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Modulation of Gain-of-function α6*-Nicotinic Acetylcholine Receptor by β3 Subunits

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**Background:** Function of physiologically important α6β3*-nicotinic receptor (nAChR) is differentially impacted by β3 subunits.

**Results:** nAChR expressed in several novel ways indicates that β3 subunits mostly potentiate gain-of-function α6*-nAChR.

**Conclusion:** Extracellular domain loop E region in α6 subunits governs the effect of β3 subunit on gain-of-function α6*-nAChR.

**Significance:** Novel α6β3*-nAChR receptor with gain-of-function, leucine- or valine-to-serine mutation could be used to assess and/or develop smoking cessation aids.

We previously have shown that β3 subunits either eliminate (e.g. for all-human (h) or all-mouse (m) α6β4β3-nAChR) or potentiate (e.g. for hybrid mα6hβ4β3- or mα6mβ4hβ3-nAChR containing subunits from different species) function of α6*-nAChR expressed in *Xenopus* oocytes, and that nAChR hα6 subunit residues Asn-143 and Met-145 in N-terminal domain loop E are important for dominant-negative effects of nAChR hβ3 subunits on hα6*-nAChR function. Here, we tested the hypothesis that these effects of β3 subunits would be preserved even if nAChR α6 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 β3 subunit incorporation. However, coexpression with β3 subunits potentiates rather than suppresses function of all-human, all-mouse, or hybrid α6β4/β3- or α6(N143D+M145V)β2*-nAChR. This contrasts with the lack of consistent function when α6(19’S) and β2 subunits are expressed alone or in the presence of wild-type β3 subunits. These results provide evidence that gain-of-function hα6hβ2*-nAChR (i.e. hα6(N143D+M145V)β2β3 nAChR) could be produced in *vitro*. These studies also indicate that nAChR β3 subunits can be assembly partners in functional α6*-nAChR and that 9’ or 13’ mutations in the nAChR α6 subunit second transmembrane domain can act as gain-of-function and/or reporter mutations. Moreover, our findings suggest that β3 subunit coexpression promotes function of α6*-nAChR.

Nicotinic acetylcholine receptors (nAChR)³ are pentameric ligand-gated ion channels expressed throughout the nervous system. Those other than the muscle-type (embryonic α1β1γδ- or adult α1β1γε-) nAChR are thought to be composed of different permutations of eight α subunits (α2-α7, α9-α10) and three β subunits (β2-β4) in humans (1). Of specific interest to us in this study are α6β3*-nAChR (where * indicates the known or possible presence of nAChR subunits other than those specified) (2–4). α6β3*-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 α6β3*-nAChR has been difficult to achieve despite strong evidence for expression of α6β3*-nAChR in rodent brain (3, 4, 6, 7, 10, 12–15). Functional expression of α6*-nAChR only has been achieved in *Xenopus* oocytes when using specific forms of mutant or chimeric subunits or in hybrid α6*-nAChR composed of subunits from different species (16–20). For example, function is achieved when chimeric, hα6/hα3 subunits (composed of the N-terminal, first extracellular domain of the hα6 subunit fused to the first transmembrane domain through to the C terminus of the hα3 subunit) are coexpressed with hβ2 or hβ4 subunits alone or in the presence of hβ3 subunits (19). α6*-nAChR are functional when expressed as hybrids of mouse and human α6 and other subunits, and there is function of some complexes containing β3 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 β3 subunits are incorporated into hybrid complexes, but this is in contrast to dominant-negative effects of coexpression with wild-type β3 subunits on function of α6β4*-nAChR when all subunits are from the same species (4, 21). There may be host cell specificity in some of these effects because nAChR hβ3 subunits promote expression and nicotine-induced up-regulation of h6*-nAChR in transfected cell lines (22).

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1 The abbreviations used are: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor(s); I_{max} peak current response; m, mouse; h, human.
Gain-of-function α6*-Nicotinic Receptor and β3 Subunits

We and others have taken advantage of gain-of-function mutations in the nAChR β3 subunit to produce functional nAChR, including those containing α6 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 β3V9S subunits increases agonist sensitivity and efficacy for α6*-nAChR. We hypothesized that similar mutations in nAChR α6 subunits would increase agonist sensitivity and efficacy of α6(V9S or V13S)(β4 or β2)*-nAChR to provide enough functional gain to facilitate evaluation of effects of wild-type β3 subunits on complexes and even to ensure that we can detect incorporation of wild-type β3 subunits into α6(V9S or V13S)h*nAChR. We also hypothesized that wild-type β3 subunits would have the same effects, dominant-negative or potentiating, depending on the subunit combination investigated, on gain-of-function α6(V9S or V13S)-nAChR as they did on wild-type α6*-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 β3 subunit incorporation facilitates formation of dead end intermediates (23). Our results indicated that whenever nAChR β3 subunits are incorporated into (α6 or ha6(N143D+M145V))V9S or V13S)-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 β3 subunits on wild-type α6*-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 α6, β2, β3, or β4 subunits were subcloned into the oocyte expression vector, pGEMHE, as earlier (4, 15). Fully synthetic, nAChR hβ2 subunit GenBank JN565027 with nucleotide sequence optimized for better heterologous expression (hβ2opt) was generated (GENEART, Burlingame, CA) and subcloned into the pCI vector (Promega, San Luis Obispo, CA) for 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 ha6 subunit (L9’S; L280S) were 5’-ccgtttgtatcgttcctcgtcagtctgttctggt3’ (forward) and 5’-gc-agaaacagcagacaagcagagttaataaagac3’ (reverse). Similarly, oligonucleotides were created for the 9’ mutant in the mβ6 subunit (L9’S; L280S) were 5’-cttcggtttatctcgttcgcgtctctcctggttctggt3’ (forward) and 5’-gcaaaacagcagacaagcagagttaataaagac3’ (reverse). Subsequently, similar oligonucleotides were used for creation of the 9’ mutant in the mβ6 subunit (L9’S; L280S) were 5’-cttcggtttatctcgttcgcgtctctcctggttctggt3’ (forward) and 5’-gcaaaacagcagacaagcagagttaataaagac3’ (reverse). Also, a 13’ mutation in the mβ6 subunit (V13’S; V284S) was created by using 5’-gc-gctctcggagtctcgtcgcctccctctcctggttctggt3’ (forward) and 5’-gcaaaacagcagacaagcagagttaataaagac3’ (reverse) oligonucleotides. Mutations in the N-terminal domain of the nAChR ha6 subunit (i.e. N143D+M145V) were introduced as earlier (4, 15). This ha6 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 ha6, ha6V9S, ha6(N143D+M145V), ha6(N143D+M145V)V9S, hβ3, hβ4, mβ6, mβ6V9S, mβ6L13S, mβ2, mβ3, and mβ4 subunit-containing plasmids, and SbfI was used for linearizing the hβ2 subunit-containing plasmid. Swal was used to linearize hβ2opt. Capped mRNA was transcribed from linearized plasmids in a reaction mixture (25 µl) containing 1X transcription buffer, 1.6 mm rNTPs (Promega, WI), 0.5 mm m7G(5’ppp(5’))G RNA Cap Structure Analog (New England Biolabs), 1 µl of RNAasin plus (New England Biolabs) and 1 µl 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 µg/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 40 µm in diameter. cRNA was drawn up into the micropipette and injected into oocytes using a Nanoject microinjection system (Drummond Scientific) at a total volume of ~60 nl. To express nAChR in oocytes, about 4 ng of cRNA corresponding to each 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 ha6(N143D+M145V)+hβ2*-nAChR in the presence or absence nAChR hβ3 subunit, about 10 ng of cRNA corresponding to each subunit including nAChR hβ2opt 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...
Gain-of-function α6*-Nicotinic Receptor and β3 Subunits

Human nAChR α6β4γδ Subunits Form Functional Receptors in Association with nAChR hα6 and hβ3 Subunits with Increased Receptor Agonist Sensitivity and Efficacy—Earlier, we observed that oocytes coinjected with nAChR hα6 and hβ4 subunit cRNAs produce functional hα6hβ4-γδnAChR 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 2 ± 3 nA for hα6hβ4-γδnAChR in response to 100 μM acetylcholine, we were unable to measure reliable and reproducible functional responses to nicotine. Also, oocytes injected with nAChR hα6, hβ4, and hβ3 subunit cRNAs do not produce reliable and reproducible functional hα6hβ4hβ3-γδnAChR, suggesting that the small amount of function seen for hβ3-γδnAChR is either reduced or completely eliminated, probably due to β3 subunits exerting a negative effect on function of hα6hβ4-γδnAChR. We replicated those findings in the current work, and we also found that oocytes coexpressing nAChR hα6γδ2 and hβ4 subunit cRNAs have marginally increased, but more reproducible, responses to nicotine (peak current of 32 ± 7 nA for hα6γδ2hβ4-γδnAChR in response to 100 μM nicotine; Fig. 1; Table 1). Thus, replacement of hα6γδ2 for hα6 subunits does not have as great of a gain-of-function effect on α6β4γδ-nAChR as does introduction of hβ3γδ2 subunits (4) into otherwise wild-type hα6hβ4γδ-nAChR.

Consistent with our previous observations regarding introduction of gain-of-function β3 subunits into α6*-nAChR (4), oocytes coexpressing nAChR hα6γδ2 and hβ4 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 α6β4γδ-nAChR channels (Table 1). Given the magnitudes of peak current responses to nicotine alone and to mecamylamine alone, about 27% of hα6β4-γδnAChR appear to be spontaneously open at any given time (12/ (12 + 32) = 0.27).

When nAChR hα6γδ2 and hβ4 were coexpressed with hβ3 subunits instead of alone, oocyte responsiveness to nicotine (EC50 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 β3 subunits incorporate into hα6β4γδ-nAChR and strongly potentiates receptor function. However, this does not occur with a change in agonist potency upon hβ3 subunit incorporation into hα6β4γδ-nAChR because there is not a significant change in nicotine EC50 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 hα6β4γδhβ3-γδnAChR, but levels of spontaneous opening are comparable with those for hα6β4γδ-nAChR in the absence of hβ3 subunits, indicating

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that a smaller proportion of h\(\alpha 6^{L.9}h\beta 4\)-nAChR is spontaneously open at any time (9.5/350 = 0.026; Table 1) than for h\(\alpha 6^{L.9}h\beta 4\)-nAChR. No function was observed in response to nicotine or mecamylamine in oocytes coexpressing nAChR h\(\alpha 6\), h\(\beta 4\), and h\(\beta 3\) subunits.

**Mouse nAChR \(\alpha 6^{V.13.5}\) Subunits Form Functional Receptors in Association with nAChR \(\beta 4\) and \(\beta 3\) 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 \(\beta 3\) subunits, indicating that nAChR \(\beta 3\) subunits exert dominant-negative effects on the function of m\(\alpha 6\)\(\beta 4\)-nAChR (4). Here, we observed that oocytes coexpressing either wild-type m\(\alpha 6\) or mutant m\(\alpha 6^{L.9.5}\) along with m\(\beta 4\) subunits give comparably modest peak current responses to 100 \(\mu M\) nicotine \((I_{\text{max}} = 27 \pm 7\) or 29 \(\pm 1\) nA, respectively; Table 1). Thus, although oocytes expressing m\(\alpha 6^{L.9.5}\) 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\(\alpha 6^{L.9.5}\), \(\beta 4\), and \(\beta 3\) subunits (peak current = 26 \(\pm 4\) nA), although...
Gain-of-function α6*-Nicotinic Receptor and β3 Subunits

**TABLE 1**

Parameters for agonist or antagonist action at nAChR containing gain-of-function α6 mutant subunits

| Drug        | nAChR subunit combinations | Potency | Peak response |
|-------------|----------------------------|---------|--------------|
|             |                           | n, Ec50 or IC50 (95% CI) |   | n, I, Max ± SE | I, Max Conc. (μM) |
| Nicotine    | h6α + h2β                 | 3       | -            | 5 | 32 ± 7   | 100 |
|             | h6αβ3 + h2β               | 5       | 2.1 (1.5-3.1) | 66 ± 0.09 | 350 ± 52 | 100 |
|             | h6αβ3 + hβ3               | 9       | -            | 9   | -      | - |
|             | h6αβ3 + h2β               | 3       | 0.89 (0.53-1.5) | 0.66 ± 0.09 | 327 ± 6 |
| [6α]β4      | 3                         | 26 (14-50) | 0.65 ± 0.13 | 327 ± 6 |
| [6αβ3]        | h2β                       | 9       | -            | 9   | -      | - |
| [6αβ3]        | hβ3                       | 3       | 1.2 (1-1.4)   | 0.97 ± 0.07 | 300 ± 190 |
| [6αβ3] + [6β4] + [β3] | 4 | 18 (12-28) | 0.62 ± 0.07 | 360 ± 130 |
| [6αβ3] + [6β4] + [β3] | 4 | 0.48 (0.33-0.7)   | 1.1 ± 0.17 | 680 ± 32 |
| [6α]β4      | 9                         | -       | -            | 9   | -      | - |
| [6αβ3] + [6β4] | 6 | 3.1 (2.1-4.6)  | 0.75 ± 0.09 | 80 ± 18 |
| [6αβ3] + [6β4] | 7 | 14 (9.6-21) | 0.70 ± 0.08 | 57 ± 10 |
| [6αβ3] + [6β4] | 4 | 2.3 (1.7-2.9) | 0.93 ± 0.09 | 870 ± 270 |
| [6αβ3] + [6β4] + [β3] | 3 | 0.02 (0.01-0.03) | 1.1 ± 0.12 | 98 ± 21 |
| [6αβ3] + [6β4] + [β3] | 3 | 0.02 (0.01-0.03) | 1.1 ± 0.12 | 98 ± 21 |
| Mecamylamine | h6αβ5 + [hβ4]            | 5       | -            | 5   | 12 ± 5  | 1000 |
|             | h6αβ5 + [hβ4] + [β3]    | 5       | -            | 5   | 9.5 ± 1.5 | 1000 |
| [6αβ3] + [6β4] | 9 | - | - | - |
| [6αβ3] + [6β4] | 3 | 7.8 ± 2.3 | 1000 |
| [6αβ3] + [6β4] | 3 | 9.0 ± 0.5 | 1000 |
| [6αβ3] + [6β4] | 3 | 8.5 ± 3.3 | 1000 |
| [6αβ3] + [6β4] | 3 | 12 ± 9 | 1000 |
| [6αβ3] + [6β4] | 3 | 41 ± 15 | 1000 |

mecamylamine-induced outward current indicated that there is spontaneous opening of mα6β3β4mβ3α6-nAChR (Table 1). By contrast, unexpectedly, and interestingly, oocytes expressing nAChR mα6β3β3α6, mβ4, and mββ3 subunits exhibit a >25-fold increase in peak current responses to nicotine (800 ± 190 nA) relative to responses of mα6β3β4mβ3-nAChR. Also, we were able to define an increase in nicotine potency when acting at mα6β3β4mβ3-nAChR (EC50 = 1.2 μM) relative to nicotine potency at mα6β4-nAChR (EC50 value of 26 μM; Table 1, Fig. 2). In addition, there also is spontaneous opening of mα6β3β4mβ3-nAChR, although responses to nicotine or mecamylamine are absent for mα6β3β4mβ3α6-nAChR (Table 1). No function was observed in response to nicotine or mecamylamine in oocytes coexpressing nAChR mα6 or mα6β3β3α6 or mα6β3βα6 subunits plus mβ2 subunits with or without mβ3 subunits.

Mouse nAChR a6β3α6 Subunits Form Functional Receptors in Association with nAChR h2β and h3β Subunits with Increased Receptor Agonist Sensitivity and Efficacy—We reported earlier that oocytes cojugated with nAChR mα6, h2β, and h3β subunit cRNAs tend to form functional mα6β4h2β3-nAChR, whereas oocytes coexpressing nAChR mα6 and h2β subunit cRNAs do not respond to nicotinic agonists (4). Here, we show that oocytes coexpressing nAChR mα6β3α6 and h2β subunits yield peak function of 80 ± 18 nA in response to 100 μM nicotine (Fig. 3, Table 1) and outward current responses (12 ± 9 nA) to mecamylamine, consistent with spontaneous channel opening of mα6β3β4-nAChR (Table 1). Moreover, oocytes coexpressing nAChR mα6β3α6, h2β, and h3β subunits respond to nicotine with an EC50 value of 2.3 μM and give an even larger peak current (870 ± 270 nA; Fig. 3; Table 1). Also, oocytes coexpressing mα6β3α6, h2β, and h3β subunits give relatively large, outward current responses (peak current of 41 ± 15 nA).
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Mouse nAChR α6\(^{L,9-S}\) Subunits Form Functional Receptors in Association with nAChR mβ4 and hβ3 Subunits with Increased Receptor Agonist Sensitivity and Efficacy—Earlier (4), we had shown that mutations in the N-terminal domain of the nAChR h6 subunit enable nAChR β3V\(^{9+S}\) subunits to exert a gain-of-function effect at h6(143D+M145V)hβ2*-nAChR (i.e. hα6(143D+M145V)hβ2hβ3V\(^{9+S}\)-nAChR are functional). This finding led us to explore effects of incorporation of hβ3 subunits into hα6(M143D+M145V)L\(^{1.9}\)hβ2-nAChR.
Oocytes injected with nAChR h6(N143D/H11001 M145V)L9/S and hβ4 subunit cRNAs did not yield functional nicotinic responses (Table 1). However, oocytes injected with hβ3 subunit cRNAs along with nAChR h6(N143D/H11001 M145V)L9/S and hβ2 subunit cRNAs yielded functional responses. Oocytes coexpressing nAChR h6(N143D+M145V)1,9S, hβ2, and hβ3 subunits responded to nicotine with an EC50 value of 0.02 μM and with a maximal peak current of 98 ± 21 nA (Fig. 5; Table 1). We also found that oocytes coexpressing nAChR h6(N143D+M145V)1,9S, hβ2, and hβ3 gave outward current responses when exposed to 1000 μM mecamylamine (data not shown).

**DISCUSSION**

Recent studies have investigated how nAChR β3 subunits might incorporate as accessory partners into nAChR subtypes, specifically into α6*-nAChR (4). To further understand how β3 subunits might incorporate into α6*-nAChR, we exploited the gain-of-function/reporter mutant strategy (4, 15, 26) to reveal whether β3 subunits integrate into α6*-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 β3 subunits into α6*-nAChR, mouse or human, has a dominant-negative effect; (ii) incorporation of nAChR hβ3 subunits into mα6hβ4*- or mα6mβ4*-nAChR leads to formation of functional nAChR; and (iii) mutations in the E1 N-terminal domain of the nAChR h6 subunit are essential for successful assembly and formation of functional hα6(N143D+M145V)-hβ2hβ31,9S-nAChR.
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 mo6 or ho6 subunits typically has a gain-of-function effect, leading to production of (α6 or α6(N143D+M145V))(19S or V13S)β2 or β4)-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 α6 subunits; (ii) that incorporation of β3 subunits into (α6 or α6(N143D+M145V))(19S or V13S)(β2 or β4)-nAChR typically increases levels of receptor function with or without concomitant increase in agonist potency; and (iii) that gain-of-function mutations in α6 or α6(N143D+M145V) subunits still do not allow for formation of functional α6(19S or V13S)β2-nAChR complexes, thus continuing to confound assessments of roles played by β3 subunits in modulation of α6β2-nAChR.

The amount of functional expression for ho6(19S)hβ4-, mo6(19S)mβ4-, or mo6(19S)hβ4-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 mo6(19S)mβ4- and mo6mβ4-nAChR, the increase in function upon expression with the α6 subunit 9' mutants is remarkable because of the lack of reliable function for wild-type, all-human ho6β4-, or hybrid mo6β4-nAChR. The little-if-any function for all-wild-type α6β4-nAChR complicates quantitative assessment of effects of α6 subunit gain-of-function mutations on agonist potency, although qualitatively, nicotine EC50 values are over 10 μM for α6β4-nAChR and never higher than 3.1 μM for α6(19S or V13S)β4-nAChR. However, gain-of-function effects manifest as increases in agonist potency and in peak current magnitudes are very clear based on comparisons of ho6h4b3- with ho6(19S)hβ4h3-nAChR and comparisons of mo6h4b3- with mo6(19S)hβ4h3-nAChR. A difference in agonist potency is also clear for comparison of mo6m4b3- with mo6(19S)mβ4h3-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 mo6m4h3-nAChR. Once again, however, all-mouse α6β4β3-nAChR are outliers because there is only modest function for mo6(19S)mβ4m3-nAChR, although there is no reliable function for the all-wild-type analog, mo6mβ4m3-nAChR.

Nevertheless, and very interestingly, for all-mouse α6*-receptors, although there is no reproducible function for mo6V13Sβ4-nAChR, there are increases both in agonist potency and in response magnitude for mo6V13Sβ4m3-nAChR when compared with those parameters for any form of mo6mβ4-nAChR or for mo6m4β3- or mo6(19S)mβ4m3-nAChR. Our initial studies of mouse α6*-nAChR were prompted because of the reported difficulties in heterologous expression of all-human α6*-nAChR and because so many data on naturally expressed α6*-nAChR function came from studies using rodents, but we have found all-mouse α6*-nAChR no easier to express than human α6*-nAChR. Expression of hybrid...
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FIGURE 5. Functional properties of gain-of-function hα6(N143D+M145V)β3*-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 hα6(N143D+M145V)β3-, β2, and hβ3 subunits. B, results for these and other studies averaged across experiments were used to produce concentration-response curves (ordinate, mean normalized current ± S.E.; abscissa, ligand concentration in log μM) for inward current responses to nicotine for oocytes expressing nAChR hα6(N143D+M145V)β3-, hβ2, and hβ3 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 hα6(N143D+M145V)β3-, hβ2, and hβ3 subunits when compared with receptors lacking hβ3 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 α6*-nAChR can be produced (4). The fact that mα6 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 mα6mβ4*-nAChR function indicates unexpectedly important roles for this channel-lining region in α6*-nAChR function. More work is warranted to more thoroughly characterize the bases for these influences.

Our findings demonstrate that α6 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 α6 subunits in functional receptor complexes, as expected. These studies also further affirm and recapture the strategy applied to exploit gain-of-function α6 subunit mutations expressed in vivo to enhance sensitivity to agonists and thus to help reveal roles played by α6*-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 β3 subunit incorporation into α6*-nAChR would be preserved when receptor functional levels at baseline were intentionally elevated by using reporter mutation/gain-of-function α6 subunits as coexpression partners. By earlier to earlier work by others (21), in which β3 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 hβ3 subunit incorporation into hα6hβ4*-nAChR has an uncertain effect on functional expression, that mβ3 subunit incorporation into mα6mβ4*-nAChR has a dominant-negative effect on receptor function, that hβ3 subunit incorporation into hybrid mα6hβ4*-nAChR potentiates function, but that there is even larger potentiation of function when hβ3 subunits are incorporated into hybrid mα6mβ4*-nAChR. However, with the exception of the lack of an obvious effect of mβ3 subunit incorporation on low functioning mα6L9’Smβ4*-nAChR, wild-type β3 subunit incorporation into any of the tested mα6L9’S or V13’S) β4-nAChR potentiates levels of function by >11-fold, notably including effects of hβ3 subunits on low functioning mα6L9’Smβ4*-nAChR and effects of mβ3 subunits on mα6V13’Smβ4*-nAChR. These findings indicate that β3 subunits do not always have dominant-negative effects on α6*-nAChR function as suggested earlier (21) and do not always promote formation of dead end, α6β4*-nAChR intermediates as suggested previously (23). Instead, based on our results shown here, we can hypothesize that β3 subunits seem to promote assembly, cell surface expression, and/or functional responsiveness of α6β4*-nAChR, at least when there is enough function for α6β4(n-β3)-nAChR to allow assessment of effects of β3 subunit incorporation. Our
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findings using the oocyte expression system are in line with observations made regarding β3 subunit effects on α6*-nAChR functional expression in cell lines (22), suggesting that successful, functional α6β4*-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 α6 subunits (or β3 subunits; see Refs. 4 and 15) occurs along with an increase in agonist potency, wild-type β3 incorporation into complexes increases peak current responses without affecting agonist potency.

In almost every case, α6(1.9S or V13S)β3β4*-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 block are quite similar across all the α6*-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 moeV13mβ4*-nAChR, which curiously have no reproducible responses to nicotine or to mecamylamine, despite being strong responses upon incorporation of mβ3 subunits to form moeV13mβ4mβ3-nAChR, and for moe6V9Sh4β3-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 β3 subunits in potentiating function of α6β4*-nAChR with at least a modicum of baseline functional activity, we were confounded in our studies of α6β2*-nAChR by a general lack of function. This made it impossible to assess effects of β3 subunit incorporation on α6β2*-nAChR, but the results indicate that any gain-of-function earned by incorporation of α6(1.9S or V13S) subunits into complexes is inadequate to reveal effects of β3 subunits, perhaps due to the surprising incompatibilities (illuminated in Refs. 4 and 15) that often occur in attempts to use α6, β2, and β3 subunits to form functional receptors. In order for us to show that in fact a variant of gain-of-function hα6 subunit can partner with hβ2 and hβ3 subunit to form functional nAChR, we took advantage of our site-directed mutagenesis work (4, 15), which has implicated α6 residues 143 and 145 in the ability of β3 subunits to affect α6β2*-nAChR function. The hα6(N143D+M145V) mutations change the indicated residues to those that are in the moα6 subunit and permit mutated hoα6 subunits to show function when coexpressed with hβ2 and hβ3 subunits when wild-type hoα6 subunits do not. Human nAChR α6 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 α6 subunit (−) face with the (+) face from either β2 subunits or β3 subunits are important for functional α6*-nAChR expression. In order for us to prove that the nAChR β3 subunit does affect the function of α6β2*-nAChR, a 9β mutation was introduced into the hoα6(N143D+M145V) subunit. Although coexpression of hoα6(N143D+M145V)1.9S and hβ2 subunits did not yield receptors with reliable function, upon inclusion of the hβ3 subunit, function was evident in all oocytes coexpressing the three subunits together. These hoα6(N143D+M145V)1.9S hβ2*-nAChR mimic the gain-of-function, high-affinity mo6*-nAChR artifically expressed in mouse midbrain dopamine neurons (13).

We conclude, based on the current and previous findings, that gain-of-function/reporter mutations introduced into α6 subunits in α6(β2 or β4)β3-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 β3 subunit incorporation into functionally competent (α6 or α6(N143D+M145V))1.9S or V13S)β4 or β2)β3-nAChR has a potentiating effect irrespective of whether there are dominant-negative, null, or potentiating effects of β3 subunits on wild-type α6β2 or β4)-nAChR. In fact, reliable expression of functional gain-of-function α6*-nAChR is achieved only in the presence of nAChR β3 subunits. These results suggest that wild-type β3 subunit coexpression is at least permissive for cell surface expression of α6β4*-nAChR and very likely promotes function of these receptors. The strategies and results demonstrated here to increase function of α6*-nAChR to levels compatible with drug screening could facilitate the development of new drugs selective for α6*-nAChR. This is of increasing importance given the potentially important roles for α6*-nAChR in movement and movement disorders, mood disorders, and drug reinforcement (5, 13, 29–31).

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