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10-10-2014

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#### Recommended Citation

Dash, Bhagirathi; Li, Ming D.; and Lukas, Ronald J., "Roles For N-Terminal Extracellular Domains Of Nicotinic Acetylcholine Receptor (NACHR)  $\alpha 3$  Subunits In Enhanced Functional Expression Of Mouse  $\alpha 6\alpha 2\alpha 3$ - And  $\alpha 6\alpha 4\alpha 3$ -NACHRs" (2014). *Translational Neuroscience*. 273.

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# Roles for N-terminal Extracellular Domains of Nicotinic Acetylcholine Receptor (nAChR) $\beta 3$ Subunits in Enhanced Functional Expression of Mouse $\alpha 6\beta 2\beta 3$ - and $\alpha 6\beta 4\beta 3$ -nAChRs\*

Received for publication, March 15, 2014, and in revised form, June 27, 2014. Published, JBC Papers in Press, July 15, 2014, DOI 10.1074/jbc.M114.566018

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**Background:** Naturally expressed mouse (m)  $\alpha 6^*$ -nAChRs have negligible functional expression *in vitro*.

**Results:** Functional expression of mouse  $\alpha 6\beta 2\beta 3$ - or  $\alpha 6\beta 4\beta 3$ -nAChRs is enhanced upon manipulation of  $\beta 3$  subunit N-terminal extracellular domain residues.

**Conclusion:** N-terminal extracellular domains in nAChR  $\beta 3$  subunits play heretofore underappreciated roles in controlling functional expression of  $\alpha 6^*$ -nAChR.

**Significance:** nAChR “accessory” subunits are critical elements in nAChR assembly and function.

Functional heterologous expression of naturally expressed mouse  $\alpha 6^*$ -nicotinic acetylcholine receptors ( $m\alpha 6^*$ -nAChRs; where “\*” indicates the presence of additional subunits) has been difficult. Here we expressed and characterized wild-type (WT), gain-of-function, chimeric, or gain-of-function chimeric nAChR subunits, sometimes as hybrid nAChRs containing both human (h) and mouse (m) subunits, in *Xenopus* oocytes. Hybrid  $m\alpha 6m\beta 4h\beta 3$ - (~5–8-fold) or WT  $m\alpha 6m\beta 4m\beta 3$ -nAChRs (~2-fold) yielded higher function than  $m\alpha 6m\beta 4$ -nAChRs. Function was not detected when  $m\alpha 6$  and  $m\beta 2$  subunits were expressed together or in the additional presence of  $h\beta 3$  or  $m\beta 3$  subunits. However, function emerged upon expression of  $m\alpha 6m\beta 2m\beta 3^{V9^S}$ -nAChRs containing  $\beta 3$  subunits having gain-of-function V9<sup>S</sup> (valine to serine at the 9<sup>′</sup>-position) mutations in transmembrane domain II and was further elevated 9-fold when  $h\beta 3^{V9^S}$  subunits were substituted for  $m\beta 3^{V9^S}$  subunits. Studies involving WT or gain-of-function chimeric mouse/human  $\beta 3$  subunits narrowed the search for domains that influence functional expression of  $m\alpha 6^*$ -nAChRs. Using  $h\beta 3$  subunits as templates for site-directed mutagenesis studies, substitution with  $m\beta 3$  subunit residues in extracellular N-terminal domain loops “C” (Glu<sup>221</sup> and Phe<sup>223</sup>), “E” (Ser<sup>144</sup> and Ser<sup>148</sup>), and “ $\beta 2$ - $\beta 3$ ” (Gln<sup>94</sup> and Glu<sup>101</sup>) increased function of  $m\alpha 6m\beta 2^*$ - (~2–3-fold) or  $m\alpha 6m\beta 4^*$ - (~2–4-fold)-nAChRs. EC<sub>50</sub> values for nicotine acting at  $m\alpha 6m\beta 4^*$ -nAChR were unaf-

ected by  $\beta 3$  subunit residue substitutions in loop C or E. Thus, amino acid residues located in primary (loop C) or complementary (loops  $\beta 2$ - $\beta 3$  and E) interfaces of  $\beta 3$  subunits are some of the molecular impediments for functional expression of  $m\alpha 6m\beta 2\beta 3$ - or  $m\alpha 6m\beta 4\beta 3$ -nAChRs.

Nicotinic acetylcholine receptors (nAChRs)<sup>2</sup> are a diverse set of pentameric, transmembrane, signal-transducing proteins found in the nervous system and elsewhere. Vertebrate nAChR subunits  $\alpha 1$ – $\alpha 10$ ,  $\beta 1$ – $\beta 4$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$  are encoded from a family of distinct genes.  $\alpha 1$ ,  $\beta 1$ ,  $\delta$ , and either  $\gamma$  or  $\epsilon$  subunits form muscle-type nAChRs, and other nAChR subtypes are formed as heteromers or homomers of the remaining subunits (1). Homomeric  $\alpha 7$ -nAChRs and heteromeric  $\alpha 4\beta 2$ - or  $\alpha 6\beta 2^*$ -nAChRs (\* indicates the known or possible presence of additional subunits) are the dominant subtypes in the central nervous system (CNS) (2).  $\alpha 6\beta 4^*$ -nAChRs do not seem to be abundant in the rodent CNS but are found in rat dorsal root ganglion neurons and in human adrenal chromaffin cells (3, 4).  $\alpha 6^*$ -nAChRs seem to participate in the modulation of dopamine release, locomotion, reward, and reinforcement and have been implicated in schizophrenia and Parkinson disease (5–12).

Beyond the known formation of  $\alpha 6\beta 2$ - or  $\alpha 6\beta 4$ -nAChRs (3, 4, 13–15), integration of nAChR  $\alpha 4$ ,  $\beta 3$ , or  $\alpha 5$  subunits can occur (14–17) to yield more complex  $\alpha 6^*$ -nAChR subtypes. nAChR  $\beta 3$  or  $\alpha 5$  subunits have been classified as “accessory” subunits because they do not form functional receptors alone or seem to combine with any other single kind of nAChR  $\alpha$  or  $\beta$  subunit in a functional way, but they can participate in trinary complexes containing other, selected  $\alpha$  and  $\beta$  subunits (15, 18–22). Integration of nAChR  $\beta 3$  subunits in the accessory

\* This work was supported, in whole or in part, by National Institutes of Health Grant R01 DA012844 and DA026356 (to M. D. L.) and DA015389 (to R. J. L.). This work was also supported by endowment and capitalization funds from the Barrow Neurological Foundation (to R. J. L.) and the External Research Program of Philip Morris USA Inc. and Philip Morris International (to R. J. L.). Portions of this work have been presented in abstract form: Dash, B., Bhakta, M., Whiteaker, P., Stitzel, J. A., Chang, Y., and Lukas, R. J. (2009) Gain-of-function mutants in human or mouse nAChR  $\beta 3$  subunits interchangeably activate either human or mouse  $\alpha 6\beta 4^*$ -nAChR but not human or mouse  $\alpha 6\beta 2^*$ -nAChR. *Soc. Neurosci. Abstr.* **35**, 34.7.

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<sup>2</sup> The abbreviations used are: nAChR, nicotinic acetylcholine receptor; ACh, acetylcholine;  $I_{max}$ , peak current response; h, human; m, mouse; AA, amino acid; TM, transmembrane domain; NTD, N-terminal domain; RC, reverse complement.

TABLE 1

## Primers and restriction sites used to create mutant/chimeric constructs

For mutants, the first amino acid (single letter code; numbering begins at the translation start methionine) designates the wild-type nAChR (human or mouse) subunit residue that is replaced with the indicated second amino acid. In the forward primer nucleic acid sequence, capitalization indicates the nucleotide(s) changed from the wild-type subunit to create the corresponding mutant or restriction site.

Primer	Mutant	Forward primer sequences
P1	h $\beta 3$ _V181_SalI m $\beta 3$ _Val187_SalI m $\beta 3$ _BgIII h $\beta 3$ _BgIII	5'-cttatgatggcaccatggtCgacctcattttgatcaatg-3' Innate Innate Innate
P2	m $\beta 3$ _R375_BsiWI	5'-gagagtaagggaaaccgtACgggggaaatttccaggg-3'
P3	h $\beta 3$ _K369_BsiWI	5'-ccagagaaagaggagagtcaaccagtCgtACGaggcaagtcctcgaaaaaagaag-3'
P4	m $\beta 3$ (V279S) <sup>a</sup>	5'-cacctcgggttttTCTctcttgacagtg-3'
P5	h $\beta 3$ (V273S) <sup>a</sup>	5'-cattatccacatcgggtcttTCTctcttgacagttttcc-3'
P6	m $\beta 3$ (Q94H)	5'-caggaatggacagaccaTaaattacgatggaatccc-3'
P7	m $\beta 3$ (E101D)	5'-caaaaattacgatggaatcccgaTgactatggtggaattaatcgc-3'
P8	m $\beta 3$ (N107H)	5'-ccgaagactatggtggaattCattcgataaagggttccatc-3'
P9	m $\beta 3$ (Q94H/E101D) m $\beta 3$ (Q94H/N107H)	5'-caTaaattacgatggaatcccgaTgactatggtggaattaatcgc-3' Use P8 on nAChR m $\beta 3$ (Q94H) construct
P10	m $\beta 3$ (S144N/S148V)	5'-ctcatgaccaaggccattgtgaaatccaAcggaaccgtcGgttgactcctcccgcagctacaaaag-3'
P11	m $\beta 3$ (E221D)	5'-gaagggcaacagaagagaCggcttttactcctatccg-3'
P12	m $\beta 3$ (E221D/F223V)	5'-gggatggaagggaacagaagagaCggcGtttactcctatccggtttgttacc-3'

<sup>a</sup> Primer created and used before (15).

position is suggested to be a critical, final step in formation, assembly, and stability of mature  $\alpha 6\beta 3^*$ -nAChRs (6, 23) and is important for function of  $\alpha 6^*$ - and other nAChRs (24–31). A positive role of the  $\beta 3$  subunit, rather than a negative role (27), in the function of  $\alpha 6^*$ -nAChRs has been revealed from knock-out animal studies (6). However, structure-function relationships and pharmacological features of  $\alpha 6^*$ -nAChRs are poorly understood because receptors thought to exist naturally have not been easily recreated in heterologous expression systems (13–15, 20).

There have been some successes in expressing functional  $\alpha 6^*$ -nAChRs using *Xenopus* oocytes (22) or cell lines when a chimeric nAChR ( $\alpha 6/\alpha 3$ ) subunit (composed of the  $\alpha 6$  subunit extracellular N-terminal domain fused to an otherwise  $\alpha 3$  subunit) was used instead of the wild-type (WT) nAChR  $\alpha 6$  subunit (32) or when enhanced GFP-tagged  $\alpha 6$ - and  $\beta 2$ -nAChR subunits were coexpressed in Neuro-2a cells (13). Other strategies have been to use gain-of-function variants of  $\beta 3$  or  $\alpha 5$  subunits (typically using subunits mutated to express serine instead of valine at the so-called 9'-position in the subunit channel-lining, second transmembrane domain (TM II); V9'S) to express functional  $\alpha 6^*$ -nAChRs in *Xenopus* oocytes (15, 27). We also recently succeeded in producing functional, hybrid  $\alpha 6\beta 3^*$ -nAChRs substituting mouse (m)  $\alpha 6$  subunits for human (h)  $\alpha 6$  subunits to express functional  $m\alpha 6h\beta 4h\beta 3$ - or  $m\alpha 6h\beta 2h\beta 3^{V9'S}$ -nAChRs (15). This kind of study leveraging innate variations in amino acid (AA) residues between subunits from different species produced valuable information regarding structure and function of invertebrate and vertebrate nAChRs (15, 21, 33, 34).

Here we report that nAChR h $\beta 3$  or h $\beta 3^{V9'S}$  (i.e. h $\beta 3$ (V273S)) subunits coexpressed in oocytes also expressing  $m\alpha 6$  subunits in the presence of m $\beta 4$  or m $\beta 2$  subunits yielded nAChRs with higher levels of function than those of  $m\alpha 6m\beta 4m\beta 3$ - or  $m\alpha 6m\beta 2m\beta 3^{V9'S}$ -nAChRs. Further studies using chimeric or gain-of-function chimeric mouse/human nAChR  $\beta 3$  subunits and site-directed mutagenesis identified AA residues in the extracellular N-terminal domain (NTD; in so-called loops "B2- $\beta 3$ ," C, and E) of m $\beta 3$  subunits that when substituted with corresponding residues from h $\beta 3$  subunits alone or in some spe-

cific combinations increased the function of  $m\alpha 6m\beta 2^*$ - and  $m\alpha 6m\beta 4^*$ -nAChRs. These studies elucidate some of the structural bases dictating roles for nAChR  $\beta 3$  subunits in functional expression of  $m\alpha 6m\beta 2^*$ - and  $m\alpha 6m\beta 4^*$ -nAChRs.

## EXPERIMENTAL PROCEDURES

**Bioinformatics and Homology Modeling**—Using several Web-available threading methods, the  $\beta 1$  subunit of the muscle nicotinic acetylcholine receptor of the marbled electric ray (*Torpedo marmorata*) (2BG9.B; Protein Data Bank code 2BG9 Chain B) (35) was identified as a suitable template for three-dimensional modeling of m $\beta 3$  subunits (SWISS-MODEL Protein Modeling Server) (36). The overall stereochemical quality of the final model was assessed by the program PROCHECK (37). The homology model for the nAChR m $\beta 3$  subunit was rendered using UCSF Chimera, a program for interactive visualization and analysis of molecular structures. Protein sequences for nAChR  $\beta 3$  subunits of several species or mouse nAChR subunits retrieved from the National Center for Biotechnology Information (NCBI) Entrez Web service were aligned with each other using the Web program ClustalW.

**Chemicals**—All chemicals for electrophysiology were obtained from Sigma. Fresh agonist (acetylcholine (ACh) or nicotine) or antagonist (atropine) stock solutions were made daily or diluted from frozen stock in Ringer's solution (OR2), which consisted of 92.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 5 mM HEPES, pH 7.5.

**Wild-type nAChR Subunits**—nAChR h $\beta 3$ ,  $m\alpha 6$ , m $\beta 2$ , m $\beta 3$ , and m $\beta 4$  subunits were subcloned into the oocyte expression vector pGEMHE as described previously (19, 30).

**nAChR  $\beta 3$  Subunit Chimeras**—Guided by an alignment of nAChR h $\beta 3$  and m $\beta 3$  subunit protein sequences (see Fig. 1A), chimeric mouse/human  $\beta 3$  subunits were designed and created (see Fig. 1B) as described below. We cared to construct chimeras in a manner that isolated domains and/or structural features in  $\beta 3$  subunits.

**Construction of the m $\beta 3$ (1–187)/h $\beta 3$ (182–458) Chimeric Subunit (SalI Site-based Construct)**—Mouse nAChR  $\beta 3$  subunits possess an innate SalI restriction site (see Fig. 1) in the NTD around AA residue Val<sup>187</sup>. A SalI restriction site (Table 1)

## Effects of $\beta 3$ Subunits on Mouse $\alpha 6^*$ -nAChR Function

was created in the pGEMHE-h $\beta 3$  construct (*i.e.* pGEMHE-h $\beta 3$ (Sall)) by site-directed mutagenesis around AA residue Val<sup>181</sup> by using primers P1 (forward) and its reverse complement (RC) (Table 1). Both pGEMHE-m $\beta 3$  and pGEMHE-h $\beta 3$ (Sall) constructs were digested with Sall and XbaI where the XbaI site is located downstream of the sequence encoding the C-terminal end of the m $\beta 3$  or h $\beta 3$  cDNA and is in the multiple cloning site. The pGEMHE-m $\beta 3$ (XbaI+Sall) (*i.e.* m $\beta 3$ (1–187)) and h $\beta 3$ (Sall+XbaI) (*i.e.* h $\beta 3$ (182–458)) cDNA fragments were gel-purified and ligated, producing a chimeric cDNA construct pGEMHE-m $\beta 3$ (1–187)/h $\beta 3$ (182–458) (*i.e.* m $\beta 3$ (Met<sup>1</sup>–Val<sup>187</sup>)/h $\beta 3$ (Asp<sup>182</sup>–His<sup>458</sup>)) (see Fig. 1).

**Construction of the m $\beta 3$ (1–329)/h $\beta 3$ (324–458) Chimera (BglII Site-based Construct)**—Both human and mouse  $\beta 3$  subunits possess an innate BglII restriction site right after the third transmembrane domain (TM III). The BglII site allows cutting through human and mouse  $\beta 3$  cDNA at equivalent and homologous residues (see Fig. 1). Both pGEMHE-m $\beta 3$  and pGEMHE-h $\beta 3$  plasmids were double digested with BglII and XbaI. pGEMHE-m $\beta 3$ (XbaI+BglII) (*i.e.* m $\beta 3$ (1–329)) and h $\beta 3$ (BglII-XbaI) (*i.e.* h $\beta 3$ (324–458)) cDNA fragments were gel-purified and ligated to produce the chimeric construct pGEMHE-m $\beta 3$ (1–329)/m $\beta 3$ (324–458) (*i.e.* m $\beta 3$ (Met<sup>1</sup>–Arg<sup>329</sup>)/h $\beta 3$ (Ser<sup>324</sup>–His<sup>458</sup>)) (see Fig. 1). This chimera could be considered as a combination of the NTD of m $\beta 3$  subunit and the rest of the h $\beta 3$  subunit because the AA residues between the 239th residue (presumably its N-terminal end) and the BglII site in the m $\beta 3$  subunit are identical to those between the 233rd residue (presumably its N-terminal end) and the BglII site in the h $\beta 3$  subunit (see Fig. 1).

**Construction of the m $\beta 3$ (1–375)/h $\beta 3$ (370–458) Chimera (BsiWI Site-based Construct)**—A BsiWI restriction site around AA residue Arg<sup>375</sup> in pGEMHE-m $\beta 3$  (using P2 and its RC; Table 1) and another one around AA residue Lys<sup>369</sup> in pGEMHE-h $\beta 3$  (using P3 and its RC) were created by site-directed mutagenesis (Table 1). Both pGEMHE-m $\beta 3$ (BsiWI) and pGEMHE-h $\beta 3$ (BsiWI) plasmids were double digested with BsiWI and XbaI. pGEMHE-m $\beta 3$ (XbaI+BsiWI) (*i.e.* m $\beta 3$ (1–375)) and h $\beta 3$ (BsiWI+XbaI) (*i.e.* h $\beta 3$ (370–458)) cDNA fragments were gel-purified and ligated to produce the chimeric pGEMHE-m $\beta 3$ (1–375)/h $\beta 3$ (370–458) (*i.e.* m $\beta 3$ (Met<sup>1</sup>–Arg<sup>375</sup>)/h $\beta 3$ (Gly<sup>370</sup>–His<sup>458</sup>)) (see Fig. 1).

**Gain-of-function Chimeric nAChR  $\beta 3$  Subunits**—TM II 9' valine-to-serine (V9'S) mutations in pGEMHE-m $\beta 3$ (1–329)/h $\beta 3$ (324–458) and pGEMHE-m $\beta 3$ (1–375)/h $\beta 3$ (370–458) constructs were introduced using primer P4 (Table 1) and its RC to produce pGEMHE-m $\beta 3$ (1–329)<sup>V279S</sup>/h $\beta 3$ (324–458) and pGEMHE-m $\beta 3$ (1–375)<sup>V279S</sup>/h $\beta 3$ (370–458) constructs, respectively (Fig. 1). Similarly, TM II V9'S mutations in the pGEMHE-m $\beta 3$ (1–187)/h $\beta 3$ (182–458) construct were introduced using primer P5 (Table 1) and its RC to produce the pGEMHE-m $\beta 3$ (1–187)/h $\beta 3$ (182–458)<sup>V273S</sup> construct.

**Point Mutants**—TM II V9'S, L9'S, or V13'S mutations in h $\beta 3$  (V9'S = V273S; V13'S = V277S), m $\beta 3$  (V9'S = V279S; V13'S = V283S), or  $\alpha 6$  (L9'S = L280S; V13'S = V284S) subunits were introduced into the pGEMHE background using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) as described previously (15, 38).

Single or double mutations in nAChR m $\beta 3$  subunit (*i.e.* Q94H, E101D, N107H, Q94H/E101D, S144N/S158V, E221D, and E221D/F223V) were introduced using the QuikChange II site-directed mutagenesis kit using their respective forward (P6, P7, P8, P9, P10, P11, and P12; Table 1) and RC primers. The pGEMHE-m $\beta 3$ (Q94H/N107H) construct was made by site-directed mutagenesis of the pGEMHE-m $\beta 3$ (Q94H) construct using primer P8 and its RC.

TM II 9' valine-to-serine mutations in plasmids encoding m $\beta 3$ (Q94H), m $\beta 3$ (E101D), m $\beta 3$ (N107H), m $\beta 3$ (Q94H/E101D), m $\beta 3$ (Q94H/N107H), m $\beta 3$ (S144N/S158V), m $\beta 3$ (E221D), or m $\beta 3$ (E221D/F223V) subunits were introduced using primer P4 (Table 1) and its RC. Construct integrity and accuracy of all subunits were confirmed by DNA sequencing.

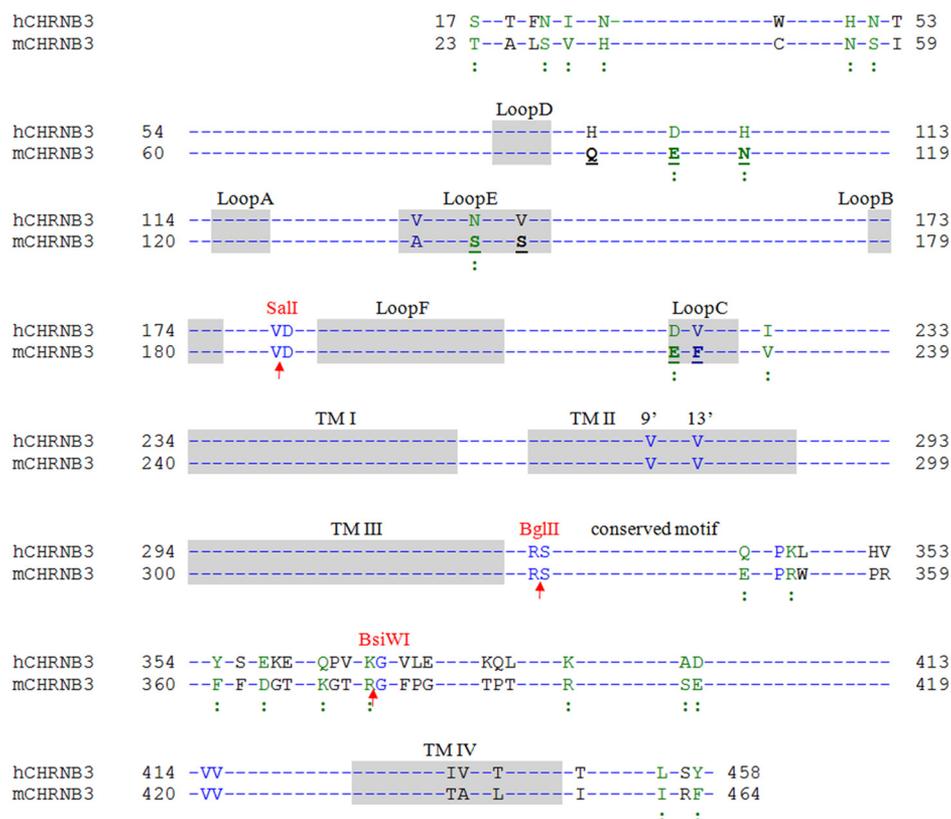
**Complementary RNA (cRNA) Preparation**—All pGEMHE plasmids were linearized immediately downstream of the 3'-polyadenylation sequence. NheI was used to linearize h $\beta 3$ , h $\beta 3$ <sup>V9'S</sup>, h $\beta 3$ <sup>V13'S</sup>,  $\alpha 6$ ,  $\alpha 6$ <sup>L9'S</sup>,  $\alpha 6$ <sup>V13'S</sup>, m $\beta 2$ , m $\beta 3$ <sup>V9'S</sup>, m $\beta 4$ , m $\beta 3$ (1–187)/h $\beta 3$ (182–458), m $\beta 3$ (1–329)/h $\beta 3$ (324–458), m $\beta 3$ (1–375)/h $\beta 3$ (370–458), m $\beta 3$ (1–187)/h $\beta 3$ (182–458)<sup>V273S</sup>, m $\beta 3$ (1–329)<sup>V279S</sup>/h $\beta 3$ (324–458), m $\beta 3$ (1–375)<sup>V279S</sup>/h $\beta 3$ (370–458), m $\beta 3$ (Q94H), m $\beta 3$ (E101D), m $\beta 3$ (N107H), m $\beta 3$ (Q94H/E101D), m $\beta 3$ (Q94H/N107H), m $\beta 3$ (S144N/S158V), m $\beta 3$ (E221D), m $\beta 3$ (E221D/F223V), m $\beta 3$ (Q94H)<sup>V9'S</sup>, m $\beta 3$ (E101D)<sup>V9'S</sup>, m $\beta 3$ (N107H)<sup>V9'S</sup>, m $\beta 3$ (Q94H/E101D)<sup>V9'S</sup>, m $\beta 3$ (Q94H/N107H)<sup>V9'S</sup>, m $\beta 3$ (S144N/S158V)<sup>V9'S</sup>, m $\beta 3$ (E221D)<sup>V9'S</sup>, and m $\beta 3$ (E221D/F223V)<sup>V9'S</sup> subunit-encoding plasmids. Capped, full-length cRNAs were prepared using individual reaction components as detailed earlier (19) or using the mMESSAGE mMACHINE® T7 kit (Ambion Inc./Invitrogen) following the manufacturer's instructions. The integrity and quality of the cRNAs were checked by electrophoresis and UV spectroscopy prior to cRNA injection.

**Preparation of cRNA Mixtures for Injection**—We planned to introduce identical amounts of cRNA, presumably producing equal amounts of each subunit protein, into oocytes largely due to lack of information about the levels of mRNA for each subunit that composes  $\alpha 6^*$ -nAChRs in neurons or cells. We provisionally assumed that  $\alpha 6$  subunits or their mutants in association with  $\beta 2$  or  $\beta 4$  subunits would form complexes having 2:3 and/or 3:2 ratios of the indicated subunits and that oocytes also injected with WT, chimeric, or other forms of  $\beta 3$  subunits would express nAChR with 2:2:1 ratios of  $\alpha$ : $\beta$ : $\beta 3$  subunits. For expression of binary nAChRs (*i.e.* nAChRs containing two subunits;  $\alpha + \beta$  but not  $\beta 3$ ), cRNA mixtures were prepared by mixing 1  $\mu$ l of cRNA for each subunit and an additional microliter of RNase-free water (*i.e.* total volume, 3  $\mu$ l). Similarly, for expression of trinary nAChRs (*i.e.* nAChRs containing three subunits;  $\alpha + \beta + \beta 3$ ) cRNA mixtures were prepared by mixing 1  $\mu$ l of cRNA for each subunit. Several preparations of each cRNA mixture were prepared and stored at  $-80^\circ\text{C}$  until further use.

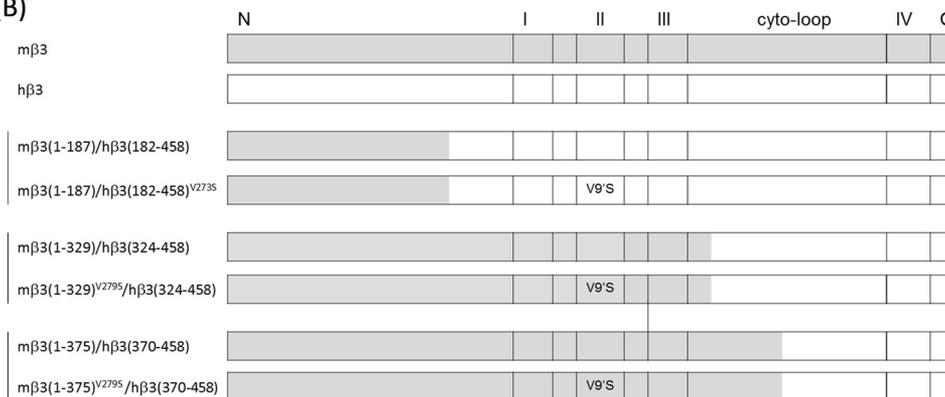
cRNA concentrations for each nAChR  $\alpha$  and  $\beta$  subunit were adjusted to 150 ng/ $\mu$ l for the first set of experiments (for data presented in Table 2 and Fig. 2). As noted above, introduction of 69 nl of cRNAs (from a 3- $\mu$ l cRNA mixture) into each oocyte would deliver  $\sim 3.5$  ng of cRNA for each  $\alpha$  and  $\beta$  subunit whether binary or trinary nAChRs are expressed. For all other

## Effects of $\beta 3$ Subunits on Mouse $\alpha 6^*$ -nAChR Function

(A)



(B)



**FIGURE 1. Construction of nAChR  $\beta 3$  subunit chimeras.** *A*, alignment of nAChR h $\beta 3$  and m $\beta 3$  subunit protein sequences. Amino acid sequences of nAChR h $\beta 3$  (accession number NP\_000740.1) and m $\beta 3$  subunits (accession number AAL75573.1) retrieved from GenBank<sup>TM</sup> were aligned using protein BLAST. AA numbering begins at the translation start methionine. Identical residues between human and mouse nAChR  $\beta 3$  subunits are indicated by a dash (-). Putative loop regions (A, B, and C in the primary face and D, E, and F in the complementary face), TM domains (I, II, III, and IV), and TM II 9' and 13' amino acid residues in human and mouse nAChR  $\beta 3$  subunits are identified. nAChR m $\beta 3$  subunit residues (Gln<sup>94</sup>, Glu<sup>101</sup>, Asn<sup>107</sup>, Ser<sup>144</sup>, Ser<sup>148</sup>, Asp<sup>221</sup>, and Phe<sup>223</sup>) given prime attention in mutagenesis studies are *underlined* and in *boldface*. An *upward arrow* ( $\uparrow$ ) indicates junctions for chimeric subunits and the restriction sites used for construction of chimeric mouse/human  $\beta 3$  subunits. *Colons* (:) below sequences indicate conserved residues. *B*, schematic diagrams of chimeric nAChR  $\beta 3$  subunits. Chimeric mouse/human nAChR  $\beta 3$  subunits with or without V9'S mutations in their respective TM II were constructed. The 273rd AA in nAChR h $\beta 3$  subunit or the 279th AA in nAChR m $\beta 3$  subunit is a valine and is noted as the 9' AA in TM II. N, N-terminal domain; I, II, III, or IV, respective TM domains; *cyto-loop*, cytoplasmic loop; C, C terminus. *Numbers in brackets* are the regions of the indicated subunits that are used for construction of mouse/human  $\beta 3$  chimeric subunits.

experiments, concentrations of cRNAs prepared for each nAChR  $\alpha$  and  $\beta$  subunit were adjusted to  $500 \text{ ng } \mu\text{l}^{-1}$ . Injection of 138 nl of cRNA of a 3- $\mu\text{l}$  cRNA mixture into each oocyte would deliver  $\sim 23 \text{ ng}$  of cRNAs for each  $\alpha$  and  $\beta$  subunit whether binary or trinary nAChRs are expressed.

**Oocyte Preparation and cRNA Injection**—Female *Xenopus laevis* (*Xenopus* I, Ann Arbor, MI or Nasco, Fort Atkinson, WI) were anesthetized using 0.2% tricaine methanesulfonate (MS-

222) (Sigma or Nasco). Ovarian lobes were surgically removed from the frogs and placed in an incubation solution that consisted of 82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.6 mM theophylline, 2.5 mM sodium pyruvate, 5 mM HEPES, 50 mg/ml gentamycin, 50 units/ml penicillin, and 50  $\mu\text{g/ml}$  streptomycin, pH 7.5. The 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

## Effects of $\beta 3$ Subunits on Mouse $\alpha 6^*$ -nAChR Function

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 or P1000 horizontal puller (Sutter Instrument Co., Novato, CA), and the tips were broken with forceps to  $\sim 40 \mu\text{m}$  in diameter. cRNAs were drawn up into the micropipette and injected into oocytes using a Nanoject or Nanoject II microinjection system (Drummond Scientific) at a total volume of 69 or 138 nl.

**Oocyte Electrophysiology**—Two to 5 days after injection, oocytes were placed in a small volume chamber and continuously perfused with OR2. The chamber was grounded through an agarose bridge. The oocytes were voltage-clamped at  $-70 \text{ mV}$  (unless otherwise noted) to measure agonist- or antagonist-induced currents using Axoclamp 900A and pClamp 10.2 software (Axon Instruments/Molecular Devices, 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 Digidata1440A and pClamp10. 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). Atropine ( $1 \mu\text{M}$ ) was always co-applied for ACh-based recordings to eliminate muscarinic acetylcholine receptor responses. nAChR  $\beta 3$  constructs were tested individually or in batches as they became available to get an estimate of their effect on the function of  $\alpha 6^*$ -nAChRs. Then, for the purpose of comparison, electrophysiological recordings were performed in a given day in a given batch of oocytes following the same order of injections. Hence data points in a figure panel were obtained under similar experimental conditions.

**Experimental Controls**—Injection of water or empty vector (used as two forms of negative controls) or of cRNA corresponding to one subunit alone or pairwise combinations of nAChR  $\beta 3$ ,  $\beta 3^{V9'S}$ ,  $\beta 3^{V13'S}$ , or other forms of  $\beta 3$  subunits with either an  $\alpha 6$  or other forms of  $\alpha 6$  or  $\beta 4$  subunit ( $\sim 7$ – $46 \text{ ng}$  of total cRNA) did not result in the expression of functional nAChRs. Current responses to  $100 \mu\text{M}$  nicotine or ACh 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) and a spreadsheet (Excel, Microsoft, Bellevue, WA) using peak current amplitudes as measures of functional nAChR expression, and results were pooled across experiments (mean  $\pm$  S.E. for data from at least three oocytes). In some cases, mean peak current amplitudes in response to a single concentration of an agonist were compared across different subunit combinations. However, assessment of true  $I_{\text{max}}$  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  $\text{EC}_{50}$  values across specific nAChR subunit combinations.

There are limitations in the ability to compare levels of functional nAChR expression even though we injected similar amounts of RNA for all constructs. This is because expression levels assessed as peak current amplitudes are affected by batch-to-batch variation in oocytes, time between cRNA injection and recording, and subunit combination-specific parameters, such as open probability (influenced by gating rate constants and rates and extents of desensitization), single channel conductance, assembly efficiency, and efficiency of receptor trafficking to the cell surface (39). 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 (15, 19, 30, 38). Therefore peak current amplitudes shown for representative traces in some figures, pooled data from limited sets of studies, and mean peak current amplitudes across all studies for a given combination of subunits given in tables or figures sometimes differ. However, when we make statements about results comparing ligand potencies and efficacies across subunit combinations, the observations are clear, significant, and in agreement whether for pooled data or for results from smaller sets of studies (one-way analyses of variance followed by Tukey's multiple comparison tests).

## RESULTS

Previously (15) we have shown that coexpression of WT nAChR  $\alpha 6$  and  $\beta 2$  subunits alone or in combination with  $\beta 3$  or  $\beta 3^{V9'S}$  subunits in oocytes, all from a single species (human or mouse), did not yield consistent and reproducible current responses to nicotinic agonists. However, under similar experimental conditions, we were able to show that coexpression of  $\alpha 6$  subunits, instead of  $\alpha 6$  subunits, with  $\beta 2$  and  $\beta 3^{V9'S}$  subunits led to expression of functional hybrid  $\alpha 6\beta 2\beta 3^{V9'S}$ -nAChRs (15). Also, hybrid  $\alpha 6\beta 4\beta 3$ -nAChRs were fully functional, although there was no function for  $\alpha 6\beta 4\beta 3$ - or  $\alpha 6\beta 4\beta 3^{V9'S}$ -nAChRs (15). These studies were carried out by injecting  $\sim 1$ – $6 \text{ ng}$  of cRNAs for each subunit into oocytes. In continuation of our earlier efforts, in this study, we substituted human  $\beta 3$  subunits for mouse  $\beta 3$  subunits. Initially we injected about  $\sim 3.5 \text{ ng}$  of cRNAs for each nAChR subunit to express hybrid nAChRs, but later we increased amounts injected to  $\sim 23 \text{ ng}$  for each subunit to emulate the approach taken by Kuryatov *et al.* (14) to express functional human  $\alpha 6\beta 4^*$ -nAChRs.

**Incorporation of nAChR  $\beta 3$ ,  $\beta 3^{V9'S}$ , or  $\beta 3^{V13'S}$  Subunits Potentiates  $\alpha 6\beta 4^*$ -nAChR Function**—Coexpression with WT  $\beta 3$  subunits significantly ( $p < 0.05$ ) potentiated ACh- or nicotine-induced current responses of  $\alpha 6\beta 4$ -nAChRs (Fig. 2 and Table 2). Also, coexpression with nAChR  $\beta 3^{V9'S}$  or  $\beta 3^{V13'S}$  subunits increased ( $p < 0.05$ ) the current responses further (Fig. 2 and Table 2). The increase in agonist sensitivities and in peak current amplitudes indicate that WT  $\beta 3$  subunits incorporate into at least some complexes containing  $\alpha 6$  and  $\beta 4$  subunits and these effects are most likely due to higher levels of functional receptor expression. Moreover, whereas not all oocytes expressing  $\alpha 6$  and  $\beta 4$  subunits yield functional

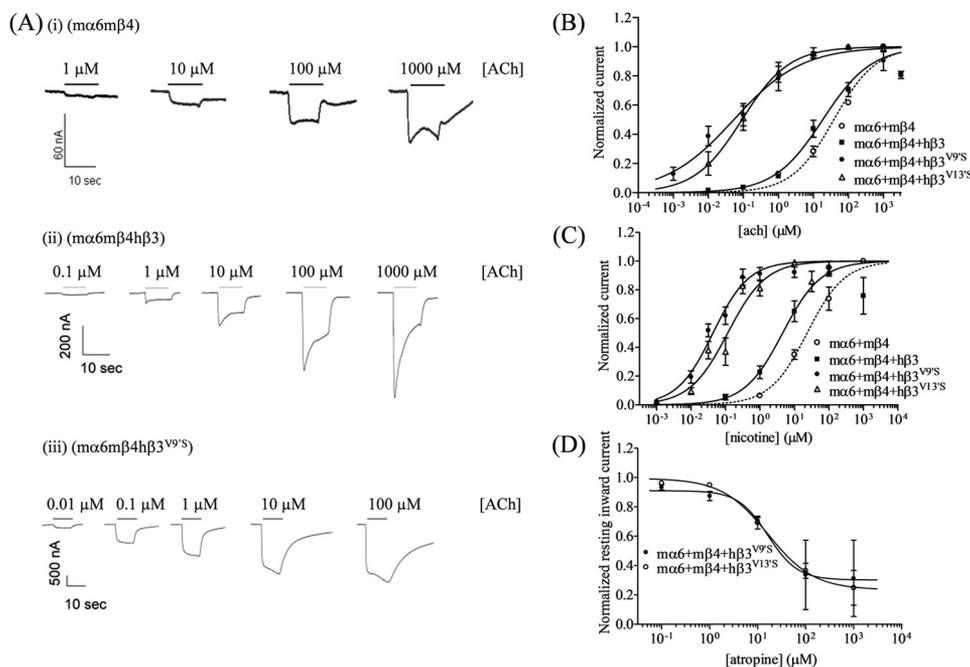


FIGURE 2. Concentration-dependent effects of agonist exposure on current responses in oocytes expressing  $\alpha 6^*$ m $\beta 4^*$ -nAChRs. A, representative traces are shown for inward currents in oocytes held at  $-70$  mV responding to application at the indicated concentrations of ACh (shown with the duration of agonist exposure as black bars above the traces) and expressing nAChR  $\alpha 6$  and  $m\beta 4$  subunits alone (panel i) or in the additional presence of wild-type  $h\beta 3$  (panel ii) or  $h\beta 3^{V9/S}$  (panel iii) subunits. Calibration bars are for 60-, 200-, or 500-nA currents (vertical) and 10 s (horizontal). Results for these and other studies averaged across experiments were used to produce agonist- or antagonist-induced concentration-response curves (ordinate, mean normalized current  $\pm$  S.E. (error bars); abscissa, ligand concentration in log  $\mu$ M) for responses to ACh (B), nicotine (C), or atropine (D) for oocytes expressing nAChR  $\alpha 6$  and  $m\beta 4$  subunits alone ( $\circ$ ) or in the additional presence of  $h\beta 3$  ( $\blacksquare$ ),  $h\beta 3^{V9/S}$  ( $\bullet$ ), or  $h\beta 3^{V13/S}$  ( $\triangle$ ) subunits as indicated. Leftward shifts in agonist concentration-response curves are evident for functional nAChR containing  $h\beta 3$ ,  $h\beta 3^{V9/S}$ , or  $h\beta 3^{V13/S}$  subunits ( $p < 0.0001$ ;  $\sim 2$ -,  $\sim 717$ -, and  $\sim 418$ -fold, respectively, for ACh  $EC_{50}$  values and  $\sim 6$ -,  $\sim 605$ -, and  $217$ -fold, respectively, for nicotine  $EC_{50}$  values relative to the respective agonist  $EC_{50}$  values for activation of  $\alpha 6^*$ m $\beta 4^*$ -nAChR function). See Table 2 for parameters.

TABLE 2

Parameters for drug action at hybrid  $\alpha 6^*$ h $\beta 4^*$ - or  $\alpha 6^*$ m $\beta 4^*$ -nAChRs

Potencies (micromolar  $EC_{50}$  or  $IC_{50}$  values with 95% confidence intervals (CI)), Hill coefficients ( $n_H \pm$  S.E.), mean  $\pm$  S.E. efficacies ( $I_{max}$  in nA), and concentrations (conc.) where maximal peak current amplitudes ( $I_{max}$ ) are achieved ( $\mu$ M) are provided for the indicated agonist (ACh or nicotine) or antagonist (atropine) acting at nAChR composed of the indicated subunits derived from the specified species and from the indicated number of independent experiments ( $n$ ) based on studies as shown in Fig. 2.  $\uparrow$  indicates a significant ( $p < 0.05$ ) increase in potency or efficacy of the specified agonist at the indicated nAChR subtype relative to nAChR upon expression in the presence of the indicated wild-type or mutant  $\beta 3$  subunit instead of in the absence of a  $\beta 3$  subunit,  $\blacktriangle$  indicates a significant increase in specified agonist potency or efficacy at the indicated nAChR subtype upon substitution of a mutant for a wild-type  $\beta 3$  subunit relative to nAChR containing the same subunits in the presence of wild-type  $\beta 3$  subunits, and  $\Delta$  or  $\nabla$  indicates a significant increase or decrease, respectively, in potency or efficacy of the specified agonist or antagonist at the indicated nAChR subtype containing  $\beta 3^{V13/S}$  instead of  $\beta 3^{V9/S}$  subunits.

Drug	nAChR subunit combinations	$n$	Potency		Peak response		
			$EC_{50}$ or $IC_{50}$ (95% CI)	$n_H \pm$ S.E.	$n$	Mean $I_{max} \pm$ S.E.	$I_{max}$ conc.
ACh	$m\alpha 6 + m\beta 4^a$	3	38 (25–58)	$0.72 \pm 0.09$	3	$65 \pm 25$	1000
	$m\alpha 6 + m\beta 4 + h\beta 3$	4	18 (12–28) $\uparrow$	$0.62 \pm 0.07$	4	$358 \pm 128$ $\uparrow$	100
	$m\alpha 6 + m\beta 4 + h\beta 3^{V9/S}$	4	0.05 (0.03–0.11) $\uparrow \blacktriangle$	$0.45 \pm 0.07$	3	$3379 \pm 189$ $\uparrow \blacktriangle$	100 $\uparrow \blacktriangle$
	$m\alpha 6 + m\beta 4 + h\beta 3^{V13/S}$	4	0.09 (0.05–0.16) $\uparrow \blacktriangle$	$0.64 \pm 0.1$	4	$2989 \pm 43$ $\uparrow \blacktriangle$	100 $\uparrow \blacktriangle$
Nicotine	$m\alpha 6 + m\beta 4^a$	3	24 (14–50)	$0.65 \pm 0.13$	5	$27 \pm 7$	1000
	$m\alpha 6 + m\beta 4 + h\beta 3$	4	4.5 (2.5–8.3) $\uparrow$	$0.78 \pm 0.16$	4	$212 \pm 5$ $\uparrow$	100 $\uparrow$
	$m\alpha 6 + m\beta 4 + h\beta 3^{V9/S}$	6	0.04 (0.03–0.06) $\uparrow \blacktriangle$	$0.84 \pm 0.10$	7	$2884 \pm 333$ $\uparrow \blacktriangle$	10 $\uparrow \blacktriangle$
	$m\alpha 6 + m\beta 4 + h\beta 3^{V13/S}$	7	0.12 (0.08–0.17) $\uparrow \blacktriangle \nabla$	$0.79 \pm 0.1$	8	$2783 \pm 622$ $\uparrow \blacktriangle$	10 $\uparrow \blacktriangle$
Atropine	$m\alpha 6 + m\beta 4 + h\beta 3^{V9/S}$	3	16 (1–241)	$-1.40 \pm 2.4$	3	$140.8 \pm 13.44$	1000
	$m\alpha 6 + m\beta 4 + h\beta 3^{V13/S}$	3	17 (4.8–58)	$-0.90 \pm 0.46$	3	$512.6 \pm 28.87$ $\Delta$	1000

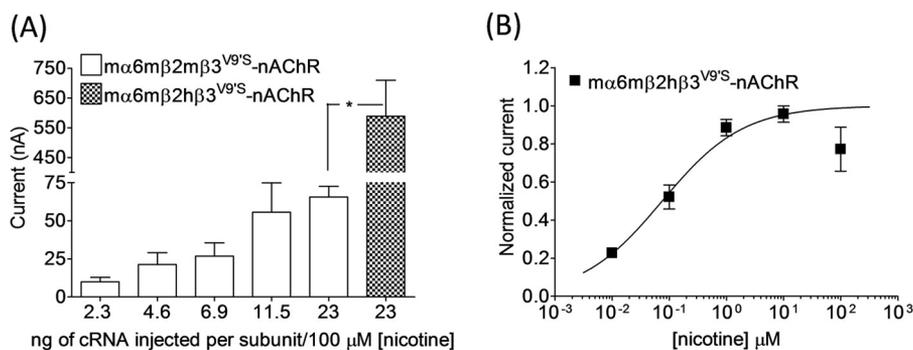
<sup>a</sup> Data from Ref. 15.

responses to nicotinic agonists, almost all oocytes expressing nAChR  $\alpha 6$ ,  $m\beta 4$ , and  $h\beta 3$  subunits produced functional responses, suggesting that nAChR  $h\beta 3$  subunits facilitate formation of functional, trinary (containing three kinds of subunits) nAChRs.

Spontaneously Opening  $\alpha 6^*$ m $\beta 4$ h $\beta 3^{V9/S}$ - or  $\alpha 6^*$ m $\beta 4$ h $\beta 3^{V13/S}$ -nAChRs Are Sensitive to Blockade by Atropine—Atropine (1  $\mu$ M) was always co-applied for ACh-based recordings to eliminate muscarinic acetylcholine receptor responses. Because

atropine at higher concentrations also can interact with different nAChR subtypes (15, 40, 41), initially as a simple control, we assessed the effects of atropine at different concentrations alone on all receptor combinations studied. Atropine alone did not produce any effect when assessed using oocytes expressing any combination of WT nAChR subunits (data not shown), but it reversibly produced outward currents when applied to oocytes expressing receptors containing  $\beta 3^{V9/S}$  or  $\beta 3^{V13/S}$  subunits. The concentration-dependent effects of atropine were

## Effects of $\beta 3$ Subunits on Mouse $\alpha 6^*$ -nAChR Function



**FIGURE 3. Effects of agonist exposure on current responses in oocytes expressing  $\alpha 6\text{m}\beta 2(\text{m}\beta 3^{\text{V9'S}}$  or  $\text{h}\beta 3^{\text{V9'S}}$ )-nAChRs.** A, current responses (mean  $\pm$  S.E. (error bars); nA) to 100  $\mu\text{M}$  nicotine obtained on the 5th day after injection from oocytes ( $n = 3-6$ ) voltage-clamped at  $-70$  mV and expressing  $\alpha 6\text{m}\beta 2\text{m}\beta 3^{\text{V9'S}}$  or  $\alpha 6\text{m}\beta 2\text{h}\beta 3^{\text{V9'S}}$ -nAChRs. Oocytes were injected with the indicated amount (ng) of cRNA for each subunit. B, concentration-response curve for responses to nicotine (ordinate, mean normalized inward current  $\pm$  S.E. (error bars); abscissa, ligand concentration in log  $\mu\text{M}$ ) for oocytes expressing  $\alpha 6\text{m}\beta 2\text{h}\beta 3^{\text{V9'S}}$ -nAChRs. \*,  $p < 0.05$ .

defined in terms of  $\text{IC}_{50}$  values for half-maximal blockade of spontaneous function, which for  $\alpha 6\text{m}\beta 4\text{h}\beta 3^{\text{V9'S}}$ - and  $\alpha 6\text{m}\beta 4\text{h}\beta 3^{\text{V13'S}}$ -nAChRs were 16 and 17  $\mu\text{M}$ , respectively (Fig. 2 and Table 2). It is estimated, based on comparisons of atropine-induced outward current peak amplitudes with the sum of those currents plus inward currents induced by fully efficacious concentrations of nicotine or ACh, that more than 4–16% of these receptors are spontaneously open at any one time.

*Function of  $\alpha 6\text{m}\beta 2^*$ -nAChRs Is Potentiated by Coexpression with nAChR  $\text{m}\beta 3^{\text{V9'S}}$  Subunits and Is Yet Higher upon Coexpression with  $\text{h}\beta 3^{\text{V9'S}}$  Instead of  $\text{m}\beta 3^{\text{V9'S}}$  Subunits*—Coexpression of  $\text{h}\beta 3$  or  $\text{h}\beta 3^{\text{V9'S}}$  subunits in oocytes also expressing  $\alpha 6$  and  $\text{m}\beta 2$  subunits did not lead to reproducible current responses to ACh or nicotine whenever they were expressed using  $\sim 3$  ng of cRNA for each subunit (data not shown). We decided to inject higher amounts of cRNA for each subunit (similar to the approach taken by Kuryatov *et al.* (14) to express  $\alpha 6\text{h}\beta 4$ - or  $\alpha 6\text{h}\beta 4\text{h}\beta 3$ -nAChRs) to see whether that would influence the functional expression of  $\alpha 6\text{m}\beta 2^*$ -nAChRs. Current responses to nicotine from oocytes expressing  $\alpha 6\text{m}\beta 2\text{m}\beta 3^{\text{V9'S}}$ -nAChRs increased with injection of increased amounts of cRNA for each subunit (Fig. 3). However, at the largest amount of cRNA for each subunit injected ( $\sim 23$  ng), there was no functional expression of  $\alpha 6\text{m}\beta 2\text{m}\beta 3$ - or  $\alpha 6\text{m}\beta 2\text{h}\beta 3$ -nAChRs. Nonetheless, oocytes expressing  $\alpha 6\text{m}\beta 2\text{h}\beta 3^{\text{V9'S}}$ -nAChRs yielded higher peak current responses ( $589 \pm 121$  versus  $66 \pm 7$  nA;  $\sim 9$ -fold;  $p < 0.05$ ) than those expressing  $\alpha 6\text{m}\beta 2\text{m}\beta 3^{\text{V9'S}}$ -nAChRs (Fig. 3). Oocytes expressing  $\alpha 6\text{m}\beta 2\text{h}\beta 3^{\text{V9'S}}$ -nAChRs, but not  $\alpha 6\text{m}\beta 2\text{m}\beta 3^{\text{V9'S}}$ -nAChRs, cultured for prolonged times produced some outward reversible currents in response to atropine (data not shown). The  $\text{EC}_{50}$  value for nicotine for activation of  $\alpha 6\text{m}\beta 2\text{h}\beta 3^{\text{V9'S}}$ -nAChRs is 0.08  $\mu\text{M}$ .

*Incorporation of Chimeric Mouse/Human  $\beta 3$  Subunits into Mouse  $\alpha 6\beta 2^*$ - and  $\alpha 6\beta 4^*$ -nAChRs*—We extended our studies to work using chimeric mouse/human nAChR  $\beta 3$  subunits to understand the molecular bases for the differential effect of  $\text{m}\beta 3$  and  $\text{h}\beta 3$  subunits on  $\alpha 6^*$ -nAChR function. We first constructed the *Bgl*III site-based nAChR chimeric subunit  $\text{m}\beta 3(1-329)/\text{h}\beta 3(324-458)$ . We injected  $\sim 23$  ng of cRNA for each subunit as we did for expression of  $\alpha 6\text{m}\beta 2(\text{m}\beta 3^{\text{V9'S}}$  or  $\text{h}\beta 3^{\text{V9'S}})$ -nAChRs to express  $\alpha 6\text{m}\beta 4$ -,  $\alpha 6\text{m}\beta 4\text{m}\beta 3$ -,  $\alpha 6\text{m}\beta 4\text{h}\beta 3$ -, or

$\alpha 6\text{m}\beta 4\text{m}\beta 3(1-329)/\text{h}\beta 3(324-458)$ -nAChRs. Initial assessments indicated that all these nAChRs were functional, but the current responses from oocytes expressing  $\alpha 6\text{m}\beta 4\text{m}\beta 3(1-329)/\text{h}\beta 3(324-458)$ -nAChRs were similar to those from oocytes expressing  $\alpha 6\text{m}\beta 4$ -nAChRs and lower than those elicited from oocytes expressing  $\alpha 6\text{m}\beta 4\text{h}\beta 3$ -nAChRs (see Fig. 4B). Coexpression of this chimeric subunit with  $\alpha 6$  and  $\text{m}\beta 2$  subunits did not produce any detectable functional nAChR. These results prompted us to construct two additional chimeras that either increased or reduced the length of the  $\text{h}\beta 3$  subunit contribution to mouse/human  $\beta 3$  subunit chimeras. This led to construction of chimeric  $\text{m}\beta 3(1-187)/\text{h}\beta 3(182-458)$  (*Sal*I restriction site-based) and  $\text{m}\beta 3(1-375)/\text{h}\beta 3(370-458)$  (*Bsi*WI restriction site-based) subunits. All three chimeras were assessed in parallel for their effects on  $\alpha 6\text{m}\beta 2^*$ - or  $\alpha 6\text{m}\beta 4^*$ -nAChRs.

Coexpression of chimeric mouse/human nAChR subunits (*i.e.*  $\text{m}\beta 3(1-187)/\text{h}\beta 3(182-458)$ ,  $\text{m}\beta 3(1-329)/\text{h}\beta 3(324-458)$ , or  $\text{m}\beta 3(1-375)/\text{h}\beta 3(370-458)$ ) with nAChR  $\alpha 6$  and  $\text{m}\beta 2$  subunits in oocytes did not result in detectable nAChR function (data not shown). However, these chimeric subunits coexpressed with  $\alpha 6$  and  $\text{m}\beta 4$  subunits yielded minimally functional nAChRs. Results indicated that peak current responses of  $\alpha 6\text{m}\beta 4[\text{m}\beta 3(1-187)/\text{h}\beta 3(182-458)]$ -,  $\alpha 6\text{m}\beta 4[\text{m}\beta 3(1-329)/\text{h}\beta 3(324-458)]$ -, or  $\alpha 6\text{m}\beta 4[\text{m}\beta 3(1-375)/\text{h}\beta 3(370-458)]$ -nAChRs are similar ( $p > 0.05$ ) to those of  $\alpha 6\text{m}\beta 4$ -( $50 \pm 13$  nA; 1000  $\mu\text{M}$  nicotine) or  $\alpha 6\text{m}\beta 4\text{m}\beta 3$ -nAChRs ( $85 \pm 15$  nA; 100  $\mu\text{M}$  nicotine) but lower ( $p < 0.05$ ) than those of  $\alpha 6\text{m}\beta 4\text{h}\beta 3$ -nAChRs ( $410 \pm 88$  nA; 100  $\mu\text{M}$  nicotine) (Fig. 4B).

*Incorporation of Gain-of-function Chimeric Mouse/Human nAChR  $\beta 3$  Subunits into Mouse  $\alpha 6\beta 2^*$ - and  $\alpha 6\beta 4^*$ -nAChRs*—To evaluate whether the chimeric nAChR  $\text{m}\beta 3/\text{h}\beta 3$  subunits are truly participating in  $\alpha 6^*$ -nAChR formation, we assessed the incorporation of gain-of-function  $\text{m}\beta 3(1-187)/\text{h}\beta 3(182-458)^{\text{V9'S}}$ ,  $\text{m}\beta 3(1-329)^{\text{V9'S}}/\text{h}\beta 3(324-458)$ , or  $\text{m}\beta 3(1-375)^{\text{V9'S}}/\text{h}\beta 3(370-458)$  subunits into  $\alpha 6\text{m}\beta 2^*$ - and  $\alpha 6\text{m}\beta 4^*$ -nAChRs. Coexpression of these gain-of-function chimeric nAChR subunits with  $\alpha 6$  and  $\text{m}\beta 2$  subunits in oocytes resulted in the production of functional nAChRs (Fig. 4A). Current responses from oocytes expressing chimeric  $\alpha 6\text{m}\beta 2^*$ -nAChRs progressively decreased when coexpressed with chi-

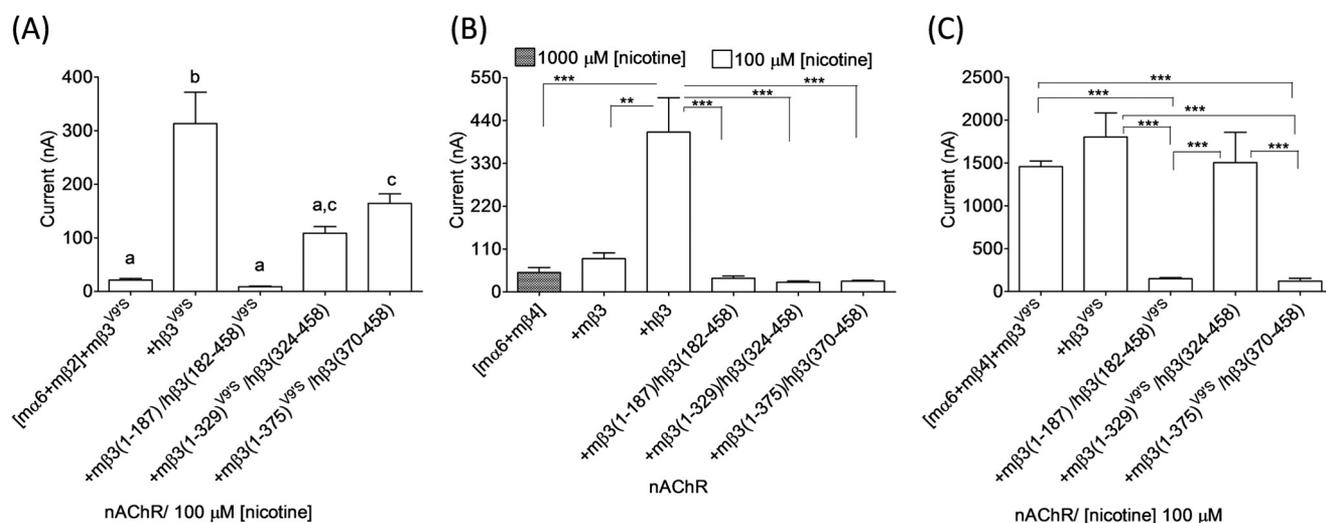


FIGURE 4. **Effects of chimeric mouse/human or gain-of-function chimeric mouse/human nAChR  $\beta 3$  subunits on functional responsiveness of  $\alpha 6^*$ -nAChRs.** A–C, mean  $\pm$  S.E. (error bars) peak inward current responses upon exposure to 100 or 1000  $\mu\text{M}$  nicotine (5-s exposure; ordinate) from oocytes ( $n = 3-10$ ) voltage-clamped at  $-70$  mV and heterologously expressing  $\alpha 6\text{m}\beta 2^*$ - (A) or  $\alpha 6\text{m}\beta 4^*$ -nAChRs (B and C) in the presence of the indicated chimeric nAChR  $\beta 3$  subunits. Note that  $\alpha 6\text{m}\beta 4$ -nAChRs reach  $I_{\text{max}}$  at 1000  $\mu\text{M}$  nicotine. The largest amplitude responses to nicotine were observed when h $\beta 3$  or h $\beta 3^{\text{V9'S}}$  subunits were present in  $\alpha 6\text{m}\beta 2^*$ - or  $\alpha 6\text{m}\beta 4^*$ -nAChRs. Current responses were compared using one-way analyses of variance with Tukey's post hoc comparison. Groups with different letters (a, b, and c) are significantly ( $p < 0.05$ ) different. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

meric  $\beta 3$  subunits containing shorter N-terminal segments from the mouse  $\beta 3$  subunit. For example,  $\alpha 6\text{m}\beta 2[\text{m}\beta 3(1-187)/\text{h}\beta 3(182-458)]^{\text{V9'S}}$ -nAChRs were least responsive to nicotine. This suggested that a combination of m $\beta 3$  subunit domains and residues contributes to impeded functional expression of  $\alpha 6\text{m}\beta 2^*$ -nAChRs.

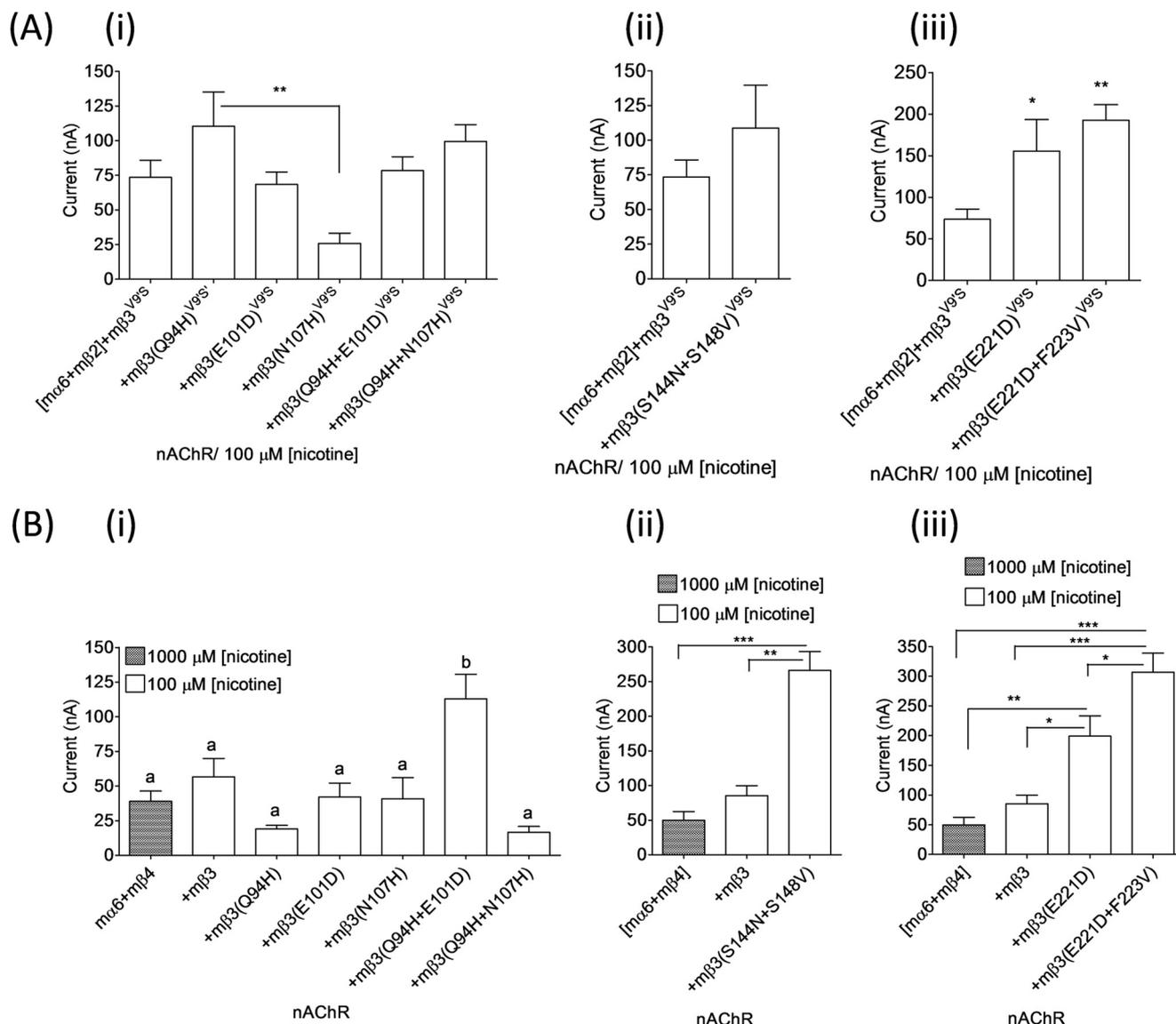
Incorporation of m $\beta 3(1-187)/\text{h}\beta 3(182-458)]^{\text{V273S}}$ , m $\beta 3(1-329)^{\text{V279S}}/\text{h}\beta 3(324-458)$ , or m $\beta 3(1-375)^{\text{V279S}}/\text{h}\beta 3(370-458)$  subunits into  $\alpha 6\text{m}\beta 4$ -nAChRs yielded functional nAChRs (Fig. 4C). Peak current responses from oocytes expressing  $\alpha 6\text{m}\beta 4[\text{m}\beta 3(1-329)/\text{h}\beta 3(324-458)]$ -nAChRs ( $1505 \pm 353$  nA) were similar ( $p > 0.05$ ) to those of  $\alpha 6\text{m}\beta 4\text{m}\beta 3^{\text{V279S}}$ - or  $\alpha 6\text{m}\beta 4\text{h}\beta 3^{\text{V273S}}$ -nAChRs. However, oocytes expressing  $\alpha 6\text{m}\beta 4[\text{m}\beta 3(1-187)/\text{h}\beta 3(182-458)]^{\text{V273S}}$ - ( $148 \pm 13$  nA) or  $\alpha 6\text{m}\beta 4[\text{m}\beta 3(1-375)^{\text{V279S}}/\text{h}\beta 3(370-458)]$ -nAChRs ( $122 \pm 33$  nA) yielded much lower ( $p < 0.001$ ) peak current responses than those expressing either  $\alpha 6\text{m}\beta 4\text{m}\beta 3^{\text{V279S}}$ - or  $\alpha 6\text{m}\beta 4\text{h}\beta 3^{\text{V273S}}$ -nAChRs. Although the presence of h $\beta 3$  subunit residues from TM III through the large, second cytoplasmic domain to the C terminus seems somehow to quell such an effect, this again suggested that a combination of m $\beta 3$  subunit domains and residues contributes to reduced functional expression of  $\alpha 6\text{m}\beta 4^*$ -nAChRs. However, strongest implications were that the extracellular N-terminal domain was involved.

**N-terminal AA Residues in the nAChR m $\beta 3$  Subunit That Influence the Function of Mouse  $\alpha 6\beta 2^*$ - and  $\alpha 6\beta 4^*$ -nAChRs—**Because effects on  $\alpha 6\text{m}\beta 2^*$ - and  $\alpha 6\text{m}\beta 4^*$ -nAChR function were most extreme in chimeras containing extracellular N-terminal domains from the m $\beta 3$  subunit, we focused on this region and on residues that differ between h $\beta 3$  and m $\beta 3$  subunits. For all nAChR subunits, there is a “primary” or (+) face and a “complementary” or (–) face where subunit extracellular N-terminal domains interact, forming a subunit interface. Interface interactions are critical for subunits to form dimers and for dimers to join with a single subunit to close the penta-

meric assembly. Interfaces involving the primary face of specific  $\alpha$  subunits and the complementary face of specific  $\beta$  subunits also are known to contain agonist binding pockets, occupancy of which leads to channel opening and where competitive antagonists also bind to affect function (the  $\alpha$  subunit was designated as that providing the primary face because it was initially thought that agonist binding sites resided solely within  $\alpha$  subunits). nAChR biologists have identified several loops at turns in  $\beta$ -strands that criss-cross subunit extracellular domains as in a woven basket. So-called loop  $\beta 2$ - $\beta 3$  (named so because the loop is formed at the tip of a turn between  $\beta$  strands  $\beta 2$  and  $\beta 3$ ) and loops D, E, and F are evident from modeling and structural studies to be on the complementary face of a given subunit, whereas loops A, B, and C are on the primary face. Loops A–F appear to be engaged in ligand recognition. For site-directed mutagenesis studies, we focused on some of the very few residues that differ between h $\beta 3$  and m $\beta 3$  subunits, AAs Gln<sup>94</sup>, Glu<sup>101</sup>, and Asn<sup>107</sup> in the  $\beta 2$ - $\beta 3$  loop; AAs Ser<sup>144</sup> and Ser<sup>148</sup> in putative loop E, and AAs Glu<sup>221</sup> and Phe<sup>223</sup> in putative loop C, to determine roles in functional expression of  $\alpha 6\text{m}\beta 2^*$ - and  $\alpha 6\text{m}\beta 4^*$ -nAChRs (Fig. 1A). Residues in the nAChR m $\beta 3$  subunit were mutated to their counterparts in the nAChR h $\beta 3$  subunit alone or in specific combinations (*i.e.* m $\beta 3$ (Q94H), m $\beta 3$ (E101D), m $\beta 3$ (N107H), m $\beta 3$ (Q94H/E101D), m $\beta 3$ (Q94H/N107H), m $\beta 3$ (S144N/S148V), m $\beta 3$ (E221D), and m $\beta 3$ (E221D/F223V)) (Fig. 5).

**AA Substitutions in the  $\beta 2$ - $\beta 3$  Loop of nAChR m $\beta 3$  Subunit Influence the Current Responses of  $\alpha 6\text{m}\beta 2^*$ - or  $\alpha 6\text{m}\beta 4^*$ -nAChRs—**Coexpression of nAChR m $\beta 3$  subunit point mutants (Q94H, E101D, or N107H) or double mutants (Q94H/E101D or Q94H/N107H) in oocytes with  $\alpha 6$  and m $\beta 2$  subunits did not lead to production of functional nAChR. However, these mutant subunits harboring the V9'S mutation in their respective TM II domains (*i.e.* m $\beta 3$ (Q94H)<sup>V9'S</sup>, m $\beta 3$ (E101D)<sup>V9'S</sup>, m $\beta 3$ (N107H)<sup>V9'S</sup>, m $\beta 3$ (Q94H/E101D)<sup>V9'S</sup>, or m $\beta 3$ (Q94H/N107H)<sup>V9'S</sup>) upon coexpression with  $\alpha 6$  and

## Effects of $\beta 3$ Subunits on Mouse $\alpha 6^*$ -nAChR Function



**FIGURE 5. Effects of nAChR  $\beta 3$  subunit amino acid substitutions on the current responses of  $\alpha 6^*$ -nAChRs.** A and B, current responses (mean  $\pm$  S.E. (error bars)) from oocytes ( $n = 3-6$ ) (voltage-clamped at  $-70$  mV) responding to the application of 100 or 1000  $\mu\text{M}$  nicotine (5-s exposure; ordinate) were measured from  $\alpha 6\text{m}\beta 2^*$ - (A) or  $\alpha 6\text{m}\beta 4^*$ -nAChRs (B) harboring WT, mutant, or gain-of-function  $\beta 3$ -,  $\beta 3(\text{Q94H})$ -,  $\beta 3(\text{E101D})$ -,  $\beta 3(\text{E107H})$ -,  $\beta 3(\text{Q94H}/\text{E101D})$ -, or  $\beta 3(\text{Q94H}/\text{N107H})$ -nAChR subunits (panel i); WT, mutant, or gain-of-function  $\beta 3$ - or  $\beta 3(\text{S144N}/\text{S148V})$ -nAChR subunits (panel ii); or WT, mutant, or gain-of-function  $\beta 3$ -,  $\beta 3(\text{E221D})$ -, or  $\beta 3(\text{E221D}/\text{F223V})$ -nAChR subunits (panel iii). Current responses were compared using Student's  $t$  test (two-tailed) or one-way analyses of variance (with Tukey's post hoc comparison). Groups with different letters (a and b) are significantly ( $p < 0.05$ ) different. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

$\beta 2$  subunits in oocytes and responding to application of 100  $\mu\text{M}$  nicotine produced peak current responses of  $110 \pm 25$ ,  $68 \pm 9$ ,  $26 \pm 7$ ,  $78 \pm 10$ , or  $99 \pm 12$  nA, respectively (Fig. 5A, panel i). The peak current responses of  $\alpha 6\text{m}\beta 2[\beta 3(\text{Q94H})^{\text{V97S}}]$ -,  $\alpha 6\text{m}\beta 2[\beta 3(\text{E101D})^{\text{V97S}}]$ -,  $\alpha 6\text{m}\beta 2[\beta 3(\text{N107H})^{\text{V97S}}]$ -,  $\alpha 6\text{m}\beta 2[\beta 3(\text{Q94H}/\text{E101D})^{\text{V97S}}]$ -, or  $\alpha 6\text{m}\beta 2[\beta 3(\text{Q94H}/\text{N107H})^{\text{V97S}}]$ -nAChRs did not differ ( $p < 0.05$ ) from those of  $\alpha 6\text{m}\beta 2\text{m}\beta 3^{\text{V97S}}$ -nAChRs ( $73 \pm 12$  nA).

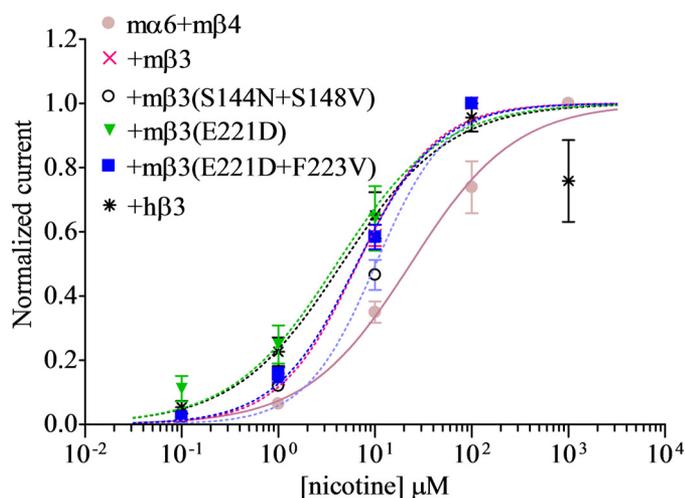
Oocytes expressing  $\alpha 6$  and  $\beta 4$  subunits alone or in the additional presence of  $\beta 3(\text{Q94H})$ ,  $\beta 3(\text{E101D})$ ,  $\beta 3(\text{N107H})$ ,  $\beta 3(\text{Q94H}/\text{E101D})$ , or  $\beta 3(\text{Q94H}/\text{N107H})$  subunits produced functional nAChRs (Fig. 5B, panel i). Peak current responses of  $\alpha 6\text{m}\beta 4\text{m}\beta 3$ -nAChRs ( $57 \pm 13$  versus  $113 \pm 18$  nA) were increased ( $\sim 2$ -fold;  $p < 0.05$ ) as a result of Q94H/N107H double substitution in the nAChR  $\beta 3$  subunit. Also, the current

responses of  $\alpha 6\text{m}\beta 4\text{m}\beta 3(\text{Q94H}/\text{N107H})$ -nAChRs were higher ( $\sim 3$ -fold;  $p < 0.05$ ) than those of  $\alpha 6\text{m}\beta 4$ -nAChRs ( $39 \pm 7$  nA).

**AA Substitutions in Loop E of the nAChR  $\beta 3$  Subunit Influence the Function of  $\alpha 6\text{m}\beta 2^*$ - or  $\alpha 6\text{m}\beta 4^*$ -nAChRs—**Coexpression of gain-of-function nAChR  $\beta 3(\text{S144N}/\text{S148V})^{\text{V97S}}$  subunits, but not nAChR  $\beta 3(\text{S144N}/\text{S148V})$  subunits, in oocytes with  $\alpha 6$  and  $\beta 2$  subunits resulted in production of functional nAChRs. Current responses of  $\alpha 6\text{m}\beta 2[\beta 3(\text{S144N}/\text{S148V})^{\text{V97S}}]$ -nAChRs to 100  $\mu\text{M}$  nicotine ( $109 \pm 31$  nA) were not different ( $p < 0.05$ ) from those of  $\alpha 6\text{m}\beta 2\text{m}\beta 3^{\text{V97S}}$ -nAChRs ( $73 \pm 12$  nA) (Fig. 5Aii). Peak current responses of  $\alpha 6\text{m}\beta 4\text{m}\beta 3$ -nAChRs were increased ( $266 \pm 27$  versus  $85 \pm 15$  nA;  $\sim 2.5$ -fold;  $p < 0.05$ ) as a result of the S144N/S148V double mutation in the nAChR  $\beta 3$  subunit (Fig. 5Bii). EC<sub>50</sub> values for nicotine at  $\alpha 6\text{m}\beta 4\text{h}\beta 3$ -,  $\alpha 6\text{m}\beta 4\text{m}\beta 3$ -, and

$\alpha 6\beta 4\beta 3$ (S144V/S148V)-nAChRs (*i.e.* 4.5, 6.6, and 10  $\mu\text{M}$ , respectively; Fig. 6 and Table 3) were essentially the same ( $p > 0.05$ ).

**AA Substitutions in Loop C of in the nAChR  $\beta 3$  Subunit Influence the Function of  $\alpha 6\beta 2^*$  or  $\alpha 6\beta 4^*$ -nAChRs**—Oocytes expressing  $\alpha 6\beta 2$ [ $\beta 3$ (E221D)<sup>V9'S</sup>] (155  $\pm$  38 nA) or  $\alpha 6\beta 2$ [ $\beta 3$ (E221D/F223V)<sup>V9'S</sup>] (193  $\pm$  18 nA)-nAChRs elicited higher ( $\sim 2$ – $3$ -fold;  $p < 0.05$ ) peak currents in response to 100  $\mu\text{M}$  nicotine than those expressing  $\alpha 6\beta 2\beta 3$ <sup>V9'S</sup>-nAChRs (73  $\pm$  12 nA; Fig. 5Aiii). However, coexpression of nAChR  $\beta 3$ (E221D) or  $\beta 3$ (E221D/F223V) subunits in oocytes with  $\alpha 6$  and  $\beta 2$  subunits did not result in expression of functional nAChR. Substitution of nAChR  $\beta 3$ (E221D) (199  $\pm$  34 nA;  $\sim 2$ -fold) or  $\beta 3$ (E221D/F223V) (307  $\pm$  32 nA;  $\sim 6$ -fold) subunits for  $\beta 3$  subunit increased ( $p < 0.05$ ) the current responses of  $\alpha 6\beta 4\beta 3$ -nAChRs (85  $\pm$  15 nA (Fig. 5Biii)). However, EC<sub>50</sub> values for nicotine at  $\alpha 6\beta 4\beta 3$ - (6.6  $\mu\text{M}$ ) or  $\alpha 6\beta 4\beta 3$ h $\beta 3$  (4.5  $\mu\text{M}$ )-nAChRs were not altered ( $p > 0.05$ ) when  $\beta 3$ (E221D) (4  $\mu\text{M}$ ) or  $\beta 3$ (E221D/F223V) (6.5  $\mu\text{M}$ ) subunits were substituted for  $\beta 3$  subunits (Fig. 6 and Table 3).



**FIGURE 6. Effects of nAChR  $\beta 3$  subunit amino acid substitutions on nicotine sensitivities of  $\alpha 6\beta 4^*$ -nAChRs.** Concentration-response curves (ordinate, mean normalized current  $\pm$  S.E. (error bars); abscissa, ligand concentration in log  $\mu\text{M}$ ) are shown for responses to nicotine for oocytes expressing nAChR  $\alpha 6$  and  $\beta 4$  subunits alone ( $\bullet$ ) or in the additional presence of h $\beta 3$  ( $*$ ),  $\beta 3$  ( $\times$ ), h $\beta 3$ (S144N/S148V) ( $\circ$ ), h $\beta 3$ (E221D) ( $\blacktriangledown$ ), or h $\beta 3$ (E221D/F223V) ( $\blacksquare$ ) subunits as indicated. Leftward shifts in agonist concentration-response curves are evident for functional nAChRs containing h $\beta 3$ ,  $\beta 3$ , h $\beta 3$ (S144N/S148V), h $\beta 3$ (E221D), h $\beta 3$ (E221D/F223V), or h $\beta 3$ (E347Q/R361K) subunits. See Table 3 for parameters.

**TABLE 3**

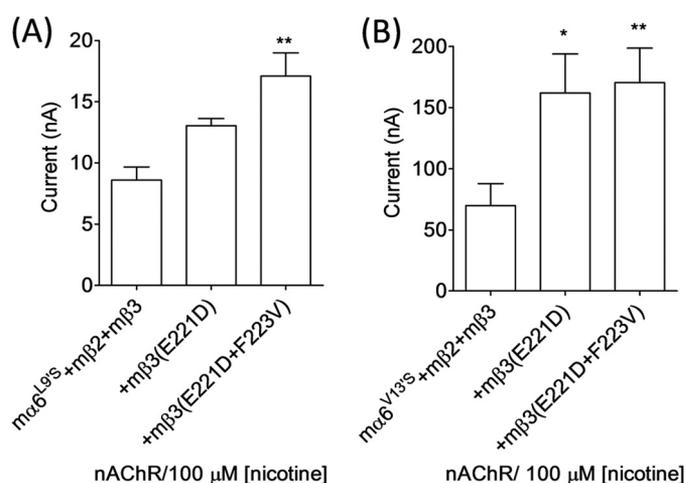
**Parameters for nicotine action at nAChRs containing  $\alpha 6$  and  $\beta 3$  mutant subunits**

Potencies (micromolar EC<sub>50</sub> values with 95% confidence intervals (CI)), Hill coefficients ( $n_H \pm$  S.E.), mean  $\pm$  S.E. efficacies ( $I_{\text{max}}$  in nA), and concentrations (conc.) where maximal peak current amplitudes ( $I_{\text{max}}$ ) are achieved ( $\mu\text{M}$ ) are provided for nicotine acting at mouse nAChR composed of the indicated subunits and from the indicated number of independent experiments ( $n$ ) based on studies as shown in Fig. 6.  $\uparrow$  indicates a significant ( $p < 0.05$ ) increase in the indicated parameter at the indicated nAChR subtype relative to wild-type  $\alpha 6\beta 4$ -nAChR, and  $\blacktriangle$  indicates a significant ( $p < 0.05$ ) increase in the indicated parameter at the indicated nAChR subtype relative to wild-type  $\alpha 6\beta 4\beta 3$ -nAChR.

nAChR subunit combinations	$n$	Potency		Peak response	
		EC <sub>50</sub> (95% CI)	$n_H \pm$ S.E.	Mean $I_{\text{max}} \pm$ S.E.	$I_{\text{max}}$ conc.
$\alpha 6 + \beta 4$	6	23 (15–37)	0.8 $\pm$ 0.13	50 $\pm$ 13	1000
$\alpha 6 + \beta 4 + \beta 3$	3	6.6 (5.1–8.5) $\uparrow$	1.1 $\pm$ 0.12	85 $\pm$ 15	100 $\uparrow$
$\alpha 6 + \beta 4 + \beta 3$ (S144N/S148V)	5	10 (8.2–13) $\uparrow$	1.2 $\pm$ 0.18	266 $\pm$ 27 $\uparrow\blacktriangle$	100 $\uparrow$
$\alpha 6 + \beta 4 + \beta 3$ (E221D)	5	4 (2.3–7.2) $\uparrow$	0.8 $\pm$ 0.18	199 $\pm$ 34 $\uparrow\blacktriangle$	100 $\uparrow$
$\alpha 6 + \beta 4 + \beta 3$ (E221D/F223V)	5	6.5 (5.1–8.2) $\uparrow$	1 $\pm$ 0.11	307 $\pm$ 32 $\uparrow\blacktriangle$	100 $\uparrow$

**AA Substitutions in Loop C of nAChR  $\beta 3$  Subunit Also Influence Function of  $\alpha 6^{L9'S}$  or  $\alpha 6^{V13'S}$   $\beta 2^*$ -nAChRs**—Previously, we could not detect functional expression of  $\alpha 6^{L9'S}\beta 2$ - or  $\alpha 6^{V13'S}\beta 2$ -nAChRs or of  $\alpha 6^{L9'S}\beta 2\beta 3$ - or  $\alpha 6^{V13'S}\beta 2\beta 3$ -nAChRs in oocytes (38). Upon increasing the amount of cRNA injected for each subunit to  $\sim 23$  ng, there was emergence of functional  $\alpha 6^{L9'S}$  or  $\alpha 6^{V13'S}\beta 2\beta 3$ -nAChRs, but not  $\alpha 6^{L9'S}$  or  $\alpha 6^{V13'S}\beta 2$ -nAChRs, in oocytes. However, nicotine-elicited (100  $\mu\text{M}$ ) peak current responses from oocytes expressing  $\alpha 6^{V13'S}\beta 2\beta 3$ -nAChRs were higher (70  $\pm$  18 versus 9  $\pm$  1 nA;  $\sim 8$ -fold;  $p < 0.05$ ) than those expressing  $\alpha 6^{L9'S}\beta 2\beta 3$ -nAChRs (Fig. 7).

We also assessed whether substitutions of AA residues in loop C of the nAChR  $\beta 3$  subunit would increase the peak current responses of  $\alpha 6^{L9'S}\beta 2^*$ - or  $\alpha 6^{V13'S}\beta 2^*$ -nAChRs. Nicotine-elicited (100  $\mu\text{M}$ ) peak current responses from oocytes expressing  $\alpha 6^{L9'S}\beta 2\beta 3$ - (9  $\pm$  1 versus 17  $\pm$  2 nA) or  $\alpha 6^{V13'S}\beta 2\beta 3$  (70  $\pm$  18 versus 170  $\pm$  28 nA)-nAChRs were increased ( $p < 0.05$ ) as a result of E221D/F223V substitution in the nAChR  $\beta 3$  subunit. Also, oocytes expressing  $\alpha 6^{V13'S}\beta 2\beta 3$ (E221D)-nAChRs (162  $\pm$  32 nA) yielded higher peak current ( $p < 0.05$ ) in response to 100  $\mu\text{M}$  nicotine



**FIGURE 7. Effects of nAChR  $\beta 3$  subunit loop C amino acid substitutions on functional responsiveness of  $\alpha 6^{L9'S}$  or  $\alpha 6^{V13'S}$   $\beta 2^*$ -nAChRs.** Mean  $\pm$  S.E. (error bars) peak inward current responses upon exposure to 100  $\mu\text{M}$  nicotine (5-s exposure; ordinate) were measured from oocytes ( $n = 3$ – $6$ ) voltage-clamped at  $-70$  mV and heterologously expressing  $\alpha 6^{L9'S}$ ,  $\beta 2$ - plus  $\beta 3$ -,  $\beta 3$ (E221D)-, or  $\beta 3$ (E221D/F223V)-nAChRs (A) or  $\alpha 6^{V13'S}$ ,  $\beta 2$ - plus  $\beta 3$ -,  $\beta 3$ (E221D)-, or  $\beta 3$ (E221D/F223V)-nAChR subunits (B). Current responses were compared using one-way analyses of variance (with Tukey's post hoc comparison).  $*$ ,  $p < 0.05$ ;  $**$ ,  $p < 0.01$ .

## Effects of $\beta 3$ Subunits on Mouse $\alpha 6^*$ -nAChR Function

than those expressing  $\alpha 6^{V13'S}m\beta 2m\beta 3$ -nAChRs. These results confirm the previous findings that TM II 13' valine-to-serine mutations in the  $\alpha 6$  subunit are more capable of attributing gain of function to  $\alpha 6^*$ -nAChRs than the TM II 9' leucine-to-serine mutation (38).

### DISCUSSION

Functional heterologous expression of human or mouse  $\alpha 6^*$ -nAChRs has been difficult. Various approaches have been undertaken to circumvent this situation for human  $\alpha 6^*$ -nAChRs (14, 15, 19, 22, 31, 38, 42). There is hardly any focus on heterologous expression of functional mouse  $\alpha 6^*$ -nAChRs, although they are known to be physiologically important (3, 8, 10, 12, 43–45). Our initial studies, using  $\sim 1$ – $6$  ng of cRNA for each subunit, indicated that mouse  $\alpha 6\beta 4$ -nAChRs expressed in oocytes are minimally functional (15). Although there was no emergence of functional mouse  $\alpha 6\beta 4\beta 3$ -nAChRs in oocytes, mouse  $\alpha 6\beta 4\beta 3^{V9'S}$ -nAChRs in oocytes were highly functional (15). Functional  $\alpha 6m\beta 2$ -,  $\alpha 6m\beta 2m\beta 3$ -, or  $\alpha 6m\beta 2m\beta 3^{V9'S}$ -nAChRs or  $\alpha 6^{L9'S}m\beta 2$ -,  $\alpha 6^{L9'S}m\beta 2m\beta 3$ -,  $\alpha 6^{V13'S}m\beta 2$ -, or  $\alpha 6^{V13'S}m\beta 2m\beta 3$ -nAChRs also were not detected in oocytes (15, 38). In continuation of these studies, here we used various approaches to produce or enhance the functional expression of mouse  $\alpha 6^*$ -nAChRs.

We noticed that upon substitution of nAChR  $h\beta 3$  subunits for nAChR  $m\beta 3$  subunits highly functional hybrid  $\alpha 6m\beta 4h\beta 3$ -nAChRs were produced in oocytes. We also noticed that functional  $\alpha 6m\beta 4m\beta 3$ -nAChRs were formed in oocytes when they were expressed using an injection of relatively larger amounts of cRNAs ( $\sim 23$  ng) for each subunit. The peak current responses of these  $\alpha 6m\beta 4m\beta 3$ -nAChRs were nonetheless severalfold lower than those of hybrid  $\alpha 6m\beta 4h\beta 3$ -nAChRs. Similar to previous observations (15), whether using relatively lower or higher amounts of injected cRNA for each subunit, functional  $\alpha 6m\beta 2$ -,  $\alpha 6m\beta 2m\beta 3$ -, or  $\alpha 6m\beta 2h\beta 3$ -nAChRs were not detected in oocytes. However, upon increasing the amount of cRNA injected for each subunit, minimally functional  $\alpha 6m\beta 2m\beta 3^{V9'S}$ - or robustly functional  $\alpha 6m\beta 2h\beta 3^{V9'S}$ -nAChRs emerged on cell surfaces. Additionally,  $\alpha 6m\beta 4^*$ -nAChRs harboring  $h\beta 3^{V9'S}$  or  $h\beta 3^{V13'S}$  subunits showed gain of function similar to those of  $\alpha 6m\beta 4m\beta 3^{V9'S}$ - or  $\alpha 6m\beta 4m\beta 3^{V13'S}$ -nAChRs (15). Therefore, incorporation of  $h\beta 3$  or  $m\beta 3$  subunits into  $\alpha 6m\beta 4^*$ -nAChRs is evident because it had a potentiation effect. These results also suggest that these WT  $\beta 3$  subunits must be facilitating assembly of functional receptors. Potentiation of agonist sensitivity and levels of functional responses also indicate that there was incorporation of mutant  $h\beta 3^{V9'S}$  or  $m\beta 3^{V9'S}$  subunits into  $\alpha 6m\beta 4^*$ - or  $\alpha 6m\beta 2^*$ -nAChRs with further facilitation of functional receptor expression, increased frequency of agonist-gated channel opening, or both. These results also are indicative of efficient incorporation of  $h\beta 3$  subunits, but not that of  $m\beta 3$  subunits, into assemblies of  $\alpha 6$  and  $m\beta 4$  subunits or of  $\alpha 6$  and  $m\beta 2$  subunits.

Our results confirm that ternary complexes involving  $\alpha 6$  plus  $m\beta 2$  or  $\alpha 6$  plus  $m\beta 4$  subunits are formed. The observation that functional  $\alpha 6m\beta 4^*$ -nAChRs, but not  $\alpha 6m\beta 2^*$ -nAChRs, were formed whenever oocytes were injected with higher amount of cRNAs is consistent with such observations

made in studies of expression of human  $\alpha 6^*$ -nAChRs in oocytes (14). We also extended this strategy to ensure expression of functional  $\alpha 6m\beta 2m\beta 3^{V9'S}$ - or  $\alpha 6m\beta 2h\beta 3^{V9'S}$ -nAChRs. The need for elevated subunit abundance in oocytes for formation of cell surface, functional  $\alpha 6^*$ -nAChRs is in contrast to the relative ease of expression of functional WT  $\alpha 2\beta 4$ -,  $\alpha 3\beta 2$ -,  $\alpha 3\beta 4$ -,  $\alpha 4\beta 2$ -, or  $\alpha 4\beta 4$ -nAChRs or hybrid  $\alpha 6h\beta 4h\beta 3$ - or  $\alpha 6h\beta 2h\beta 3^{V9'S}$ -nAChRs in oocytes using  $\sim 1$ – $2$  ng of cRNAs for each subunit (27, 30). This also suggests that differences in features or AA sequence between  $m\beta 3$  and  $h\beta 3$  subunits accounts for increased efficiency of subunit assembly to closure of pentameric and functional  $\alpha 6^*$ -nAChRs.

Transmembrane II 9' or 13' valine-to-serine mutants of  $h\beta 3$  or  $m\beta 3$  ( $h\beta 3$  or  $m\beta 3$ )<sup>(V9'S or V13'S)</sup> subunits whenever coexpressed with  $\alpha 6m\beta 4^*$ -nAChRs almost always yielded oocytes giving apparently outward current responses to atropine, indicating integration of  $\beta 3$  subunits into  $\alpha 6m\beta 4^*$ -nAChRs. This also indicates that these channels are opening spontaneously, a feature commonly seen for receptors of the ligand-gated ion channel family containing gain-of-function mutations (TM II V9'S or V13'S) (15, 19, 38, 46). Effects of atropine at high concentrations reflect its open channel blocking ability, which is seen for oocytes expressing  $\alpha 6m\beta 2h\beta 3^{V9'S}$ -nAChRs but not  $\alpha 6m\beta 2m\beta 3^{V9'S}$ -nAChRs (data not shown).

Differences in amino acid composition between  $h\beta 3$  and  $m\beta 3$  subunit extracellular N-terminal and second cytoplasmic loop regions (e.g. as opposed to their nearly identical transmembrane domains) that influence effects on  $\alpha 6^*$ -nAChR function were revealed based on studies of chimeric nAChR mouse/human  $\beta 3$  subunits or their gain-of-function variants. The involvement of the NTD of  $\beta 3$  subunits in these effects echoes previous findings that the NTD of  $h\alpha 6$  subunits influences assembly and function of human  $\alpha 6\beta 3^*$ -nAChRs (15, 19, 38).

The current site-directed mutagenesis studies indicate that substitution of  $m\beta 3$  subunit AA residues in primary face loop C with  $h\beta 3$  subunit residues enhanced functional expression of  $\alpha 6m\beta 2m\beta 3^{V9'S}$ -nAChRs. In addition,  $h\beta 3$  subunit AA substitutions in complementary face  $\beta 2$ - $\beta 3$  and E loops for residues in  $m\beta 3$  subunits increased functional expression of  $\alpha 6m\beta 4m\beta 3$ -nAChRs. These results are in agreement with the previous observations that substitutions at extracellular N-terminal loops influence functional expression of  $h\alpha 6^*$ -nAChRs and other subtypes of nAChRs (15, 19, 38, 47).

The increased functional expression of  $\alpha 6^*$ -nAChRs seen upon AA substitution in  $m\beta 3$  subunits must be due to some combination of increases in efficiency of incorporation of subunits into receptor complexes, trafficking to the cell surface, and/or preservation of cell surface receptors. nAChR  $m\beta 3$  subunit loop E residues Ser<sup>144</sup> and Ser<sup>148</sup> differ from Asn or Val residues, respectively, in  $h\beta 3$  subunits in side chain length and possibility of engaging in glycosylation (Ser *versus* Asn) and hydrophobicity (Ser *versus* Val) (48). nAChR  $m\beta 3$  subunit loop C residues Glu<sup>221</sup> and Phe<sup>223</sup> differ from Asp or Val AAs in  $h\beta 3$  subunits in side chain length (Glu *versus* Asp and Phe *versus* Val) and to some degree in hydrophobicity (Phe *versus* Val). These differences in AAs could influence interactions with

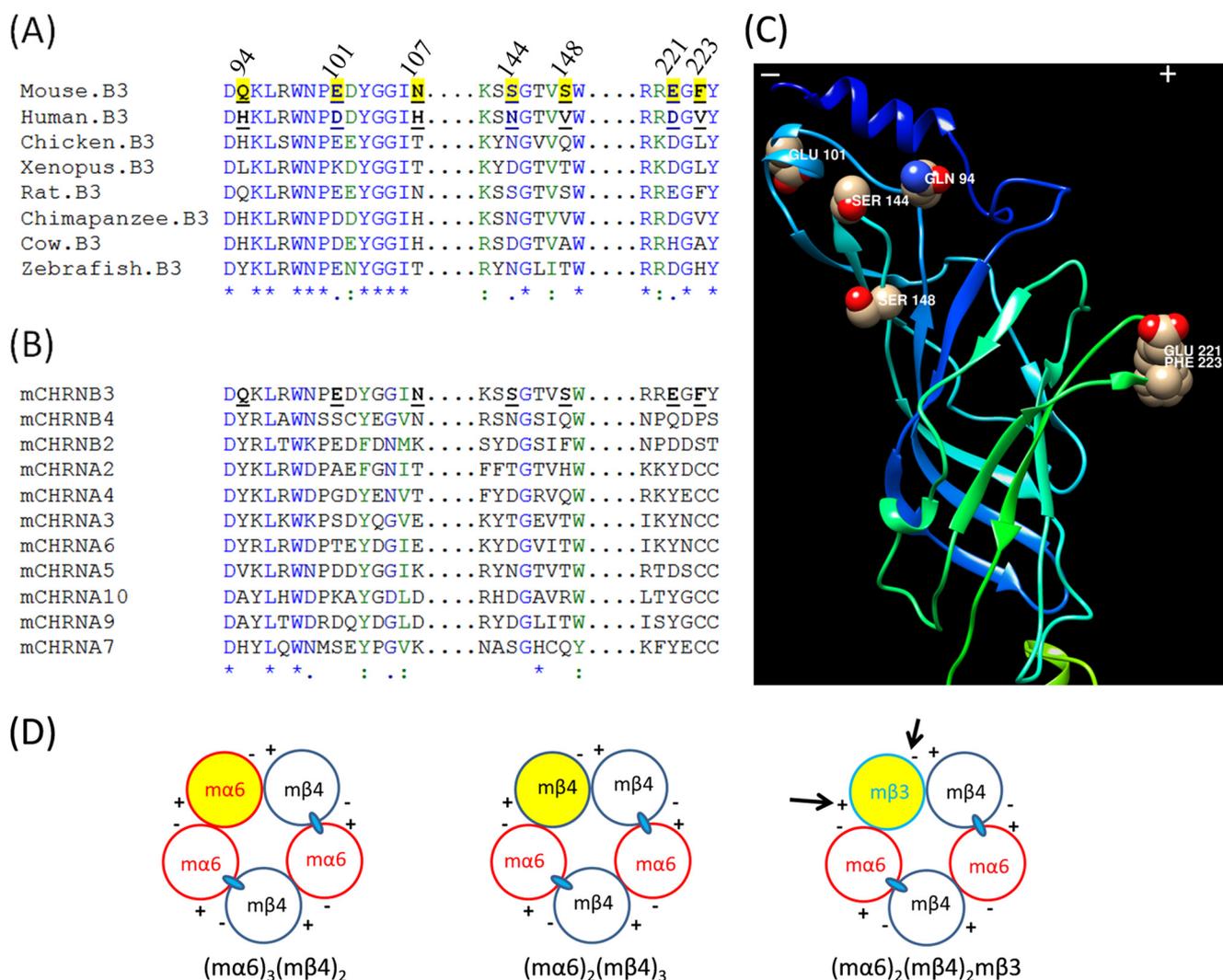


FIGURE 8. Illustration of nAChR  $\beta 3$  subunit residues and its interfaces that are important in the function of  $\alpha 6^*$ -nAChRs. A, sequence alignment of nAChR  $\beta 3$  subunit proteins from several species. nAChR  $\beta 3$  protein sequences extracted from GenBank accession numbers NP\_775304.1 (*Mouse; Mus musculus*), NP\_000740.1 (*Human; Homo sapiens*), NP\_990143.1 (*Chicken; Gallus gallus*), NP\_001080652.1 (*Frog; X. laevis*), NP\_598281.1 (*Rat; Rattus norvegicus*), NP\_001029105.1 (*Chimpanzee; Pan troglodytes*), XP\_599970.2 (*Cow; Bos taurus*), and NP\_775394.1 (*Zebrafish; Danio rerio*) were aligned using ClustalW. B, sequence alignment of mouse nAChR  $\beta 3$  subunit proteins. Mouse nAChR subunits were aligned using ClustalW. For both A and B, numbering begins at the translation start methionine of the mouse nAChR  $\beta 3$  subunit protein and is shown in the N-terminal domain region of interest. Symbols below sequences indicate fully (\*), strongly (:), or weakly (.) conserved residues, and underlining in shaded face indicates numbered residues in nAChR  $m\beta 3$  subunit targeted for mutagenesis studies. C, a three-dimensional model of the N-terminal domain of the mouse nAChR  $\beta 3$  subunit. A three-dimensional model of the mouse nAChR  $\beta 3$  subunit was generated based on the crystal structure of *Torpedo* muscle nAChR  $\beta$  subunit (Protein Data Bank code 2BG9:B). The N-terminal domain of the nAChR  $m\beta 3$  subunit possesses  $\beta$  strands that form a  $\beta$  sandwich and conforms to an immunoglobulin fold. AA residues in the  $\beta 2$ - $\beta 3$  loop (Gln<sup>94</sup> and Glu<sup>101</sup>), loop E (Ser<sup>144</sup> and Ser<sup>148</sup>), or loop C (Glu<sup>221</sup> and Phe<sup>223</sup>) that positively influence the current responses of  $\alpha 6^*$ -nAChRs are identified. The figure displayed was drawn using the program Chimera. D, schematic illustration of the composition of  $m\alpha 6m\beta 4^*$ -nAChRs. Adhering to the canonical rule of pentamer formation,  $m\alpha 6m\beta 4^*$ -nAChRs would be formed of three  $\alpha 6$  and two  $\beta 4$  subunits (left) or two  $\alpha 6$  and three  $\beta 4$  subunits (middle). In the event  $m\beta 3$  subunits are integrated into  $m\alpha 6m\beta 4^*$ -nAChRs, they would substitute for the third  $\alpha 6$  subunit in the first (left) configuration or the third  $m\beta 4$  subunit in the second (middle) configuration, occupying what is labeled as the fifth position (yellow). Agonist (ACh or nicotine and others) binding sites at the interfaces between  $\alpha 6$  and either  $\beta 2$  or  $\beta 4$  subunits are shown as ovals. Results from the current study (right) support the idea that the  $\beta 2$ - $\beta 3$  loop and loop E residues in the (-) face and/or loop C residues in the (+) face (arrows) of the  $m\beta 3$  subunit are important in higher functional expression of  $m\alpha 6m\beta 4^*$ -nAChRs. Mouse  $\alpha 6\beta 2^*$ -nAChRs would attain similar configurations, but the  $\beta 2$  subunit would substitute for the  $\beta 4$  subunit.

adjacent (or distant?)  $\beta 2$ ,  $\beta 4$ , or  $\alpha 6$  subunits that are important for  $m\alpha 6m\beta 2^*$ - and  $m\alpha 6m\beta 4^*$ -nAChR assembly (Fig. 8D).

$\beta 2$ - $\beta 3$  or E loop residues in the negative (-) or complementary face of the  $m\beta 3$  subunit would be involved in presumed interactions with residues on the positive (+) or primary faces of the neighboring  $m\beta 4$  or  $m\beta 2$  subunit, and loop C residues in the positive (+) or primary face of the  $\beta 3$  subunit would be involved in presumed interactions with residues on the negative (-) or complementary faces of the neighboring  $m\alpha 6$  subunit in a complex that has the presumed arrangement of  $-(\beta 3$

or  $\beta 3^{V9'5})_+; -\alpha 6_+; -(\beta 2 \text{ or } \beta 4)_+; -\alpha 6_+; -(\beta 2 \text{ or } \beta 4)_+$  where ligand binding pockets are thought to be located between the primary (+) face of  $m\alpha 6$  and complementary (-) face of the  $m\beta 2$  or  $m\beta 4$  subunits (i.e.  $\alpha 6_+; -\beta 4$  or  $\alpha 6_+; -\beta 2$ ) (Fig. 8). Agonist binding is not expected occur at  $\beta 3_+; -(\beta 2 \text{ or } \beta 4)$  or  $\beta 3_+; -\alpha 6$  subunit interfaces. However, recent evidence suggests that interfaces involving subunits in the accessory subunit position where the  $\beta 3$  subunit would be situated can engage in allosteric or co-agonist effects (49–51). Residues in or equivalent to those at  $m\beta 3$  subunit positions 94, 101, 107, 144, 148, 221, and 223 are

## Effects of $\beta 3$ Subunits on Mouse $\alpha 6^*$ -nAChR Function

conserved with those in rats but differ from those that are conserved within primates (human and chimp; Fig. 8A). These m $\beta 3$  subunit AAs also are unique across mouse nAChR subunits (Fig. 8B). We have advanced the possibility that these residues could affect efficiency of  $\alpha 6^*$ -nAChR assembly (not altering agonist potency but affecting peak current responses as for m $\alpha 6^*$ -nAChRs harboring m $\beta 3$ (S144N/S148V), m $\beta 3$ (E221D), or m $\beta 3$ (E221D/F223V) subunits). Another intriguing possibility is that these unique residues could allow formation of novel classes of ligand binding sites at  $\beta 2/\beta 4:\beta 3$  or  $\beta 3:\alpha 6$  subunit interfaces that also could lead to changes in levels of receptor function as for ligand occupancy of the  $\alpha 4:\alpha 4$  subunit interface in low sensitivity  $\alpha 4\beta 2^*$ -nAChR (51).

Plenty of information is available on the role of primary face loops (A, B, and C) from  $\alpha$  subunits and complementary face loops (D, E, and F) from  $\beta$  subunits that participate in ligand binding largely from structural and/or mutagenesis studies of muscle-type,  $\alpha 7$ -, or other nAChRs and from lower eukaryotic and prokaryotic proteins structurally homologous to the extracellular domain of nAChRs (52–54). Our results presented here, for the first time, show that extracellular N-terminal domain loops of the accessory subunit,  $\beta 3$ , regulate the functional expression of m $\alpha 6^*$ -nAChRs. These results also provide further evidence that nAChR  $\beta 3$  subunits not only form functional receptors in combination with nAChR  $\alpha 6$  subunits but also can enhance their function by interacting with adjacent subunits mediated by N-terminal loop residues. Current findings lay a foundation for enhanced functional expression of m $\alpha 6^*$ -nAChRs that could facilitate the discovery and development of nicotinic ligands that selectively interact with  $\alpha 6^*$ -nAChRs. These results could be useful to fuel and inform emerging interest in  $\alpha 6$  and  $\beta 3$  subunits and the receptors they compose with specific reference to possible roles in locomotion, reward and reinforcement behavior, schizophrenia, and Parkinson disease (5, 6, 55).

**Acknowledgments**—We thank Dr. Jerry A. Stitzel (Department of Integrative Physiology, University of Colorado, Boulder, CO) for providing mouse nAChR subunit constructs. We also thank Drs. Doug DeSimone and Todd Stukenberg and Fred Simon of Dr. DeSimone's laboratory (Department of Biology, University of Virginia, Charlottesville, VA) for kind help in establishing and maintaining an *X. laevis* colony at the University of Virginia Aquatic Animal Center.

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**Roles for N-terminal Extracellular Domains of Nicotinic Acetylcholine Receptor (nAChR)  $\beta$ 3 Subunits in Enhanced Functional Expression of Mouse  $\alpha$ 6 $\beta$ 2 $\beta$ 3- and  $\alpha$ 6 $\beta$ 4 $\beta$ 3-nAChRs**

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*J. Biol. Chem.* 2014, 289:28338-28351.

doi: 10.1074/jbc.M114.566018 originally published online July 15, 2014

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Access the most updated version of this article at doi: [10.1074/jbc.M114.566018](https://doi.org/10.1074/jbc.M114.566018)

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