.e.* $\alpha 6(N143D+M145V)h\beta 2h\beta 3^{V9'S}$ -nAChR are functional). This finding led us to explore effects of incorporation of $h\beta 3$ subunits into $\alpha 6(N143D+M145V)^{L9'S}h\beta 2^*$ -nAChR.

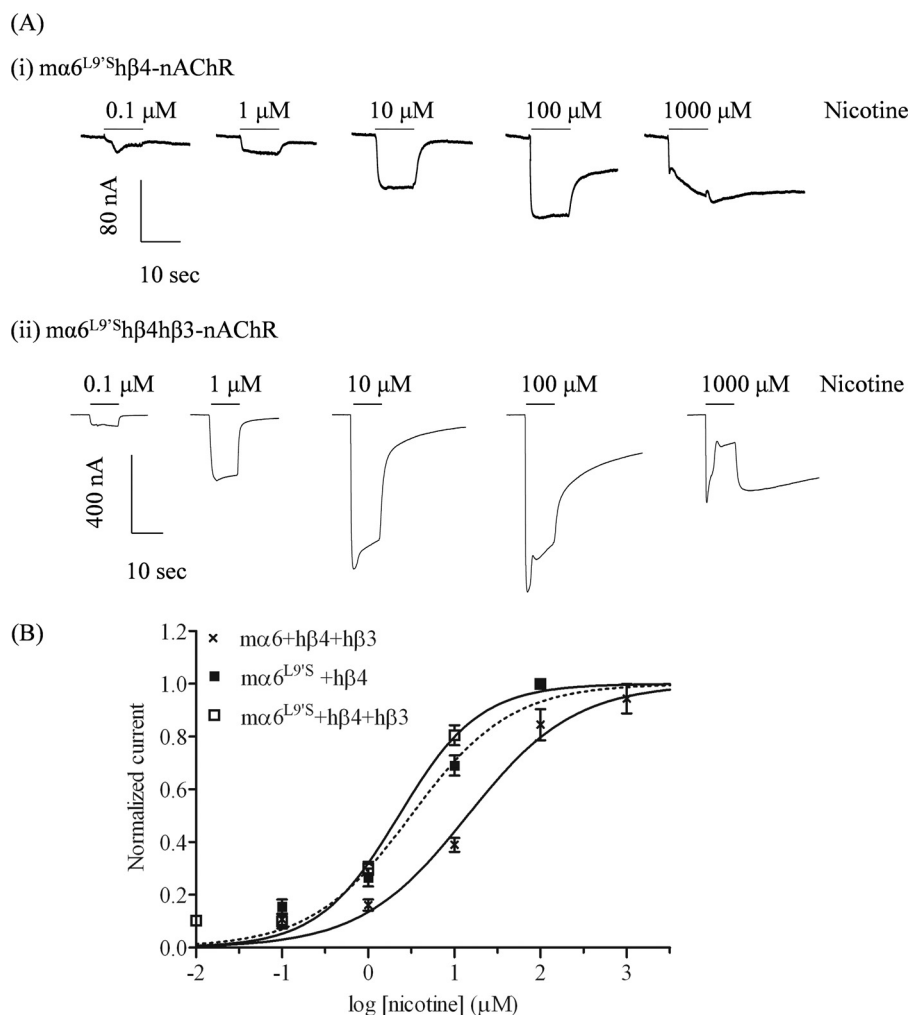


FIGURE 3. **Functional properties of hybrid $\alpha 6^*h\beta 4^*$ -nAChR.** *A*, representative traces are shown for inward currents in oocytes held at -70 mV, responding to application at the indicated concentrations of nicotine (shown with the duration of drug exposure as *black bars* above the traces), and expressing nAChR $\alpha 6^{L9'S}$ and $h\beta 4$ subunits (*i*) or nAChR $\alpha 6^{L9'S}$, $h\beta 4$, and $h\beta 3$ subunits (*ii*). *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 inward current responses to nicotine as indicated for oocytes expressing nAChR $\alpha 6^{L9'S}$ and $h\beta 4$ subunits alone (\blacksquare) or with $h\beta 3$ subunits (\square) or expressing $\alpha 6$ and $h\beta 4$ and $h\beta 3$ subunits (\times ; data from Ref. 4), where current amplitudes are represented as a fraction of the peak inward current amplitude in response to the most efficacious concentration of nicotine. Much higher levels of evoked currents are evident for functional nAChR containing $\alpha 6^{L9'S}$, $h\beta 4$, and $h\beta 3$ subunits when compared with receptors lacking $h\beta 3$ subunits. See Table 1 for parameters.

Oocytes injected with nAChR $\alpha 6(N143D+M145V)^{L9'S}$ and $h\beta 2$ subunit cRNAs did not yield functional nicotinic responses (Table 1). However, oocytes injected with $h\beta 3$ subunit cRNAs along with nAChR $\alpha 6(N143D+M145V)^{L9'S}$ and $h\beta 2$ subunit cRNAs yielded functional responses. Oocytes coexpressing nAChR $\alpha 6(N143D+M145V)^{L9'S}$, $h\beta 2$, and $h\beta 3$ subunits responded to nicotine with an EC_{50} value of $0.02 \mu\text{M}$ and with a maximal peak current of 98 ± 21 nA (Fig. 5; Table 1). We also found that oocytes coexpressing nAChR $\alpha 6(N143D+M145V)^{L9'S}$, $h\beta 2$, and $h\beta 3$ gave outward current responses when exposed to $1000 \mu\text{M}$ mecamylamine (data not shown).

DISCUSSION

Recent studies have investigated how nAChR $\beta 3$ subunits might incorporate as accessory partners into nAChR subtypes, specifically into $\alpha 6^*$ -nAChR (4). To further understand how $\beta 3$ subunits might incorporate into $\alpha 6^*$ -nAChR, we exploited the gain-of-function/reporter mutant strategy

(4, 15, 26) to reveal whether $\beta 3$ subunits integrate into $\alpha 6^*$ -nAChR complexes that are on the cell surface and functional. This approach allows us to 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 (15). In addition, we based the current studies on our findings (4) that (i) incorporation of nAChR $\beta 3$ subunits into $\alpha 6^*$ -nAChR, mouse or human, has a dominant-negative effect; (ii) incorporation of nAChR $h\beta 3$ subunits into $\alpha 6^*h\beta 4^*$ - or $\alpha 6^*h\beta 4^*$ -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 $\alpha 6(N143D+M145V)$ - $h\beta 2h\beta 3^{V9'S}$ -nAChR.

Gain-of-function $\alpha 6^*$ -Nicotinic Receptor and $\beta 3$ Subunits

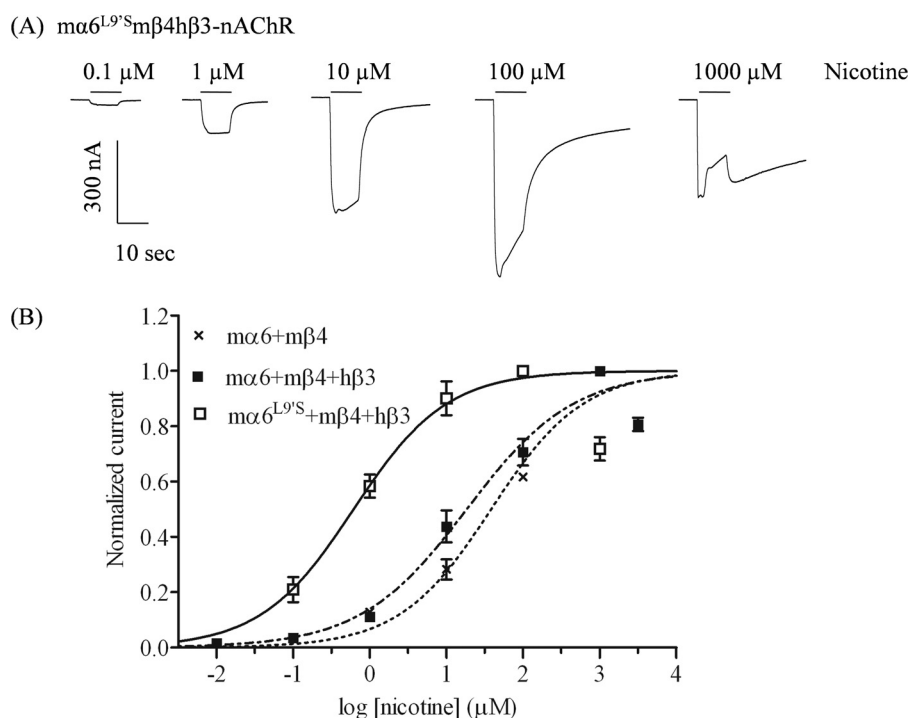


FIGURE 4. Functional properties of gain-of-function hybrid $\alpha 6^*h\beta 3^*$ -nAChR. *A*, representative traces are shown for inward currents in oocytes held at -70 mV, responding to application at the indicated concentrations of nicotine (shown with the duration of drug exposure as *black bars* above the traces), and expressing nAChR $\alpha 6^{L9'S}$, $m\beta 4$ and $h\beta 3$ subunits. *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 inward current responses to nicotine as indicated for oocytes expressing nAChR $\alpha 6$ and $m\beta 4$ subunits alone (\times) or with $h\beta 3$ subunits (\blacksquare) or expressing $\alpha 6^{L9'S}$ and $m\beta 4$ and $h\beta 3$ subunits (\square), where current amplitudes are represented as a fraction of the peak inward current amplitude in response to the most efficacious concentration of nicotine. Much higher levels of evoked currents are evident for functional nAChR containing $\alpha 6^{L9'S}$, $h\beta 4$, and $h\beta 3$ subunits when compared with receptors lacking $h\beta 3$ subunits. See Table 1 for parameters.

The principal findings of this study, whenever functional expression levels are adequate to allow comparisons, and with exceptions that could be informative as discussed below, are: (i) that introduction of 9' or 13' mutations into the second transmembrane domain of $\alpha 6$ or $h\alpha 6$ subunits typically has a gain-of-function effect, leading to production of ($\alpha 6$ or $\alpha 6(\text{N143D}+\text{M145V})^{(L9'S \text{ or } V13'S)}$)($\beta 2$ or $\beta 4$)*-nAChR that have 6–34-fold higher sensitivity to nicotine and much higher levels of function than do nAChR containing the same subunit combinations but with wild-type $\alpha 6$ subunits; (ii) that incorporation of $\beta 3$ subunits into ($\alpha 6$ or $\alpha 6(\text{N143D}+\text{M145V})^{(L9'S \text{ or } V13'S)}$)($\beta 2$ or $\beta 4$)*-nAChR typically increases levels of receptor function with or without concomitant increase in agonist potency; and (iii) that gain-of-function mutations in $\alpha 6$ or $\alpha 6(\text{N143D}+\text{M145V})$ subunits still do not allow for formation of functional $\alpha 6^{(L9'S \text{ or } V13'S)}$ $\beta 2$ -nAChR complexes, thus continuing to confound assessments of roles played by $\beta 3$ subunits in modulation of $\alpha 6\beta 2^*$ -nAChR.

The amount of functional expression for $h\alpha 6^{L9'S}h\beta 4$ -, $\alpha 6^{L9'S}m\beta 4$ -, or $\alpha 6^{L9'S}h\beta 4$ -nAChR is modest in absolute terms (27–80-nA peak current). However, with the exception of the insignificant difference in the magnitude of function seen for all-mouse $\alpha 6^{L9'S}m\beta 4$ - and $\alpha 6m\beta 4$ -nAChR, the increase in function upon expression with the $\alpha 6$ subunit 9' mutants is remarkable because of the lack of reliable function for wild-type, all human $h\alpha 6h\beta 4$ -, or hybrid $\alpha 6h\beta 4$ -nAChR. The little-if-any function for all-wild-type $\alpha 6\beta 4$ -nAChR complicates quantitative assessment of effects of $\alpha 6$ subunit gain-

of-function mutations on agonist potency, although qualitatively, nicotine EC_{50} values are over $10 \mu\text{M}$ for $\alpha 6\beta 4$ -nAChR and never higher than $3.1 \mu\text{M}$ for $\alpha 6^{(L9'S \text{ or } V13'S)}\beta 4$ -nAChR. However, gain-of-function effects manifest as increases in agonist potency and in peak current magnitudes are very clear based on comparisons of $h\alpha 6h\beta 4h\beta 3$ - with $h\alpha 6^{L9'S}h\beta 4h\beta 3$ -nAChR and comparisons of $\alpha 6h\beta 4h\beta 3$ - with $\alpha 6^{L9'S}h\beta 4h\beta 3$ -nAChR. A difference in agonist potency is also clear for comparison of $\alpha 6m\beta 4h\beta 3$ - with $\alpha 6^{L9'S}m\beta 4h\beta 3$ -nAChR, although there is only a 2-fold difference in peak current response across these receptors, partly due to the relatively high absolute levels of function for the hybrid $\alpha 6m\beta 4h\beta 3$ -nAChR. Once again, however, all-mouse $\alpha 6\beta 4\beta 3$ -nAChR are outliers because there is only modest function for $\alpha 6^{L9'S}m\beta 4m\beta 3$ -nAChR, although there is no reliable function for the all-wild-type analog, $\alpha 6m\beta 4m\beta 3$ -nAChR.

Nevertheless, and very interestingly, for all-mouse $\alpha 6^*$ -receptors, although there is not reproducible function for $\alpha 6^{V13'S}m\beta 4$ -nAChR, there are increases both in agonist potency and in response magnitude for $\alpha 6^{V13'S}m\beta 4m\beta 3$ -nAChR when compared with those parameters for any form of $\alpha 6m\beta 4$ -nAChR or for $\alpha 6m\beta 4m\beta 3$ - or $\alpha 6^{L9'S}m\beta 4m\beta 3$ -nAChR. Our initial studies of mouse $\alpha 6^*$ -nAChR were prompted because of the reported difficulties in heterologous expression of all-human $\alpha 6^*$ -nAChR and because so many data on naturally expressed $\alpha 6^*$ -nAChR function came from studies using rodents, but we have found all-mouse $\alpha 6^*$ -nAChR no easier to express than human $\alpha 6^*$ -nAChR. Expression of hybrid

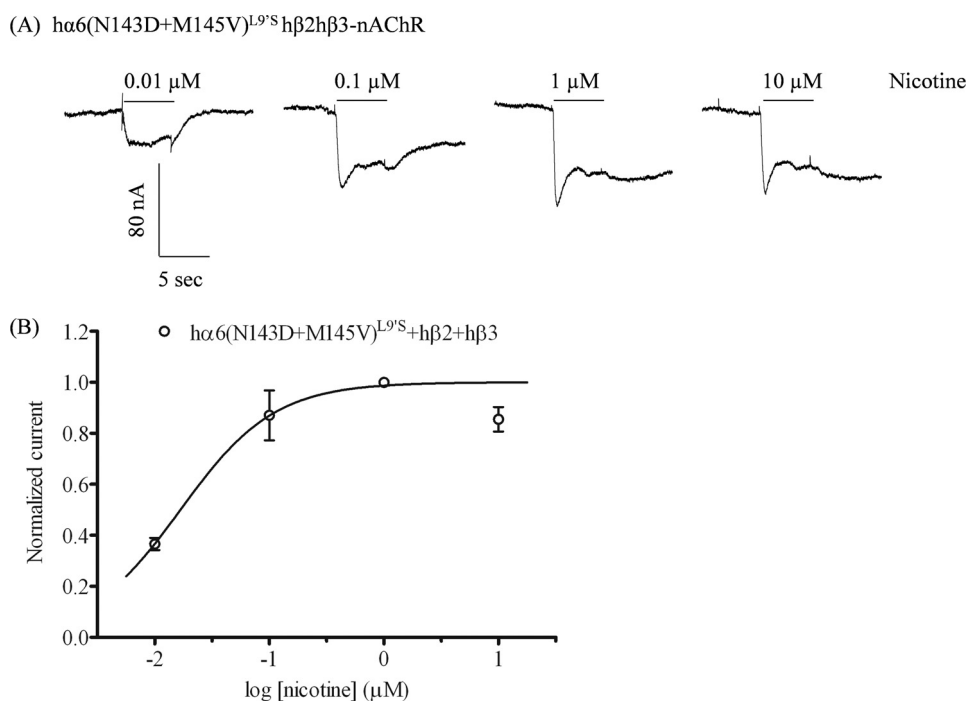


FIGURE 5. **Functional properties of gain-of-function $\text{h}\alpha 6(\text{N143D}+\text{M145V})^{\text{L9'S}}\text{h}\beta 3^*\text{-nAChR}$.** *A*, representative traces are shown for inward currents in oocytes held at -80 mV, responding to application at the indicated concentrations of nicotine (shown with the duration of drug exposure as *black bars* above the traces), and expressing nAChR $\text{h}\alpha 6(\text{N143D}+\text{M145V})^{\text{L9'S}}$, $\text{h}\beta 2$, and $\text{h}\beta 3$ subunits. *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 for oocytes expressing nAChR $\text{h}\alpha 6(\text{N143D}+\text{M145V})^{\text{L9'S}}$, $\text{h}\beta 2$, and $\text{h}\beta 3$ subunits (○), where current amplitudes are represented as a fraction of the peak inward current amplitude in response to the most efficacious concentration of nicotine. Much higher levels of evoked currents are evident for functional nAChR containing $\text{h}\alpha 6(\text{N143D}+\text{M145V})^{\text{L9'S}}$, $\text{h}\beta 2$, and $\text{h}\beta 3$ subunits when compared with receptors lacking $\text{h}\beta 3$ subunits. See Table 1 for parameters.

nAChR made up of subunits from different species has been more productive, suggesting that subtle differences for a given subunit across species in amino acid sequences in N-terminal, extracellular domains, but also in cytoplasmic and perhaps transmembrane domains, and at what must be at subunit interfaces not heretofore recognized as being functionally important, can strongly influence whether functional $\alpha 6^*$ -nAChR can be produced (4). The fact that $\text{m}\alpha 6^{\text{L9'S}}$ and V13'S mutations differing in position by just one turn in the second transmembrane domain α -helix can have such a large difference in their impact on $\text{m}\alpha 6\text{h}\beta 4^*\text{-nAChR}$ function indicates unexpectedly important roles for this channel-lining region in $\alpha 6^*\text{-nAChR}$ function. More work is warranted to more thoroughly characterize the bases for these influences.

Our findings demonstrate that $\alpha 6$ subunit L9'S or V13'S modifications can function as reporter and/or gain-of-function mutations, leading to production of receptors with heightened sensitivity to agonists, thus confirming the presence of $\alpha 6$ subunits in functional receptor complexes, as expected. These studies also further affirm and recapture the strategy applied to exploit gain-of-function $\alpha 6$ subunit mutations expressed *in vivo* to enhance sensitivity to agonists and thus to help reveal roles played by $\alpha 6^*\text{-nAChR}$ in dopaminergic pathways relevant to movement disorders and nicotine dependence (13).

This study was also initiated largely to assess whether effects previously described of nAChR $\beta 3$ subunit incorporation into $\alpha 6^*\text{-nAChR}$ would be preserved when receptor functional levels at baseline were intentionally elevated by using reporter mutation/gain-of-function $\alpha 6$ subunits as coexpression part-

ners. By contrast to earlier work by others (21), in which $\beta 3$ subunits were coexpressed in excess over other subunits, we chose to introduce equal amounts of subunit cRNAs into oocytes for the current work, anticipating that approximately equal amounts of subunit proteins would be made and that this more closely approximates conditions *in vivo*. We confirmed our previous observations (4, 15) that $\text{h}\beta 3$ subunit incorporation into $\text{h}\alpha 6\text{h}\beta 4^*\text{-nAChR}$ has an uncertain effect on functional expression, that $\text{m}\beta 3$ subunit incorporation into $\text{m}\alpha 6\text{h}\beta 4^*\text{-nAChR}$ has a dominant-negative effect on receptor function, that $\text{h}\beta 3$ subunit incorporation into hybrid $\text{m}\alpha 6\text{h}\beta 4^*\text{-nAChR}$ potentiates function, but that there is even larger potentiation of function when $\text{h}\beta 3$ subunits are incorporated into hybrid $\text{m}\alpha 6\text{h}\beta 4^*\text{-nAChR}$. However, with the exception of the lack of an obvious effect of $\text{m}\beta 3$ subunit incorporation on low functioning $\text{m}\alpha 6^{\text{L9'S}}\text{m}\beta 4^*\text{-nAChR}$, wild-type $\beta 3$ subunit incorporation into any of the tested $\alpha 6^{(\text{L9'S or V13'S})}\beta 4\text{-nAChR}$ potentiated levels of function by >11 -fold, notably including effects of $\text{h}\beta 3$ subunits on low functioning $\text{m}\alpha 6^{\text{L9'S}}\text{m}\beta 4^*\text{-nAChR}$ and effects of $\text{m}\beta 3$ subunits on $\text{m}\alpha 6^{\text{V13'S}}\text{m}\beta 4^*\text{-nAChR}$. These findings indicate that $\beta 3$ subunits do not always have dominant-negative effects on $\alpha 6^*\text{-nAChR}$ function as suggested earlier (21) and do not always promote formation of dead end, $\alpha 6\beta 4^*\text{-nAChR}$ intermediates as suggested previously (23). Instead, based on our results shown here, we can hypothesize that $\beta 3$ subunits seem to promote assembly, cell surface expression, and/or functional responsiveness of $\alpha 6\beta 4^*\text{-nAChR}$, at least when there is enough function for $\alpha 6\beta 4(\text{non-}\beta 3)\text{-nAChR}$ to allow assessment of effects of $\beta 3$ subunit incorporation. Our

findings using the oocyte expression system are in line with observations made regarding $\beta 3$ subunit effects on $\alpha 6^*$ -nAChR functional expression in cell lines (22), suggesting that successful, functional $\alpha 6\beta 4^*$ -nAChR expression in oocytes does not require coexpression with chaperones missing from oocytes but present in neurons or selected cell lines. Notably, although peak current potentiation upon substitution of gain-of-function $\alpha 6$ subunits (or $\beta 3$ subunits; see Refs. 4 and 15) occurs along with an increase in agonist potency, wild-type $\beta 3$ incorporation into complexes increases peak current responses without affecting agonist potency.

In almost every case, $\alpha 6^{(L9'S \text{ or } V13'S)^*}$ -nAChR spend a finite amount of time in a spontaneously open channel state, as judged by the ability of mecamylamine to block those open channels, giving the appearance of production of outward currents. This is a common feature for nAChR containing subunits with second transmembrane domain mutations that give gain-of-function effects (27, 28). Interestingly, the absolute magnitudes of responses to mecamylamine generally are quite similar across all the $\alpha 6^*$ -nAChR studied (7.8–12 nA), even when magnitudes of agonist-induced inward currents varied much more widely (26–800 nA). The only exceptions are for $m\alpha 6^{V13'S}m\beta 4$ -nAChR, which curiously have no reproducible responses to nicotine or to mecamylamine, despite there being strong responses upon incorporation of $m\beta 3$ subunits to form $m\alpha 6^{V13'S}m\beta 4m\beta 3$ -nAChR, and for $\alpha 6^{L9'S}h\beta 4h\beta 3$ -nAChR, which have slightly larger responses to mecamylamine (41 nA) but also have the largest responses to nicotine (870 nA).

Although the current findings support a role for $\beta 3$ subunits in potentiating function of $\alpha 6\beta 4^*$ -nAChR with at least a modicum of baseline functional activity, we were confounded in our studies of $\alpha 6\beta 2^*$ -nAChR by a general lack of function. This made it impossible to assess effects of $\beta 3$ subunit incorporation on $\alpha 6\beta 2^*$ -nAChR, but the results indicate that any gain-of-function earned by incorporation of $\alpha 6^{(L9'S \text{ or } V13'S)}$ subunits into complexes is inadequate to reveal effects of $\beta 3$ subunits, perhaps due to the surprising incompatibilities (illuminated in Refs. 4 and 15) that often occur in attempts to use $\alpha 6$, $\beta 2$, and $\beta 3$ subunits to form functional receptors. In order for us to show that in fact a variant of gain-of-function $\alpha 6$ subunit can partner with $h\beta 2$ and $h\beta 3$ subunit to form functional nAChR, we took advantage of our site-directed mutagenesis work (4, 15), which has implicated $\alpha 6$ residues 143 and 145 in the ability of $\beta 3$ subunits to affect $\alpha 6\beta 2^*$ -nAChR function. The $h\alpha 6(N143D+M145V)$ mutations change the indicated residues to those that are in the $m\alpha 6$ subunit and permit mutated $\alpha 6$ subunits to show function when coexpressed with $h\beta 2$ and $h\beta 3$ subunits when wild-type $h\alpha 6$ subunits do not. Human nAChR $\alpha 6$ subunit residues 143 and 145 are in the E1 domain, in loop E, on the (–) or complementary face of the subunit. This suggests that interactions between the $\alpha 6$ subunit (–) face with the (+) face from either $\beta 2$ subunits or $\beta 3$ subunits are important for functional $\alpha 6^*$ -nAChR expression. In order for us to prove that the nAChR $\beta 3$ subunit does affect the function of $\alpha 6\beta 2^*$ -nAChR, a 9' mutation was introduced into the $h\alpha 6(N143D+M145V)$ subunit. Although coexpression of $h\alpha 6(N143D+M145V)^{L9'S}$ and $h\beta 2$ subunits did not yield receptors with reliable function, upon inclusion of the $h\beta 3$ sub-

unit, function was evident in all oocytes coexpressing the three subunits together. These $h\alpha 6(N143D+M145V)^{L9'S}h\beta 2^*$ -nAChR mimic the gain-of-function, high-affinity $m\alpha 6^*$ -nAChR artificially expressed in mouse midbrain dopamine neurons (13).

We conclude, based on the current and previous findings, that gain-of-function/reporter mutations introduced into $\alpha 6$ subunits in $\alpha 6(\beta 2 \text{ or } \beta 4)\beta 3$ -nAChR are effective in potentiating receptor function. This potentiation yields receptors with higher agonist potency and larger magnitude responses to agonists, and also a finite likelihood of existing in a spontaneously open channel state. We also conclude from the present studies that wild-type $\beta 3$ subunit incorporation into functionally competent ($\alpha 6$ or $\alpha 6(N143D+M145V)$) $(L9'S \text{ or } V13'S)(\beta 4 \text{ or } \beta 2)^*$ -nAChR has a potentiating effect irrespective of whether there are dominant-negative, null, or potentiating effects of $\beta 3$ subunits on wild-type $\alpha 6(\beta 2 \text{ or } \beta 4)^*$ -nAChR. In fact, reliable expression of functional gain-of-function $\alpha 6^*$ -nAChR is achieved only in the presence of nAChR $\beta 3$ subunits. These results suggest that wild-type $\beta 3$ subunit coexpression is at least permissive for cell surface expression of $\alpha 6\beta 4^*$ -nAChR and very likely promotes function of these receptors. The strategies and results demonstrated here to increase function of $\alpha 6^*$ -nAChR to levels compatible with drug screening 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 (5, 13, 29–31).

Acknowledgments—We thank Dr. Jerry A. Stitzel (Department of Integrative Physiology, University of Colorado, Boulder, CO) for providing mouse nAChR subunits. We also thank Drs. Yongchang Chang and Paul Whiteaker of the Barrow Neurological Institute for comments about the project and manuscript and for technical advice and Minoti Bhakta for assistance.

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