Fourier transform coupled tryptophan scanning mutagenesis identifies a bending point on the lipid-exposed δM3 transmembrane domain of the *Torpedo californica* nicotinic acetylcholine receptor

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Abbreviations: ACh, acetylcholine; α-BgTX, α-bungarotoxin; C-R, concentration response; FT-TrpScanM, fourier transform tryptophan scanning mutagenesis; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; ¹²⁵I-TID, 3-trifluoromethyl-3-(m-[¹²⁵I]iodophenyl)diazirine; MENM, mixed elastic network model; nAChR, nicotinic acetylcholine receptor; NMDA, N-methyl-D-aspartate; pLGIC, pentameric ligand-gated ion channel; TMD, transmembrane domain; TrpPP, tryptophan periodicity profile

The nicotinic acetylcholine receptor (nAChR) is a member of a family of ligand-gated ion channels that mediate diverse physiological functions, including fast synaptic transmission along the peripheral and central nervous systems. Several studies have made significant advances toward determining the structure and dynamics of the lipid-exposed domains of the nAChR. However, a high-resolution atomic structure of the nAChR still remains elusive. In this study, we extended the Fourier transform coupled tryptophan scanning mutagenesis (FT-TrpScanM) approach to gain insight into the secondary structure of the δ M3 transmembrane domain of the *Torpedo californica* nAChR, to monitor conformational changes experienced by this domain during channel gating, and to identify which lipid-exposed positions are linked to the regulation of ion channel kinetics. The perturbations produced by periodic tryptophan substitutions along the δ M3 transmembrane domain were characterized by two-electrode voltage clamp and ¹²⁵I-labeled α -bungarotoxin binding assays. The periodicity profiles and Fourier transform spectra of this domain revealed similar helical structures for the closed- and open-channel states. However, changes in the oscillation patterns observed between positions Val-299 and Val-304 during transition between the closed- and open-channel states can be explained by the structural effects caused by the presence of a bending point introduced by a Thr-Gly motif at positions 300–301. The changes in periodicity and localization of residues between the closed-and open-channel states could indicate a structural transition between helix types in this segment of the domain. Overall, the data further demonstrate a functional link between the lipid-exposed transmembrane domain and the nAChR gating machinery.

Introduction

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The nicotinic acetylcholine receptor (nAChR) is a well-characterized integral membrane protein that belongs to the Cys-loop superfamily of ligand-gated ion channels, and it mediates chemical communication at the neuromuscular junction. Topological studies have revealed that it is composed of four homologous subunits in the stoichiometry of $2\alpha 1:\beta 1:\gamma$ or $\varepsilon:\delta$ arranged pseudo-symmetrically around a central axis, forming a cation-selective ion channel.^{1,2} Each subunit contains a large hydrophilic extracellular N-terminal

domain that bears the ligand binding domains; four hydrophobic segments of 19–25 amino acids, denoted as M1–M4, that are proposed to be membrane spanning domains; a large cytoplasmic loop between the M3 and M4 domains that bears phosphorylation sites; and a short hydrophilic extracellular C terminus.³ The M2 transmembrane domain (TMD) delimits the ion channel pore in the closed-channel state, whereas the M1 and M2 TMDs contribute to the formation of the ion channel pore in the open-channel state.^{2,4,5} The M3 and M4 domains, which have the lowest protein sequence conservation across muscle and neuronal nAChR species,

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form the outer contour and are proposed to have the largest contact with the lipid membrane. $^{\rm 6}$

A high-resolution atomic structure of the nAChR has remained elusive to date as approaches traditionally used to probe the structure and dynamics of proteins, such as X-ray crystallography and multi-dimensional ¹H-NMR, have not been able to solve the structure of such a large macromolecule. Currently, the best approximation of the nAChR structure comes from cryoelectron microscopy of the Torpedo marmorata nAChR at 4.0 Å resolution (PDB 2BG9), which provides information on the secondary structure and global arrangement of the transmembrane domains.^{2,5} The insight generated by this structure has been complemented by the elucidation of the structures of the soluble acetylcholine-binding protein from Lymnaea stagnalis, a structural and functional homologue to the N-terminal extracellular domain of the nAChR α 1 subunit, and the extracellular domain of the nAChR α 1 subunit while bound to α -bungarotoxin.^{7,8} Furthermore, several studies have made significant advances toward determining the structure and dynamics of the lipidexposed domains of the nAChR. Studies using photoaffinity labeling,⁹⁻¹¹ Fourier transform infrared spectroscopy,¹² circular dichroism,13 two-dimensional 1H-NMR,14 and tryptophan scanning mutagenesis¹⁵⁻²¹ have suggested that the TMDs are organized as helical structures and provided evidence for the dramatic effects of mutations of lipid-exposed domains on the functional states of the nAChR. However, atomic resolution data from the nAChR TMDs in the closed- and open-channel states remain unavailable. Nevertheless, the nAChR has been extensively used as a model system in the study of lipid-protein interactions due to existing knowledge of channel structure, gating mechanics, and the overall spatial orientation of the lipid-exposed domains.^{2,10,22,23}

In this study, we extend the Fourier transform coupled tryptophan scanning mutagenesis (FT-TrpScanM) approach to gain insight into the secondary structure of the $\delta M3$ TMD of the Torpedo californica nAChR, to monitor conformational changes experienced by this TMD during channel gating, and to identify which lipid-exposed positions on this domain are potentially linked to the regulation of ion channel kinetics. This approach has been used successfully for inward rectifier potassium channels,²⁴⁻²⁶ nAChRs,¹⁵⁻²¹ voltage-gated potassium channels,²⁷⁻²⁹ glutamate receptors,³⁰ γ -aminobutyric acid type A (GABA_A) receptors,³¹ voltage-gated sodium channels,³² N-methyl-Daspartate (NMDA) receptors,³³ P2X4 receptors,³⁴ and the large conductance mechanosensitive channels (MscL),³⁵ among others. Eighteen residues (Met-293 to Leu-310) along the δ M3 TMD were periodically substituted for tryptophan, and these mutant receptors were characterized by two-electrode voltage clamp and ¹²⁵I-labeled α -bungarotoxin (¹²⁵I-labeled α -BgTx) binding assays. Our results suggest helical secondary structures for the closedand open-channel states of the $\delta M3$ TMD, while the difference in oscillation patterns between both states show a discrete and/ or localized conformational change (~0.4 amino acids per helical turn) along this domain as a consequence of channel activation. The oscillation pattern observed between positions Val-299 and Val-304 in the closed-channel state suggests that this TMD exhibits a wide-turn caused by the presence of a bending point introduced by a Thr-Gly motif at positions 300–301. The changes in periodicity and localization of residues between the closed-and open-channel states in this segment of the δ M3 TMD could indicate a structural transition between helix types during channel activation. Overall, the data emphasizes the functional link between this lipid-exposed transmembrane domain and the nAChR gating machinery.

Results

Cell-surface expression levels of the nAChR δ M3 TMD mutants. Eighteen mutations along the core of the Torpedo californica nAChR 8M3 TMD (Met-293 to Leu-310) were successfully engineered by replacing the wild type (WT) codon for a tryptophan codon at the desired position (Fig. 1A). Analysis of the ¹²⁵I-labeled α-BgTx binding sites revealed different cell-surface nAChR expression levels for the mutations along the $\delta M3$ TMD (Fig. 2 and Table 1). Five mutant receptors (L298W, T300W, G301W, N305W and I308W) displayed statistically significant increases in nAChR expression levels (2.3-, 3.1-, 4.3-, 3.1- and 5.9-fold increases, respectively) as compared with the WT receptor, suggesting an increase in the efficiency of assembly and/or oligomerization induced by these mutations. Two mutant receptors (I295W and C306W) displayed dramatic statistically significant reductions in nAChR expression levels (60.6- and 29.4-fold reduction, respectively), while the remaining 11 mutant receptors (M293W, F294W, M296W, S297W, V299W, V302W, I303W, V304W, G307W, V309W and L310W) exhibited statistically similar expression levels as the WT receptor. It is noteworthy that the mutation with the lowest nAChR expression level (I295W) produced a significant normalized macroscopic response. These results demonstrate that a bulky aromatic side chain can be accommodated at any position along the $\delta M3$ TMD of the Torpedo californica nAChR without inhibiting nAChR assembly.

Electrophysiological characterization of the nAChR 8M3 TMD mutants: macroscopic and normalized responses. All 18 mutant receptors were able to elicit ACh-induced currents when characterized by two-electrode voltage clamp (Fig. 1B). Thirteen mutant receptors (M293W, F294W, I295W, M296W, S297W, L298W, V299W, I303W, V304W, C306W, G307W, V309W and L310W) displayed statistically significant reductions of the maximum macroscopic response (1.3-, 1.6-, 2.5-, 1.5-, 1.3-, 1.3-, 1.3-, 3.4-, 1.3-, 73.3-, 1.7-, 1.5- and 1.4-fold reductions, respectively) as compared with the WT receptor (Table 1). The maximum macroscopic responses of the remaining five mutant receptors (T300W, G301W, V302W, N305W and I308W) were statistically similar to that of the WT receptor. The normalized macroscopic response (-nA/fmol) was drastically increased by 8.9-fold for the I295W mutant receptor as compared with WT (Table 1). The normalized macroscopic responses of the remaining 17 mutant receptors were statistically similar to that of the WT nAChR.

Mutant receptors with expression levels similar to the WT nAChR displayed maximum macroscopic responses that were either lower (e.g., M293W, among others) than or similar (e.g., V302W) to those of the WT nAChR. In addition, mutant receptors with higher expression levels than the WT were able to



Figure 1. Sequence alignment of the δ M3 transmembrane domain and functional response of wild type and δ M3 mutant nAChRs. (A) Positions Met-293 to Leu-310 were examined in this study. Positions Met-293, Ser-297, Gly-301, Val-304 and Asn-305 (highlighted in green) were labeled as lipid-exposed using 3-trifluoromethyl-3-(m-[¹²⁵1]iodophenyl)diazirine (¹²⁵1-TID) photolabeling affinity.¹⁰ Residues highlighted in red are conserved residues among all species. The numbers at the bottom indicate the position in the Torpedo δ subunit. (B) Representative families of macroscopic ionic current traces evoked by 1–300 μ M ACh and recorded through voltage clamp. Bar scale: 5,000 nA (abscissa), 5 s (ordinate). (C) Concentration-response curves that were normalized to maximum ionic current for wild type-like (top), gain-of-function (middle) and loss-of-function (bottom) δ M3 mutant nAChRs.

display lower (e.g., L298W) or similar (e.g., T300W, G301W, N305W and I308W) maximum macroscopic responses as compared with the WT nAChR. Previous reports from our group have found mutant receptors with similar behavior in the Torpedo α M3, α M4, β M3, β M4, γ M4 TMDs, and the *Mus musculus* α M3 TMD.^{15-17,20,21} It is noteworthy that the I295W mutant receptor displayed a dramatic increase in its normalized macroscopic response while displaying a significant reduction in both macroscopic current and expression levels, and a similar potency to ACh as compared with the WT. These results show that the functional effects caused by the tryptophan substitutions are due to the mutation itself rather than the variation in the nAChR expression levels.

Electrophysiological characterization of the nAChR δ M3 TMD mutants: potency to ACh and cooperativity. All mutant nAChRs showed typical sigmoidal concentration-response (C-R) curves (Fig. 1C). Seven mutant receptors (M293W, F294W, G301W, V302W, I303W, V304W and G307W) exhibited statistically significant reductions in potency to ACh by 1.5- to 2.5-fold relative to WT, as evident from the displacement of the C-R curves to higher EC₅₀ values (Table 1 and Fig. 1C). In contrast, six mutant receptors (V299W, T300W, N305W, C306W,



Figure 2. Expression level profile for the δ M3 nAChR mutants. ¹²⁵I-labeled α -BgTx binding assays were performed to determine nAChR expression levels in the plasmatic membrane. Each bar represents the mean level of nAChR expression (fmol) \pm SEM. *p < 0.05 compared to WT nAChR.

1308W and V309W) displayed a gain-of-function response relative to the WT receptor, as their EC_{50} values were shifted to lower ACh concentrations. In particular, the potency to ACh for the 1308W mutant receptor was enhanced 3.0-fold, while its cell-surface expression was enhanced 5.9-fold. The above results could be due to changes in the affinity and/or efficacy of the agonist. The potency to ACh for each of the other five mutant receptors (1295W, M296W, S297W, L298W and L310W) was statistically similar to that of the WT nAChR.

Several mutations altered the cooperativity of ACh binding to the nAChR as evidenced by changes in the Hill coefficient (nH) of the mutant receptors, relative to the WT (**Table 1**). All mutant nAChRs displayed positive cooperativity (nH > 1); however, the S297W, L298W and N305W mutant receptors seemed to be in a higher-affinity state as evidenced by the significant increases (2.0-, 2.1- and 1.2-fold) in the Hill coefficient as compared with the WT. In contrast, five mutant receptors (I295W, M296W, V299W, T300W and V309W) appeared to be in a lower-affinity state as compared with WT (1.2- to 1.5-fold decreases in nH). The cooperativity for the remaining ten mutants was statistically similar to that of the WT nAChR.

Tryptophan periodicity profiles for the open- and closedchannel states. The tryptophan periodicity profile for nAChR expression (fmol/Å³) reveals structural information about the closed-channel state of the δ M3 TMD. ¹²⁵I-labeled α -BgTx binding assays were performed in the absence of agonist and with an excess of toxin to assure that all nAChRs on the oocyte surface were bound and completely blocked by the toxin. Furthermore, after α -BgTx incubation oocytes did not display ACh-induced currents at any concentration of the agonist, thus confirming that all nAChRs on the oocyte surface were locked in the closed-channel state conformation. In contrast, the tryptophan periodicity profile produced from the EC₅₀ values for ACh activation revealed structural information about the open-channel state of the δ M3 TMD, given that the EC₅₀ value estimates the functional state of the nAChR. The tryptophan periodicity profile of the closed-channel state showed an ordered oscillation pattern between positions Asn-305 and Leu-310, and a more irregular pattern between positions Met-293 and Val-304 (Fig. 3A). These different oscillation patterns suggest that the δ M3 domain in the closed-channel state is structurally composed of a combination of different helix types: α -helix and 3₁₀-helix (Fig. 3A). However, this periodicity profile exhibited a short segment between positions Val-299 and Val-304 with an oscillation pattern of approximately 4.51 ± 0.09 residues per helical turn. This segment had a different oscillation pattern in the open-channel state. In contrast, the tryptophan periodicity profile of the open-channel state displayed a more ordered oscillation along the TMD with periodicities that suggest segments of α -helix and 3₁₀-helix (Fig. 3D), thus suggesting a helical secondary structure for the open-channel state.

The periodicity profiles for the $\delta M3$ domain in the closed- and open-channel states illustrated oscillatory patterns of 2.9 ± 0.3 and 2.6 ± 0.2 amino acids per helical turn, respectively, revealing similar helical structures for the closed- and open-channel states (**Table 2**). The difference in oscillation patterns between the closed- and open-channel states showed a discrete conformational change (-0.4 amino acids per helical turn) along this domain as a consequence of channel activation. These results for the closed- and open-channel states suggest that the *Torpedo californica* nAChR $\delta M3$ TMD displays similar helical structures in the closed- and open-channel states, akin to the Torpedo $\beta M4$, $\gamma M4$ and $\alpha M4$ TMDs.^{15,19,21} In contrast, the $\alpha M3$ TMD of the *Mus musculus* nAChR displayed a substantial conformational change during transition between the closed- and open-channel states.²⁰

Fourier transform power spectra. To corroborate the estimated periodicity of the perturbations caused by systematic tryptophan substitutions along the $\delta M3$ TMD, we used a discrete Fourier transform analysis (see Materials and Methods). Fourier transform is a mathematical tool that is generally used to detect periodic variations in a sequence of values through the formation of a frequency spectrum. The tryptophan periodicity profiles for nAChR expression and the EC₅₀ for ACh activation values were converted into Fourier transform power spectra (Fig. 3A, B, D and E). Fourier transform power spectra show the decomposition of the tryptophan periodicity profiles into multiple peaks with different abundances, suggesting that the tryptophan periodicity profiles can be divided into various periodicities with several oscillatory patterns. Fourier transform power spectra show the peaks corresponding to mean periodicities (residues per turn) predicted by the tryptophan-periodicity profiles (see Table 2). An important aspect of these peaks, identified by an asterisk in Figure 3B and E, is that, although they are not the predominant peaks due to the disparity in the oscillatory amplitudes of the tryptophan periodicity profiles, they represent the mean periodicities of the tryptophan periodicity profiles.^{20,21,36} The mean periodicities estimated from the Fourier transform power spectra for the closed- and open-channel states were determined to be 2.96 and 2.63 amino acids per helical turn, respectively. These results correlate well with the mean periodicities obtained from the analysis of the tryptophan periodicity profiles (Table 2).

 α -helical character curves. To reliably predict the secondary structure of the δ M3 TMD, we built α -helical character curves.

Table 1. Biophysical parameters of the Torpedo nAChR &M3 TMD mutants

ЕС₅₀ (µМ)	Hill coefficient	I _{max} (-nA)	¹²⁵ I- Labeled α-BgTx binding sites (fmol)	Normalized response (-nA/fmol)
20 ± 1 (17.24–22.81)	1.58 ± 0.07	13552 ± 542	10.3 ± 0.4	1337 ± 83
34 ± 4* (24.04–44.36)	1.44 ± 0.02	$10246 \pm 226^{*}$	16.4 ± 0.8	647 ± 71
49 ± 6 [*] (31.33–67.24)	1.57 ± 0.03	8513 ± 159*	7.3 ± 0.9	1175 ± 138
15.3 ± 0.9 (13.15–17.53)	$1.29 \pm 0.05^{*}$	$5452 \pm 287^{*}$	$0.17\pm0.05^{\$}$	$11920 \pm 3356^{*}$
17.3 ± 0.8 (15.32–19.19)	$1.31 \pm 0.04^{\circ}$	$8783 \pm 252^{*}$	9 ± 2	956 ± 170
16.6 ± 0.7 (14.80–18.37)	$3.2 \pm 0.1^{*}$	$10391 \pm 330^{*}$	15 ± 1	696 ± 46
20.9 ± 0.9 (18.46-23.36)	$3.29\pm0.05^{*}$	$10130 \pm 141^{*}$	$24 \pm 3^{*}$	423 ± 41
$10.9 \pm 0.8^{*} (8.695 {-} 13.05)$	$1.09 \pm 0.03^{*}$	$10335 \pm 294^{*}$	15 ± 3	481 ± 64
10 ± 1 [*] (6.321–13.24)	$1.05 \pm 0.05^{*}$	13913 ± 724	32 ± 1*	454 ± 18
44 ± 2 [*] (37.73–49.78)	1.63 ± 0.06	14851 ± 268	$44 \pm 6^{*}$	327 ± 28
29 ± 3 [§] (22.07–34.96)	1.64 ± 0.08	12332 ± 566	6.6 ± 0.5	1925 ± 145
45 ± 2 [*] (40.95-49.15)	1.48 ± 0.06	$4024 \pm 309^{*}$	1.4 ± 0.1	3418 ± 446
41 ± 2 [*] (35.42-46.55)	1.57 ± 0.03	$10589 \pm 477^{*}$	4.0 ± 0.5	2207 ± 223
$7.3 \pm 0.6^{*} (5.658 {-} 8.917)$	$1.9 \pm 0.1^{*}$	14210 ± 544	$32 \pm 3^{*}$	476 ± 43
14 ± 1* (10.85–17.49)	1.61 ± 0.04	$185 \pm 33^{*}$	$0.35\pm0.05^{\$}$	399 ± 109
33 ± 2 [*] (28.97–36.76)	1.52 ± 0.08	$8068\pm415^{*}$	2.7 ± 0.4	3737 ± 683
$6.7\pm0.5^{*}(5.3237.990)$	1.47 ± 0.06	14540 ± 797	61 ± 4*	236 ± 16
$10.9 \pm 0.8^{*} (8.732 13.11)$	$1.27 \pm 0.05^{*}$	$8892 \pm 1242^{*}$	3.8 ± 0.6	2032 ± 352
23 ± 1 (20.08–26.21)	1.46 ± 0.07	$9658 \pm 986^{*}$	12 ± 1	963 ± 148
	EC ₅₀ (μM) 20 ± 1 (17.24-22.81) 34 ± 4* (24.04-44.36) 49 ± 6* (31.33-67.24) 15.3 ± 0.9 (13.15-17.53) 17.3 ± 0.8 (15.32-19.19) 16.6 ± 0.7 (14.80-18.37) 20.9 ± 0.9 (18.46-23.36) 10.9 ± 0.8* (8.695-13.05) 10.9 ± 0.8* (8.695-13.05) 10.9 ± 0.8* (8.695-13.05) 10.9 ± 0.8* (8.695-13.05) 10.9 ± 3 [§] (22.07-34.9.78) 29.9 ± 3 [§] (22.07-34.9.78) 29.5 ± 2* (40.95-49.15) 41 ± 2* (35.42-46.55) 41 ± 2* (35.42-46.55) 13.5 ± 0.6* (5.658-8.917) 14 ± 1* (10.85-17.49) 33 ± 2* (28.97-36.76) 6.7 ± 0.5* (5.323-7.990) 10.9 ± 0.8* (8.732-13.11) 23.5 ± 1 (20.08-26.21)	EC_{50} (μ M)Hill coefficient 20 ± 1 (17.24–22.81) 1.58 ± 0.07 $34 \pm 4'$ (24.04–44.36) 1.44 ± 0.02 $49 \pm 6'$ (31.33–67.24) 1.57 ± 0.03 15.3 ± 0.9 (13.15–17.53) $1.29 \pm 0.05'$ 17.3 ± 0.8 (15.32–19.19) 1.31 ± 0.04^{5} 16.6 ± 0.7 (14.80–18.37) $3.2 \pm 0.1'$ 20.9 ± 0.9 (18.46–23.36) $3.29 \pm 0.05'$ $10.9 \pm 0.8'$ (8.695–13.05) $1.09 \pm 0.03'$ $10 \pm 1'$ (6.321–13.24) $1.05 \pm 0.05'$ $10 \pm 1'$ (6.321–13.24) 1.63 ± 0.06 29 ± 3^{5} (22.07–34.96) 1.64 ± 0.08 $45 \pm 2'$ (40.95–49.15) 1.48 ± 0.06 $41 \pm 2'$ (35.42–46.55) 1.57 ± 0.03 $7.3 \pm 0.6'$ (5.658–8.917) $1.9 \pm 0.1'$ $14 \pm 1'$ (10.85–17.49) 1.61 ± 0.04 $33 \pm 2'$ (28.97–36.76) 1.52 ± 0.08 $6.7 \pm 0.5'$ (5.323–7.990) 1.47 ± 0.06 $10.9 \pm 0.8'$ (8.732–13.11) $1.27 \pm 0.05'$ 23 ± 1 (20.08–26.21) 1.46 ± 0.07	EC_50 (μM)Hill coefficientI max (-nA)20 ± 1 (17.24-22.81)1.58 ± 0.0713552 ± 54234 ± 4' (24.04-44.36)1.44 ± 0.0210246 ± 226'49 ± 6' (31.33-67.24)1.57 ± 0.038513 ± 159'15.3 ± 0.9 (13.15-17.53)1.29 ± 0.05'5452 ± 287'17.3 ± 0.8 (15.32-19.19)1.31 ± 0.04 [§] 8783 ± 252'16.6 ± 0.7 (14.80-18.37)3.2 ± 0.1'10391 ± 330'20.9 ± 0.9 (18.46-23.36)3.29 ± 0.05'10130 ± 141'10.9 ± 0.8' (8.695-13.05)1.09 ± 0.03'10335 ± 294'10 ± 1' (6.321-13.24)1.05 ± 0.05'13913 ± 72444 ± 2' (37.73-49.78)1.63 ± 0.0614851 ± 26829 ± 3 [§] (22.07-34.96)1.64 ± 0.0812332 ± 56645 ± 2' (40.95-49.15)1.48 ± 0.064024 ± 309'41 ± 2' (35.42-46.55)1.57 ± 0.0310589 ± 477'7.3 ± 0.6' (5.658-8.917)1.9 ± 0.1'14210 ± 54414 ± 1' (10.85-17.49)1.61 ± 0.04185 ± 33'33 ± 2' (28.97-36.76)1.52 ± 0.088068 ± 415'6.7 ± 0.5' (5.323-7.990)1.47 ± 0.05'8892 ± 1242'23 ± 1 (20.08-26.21)1.46 ± 0.079658 ± 986'	EC s0 (μM)Hill coefficientI max (nA)" ^{251-Labeled α-BgTx binding sites (fmol)20±1 (17.24-22.81)1.58±0.0713552±54210.3±0.434±4' (24.04-44.36)1.44±0.0210246±226'16.4±0.849±6' (31.33-67.24)1.57±0.038513±159'7.3±0.915.3±0.9 (13.15-17.53)1.29±0.05'5452±287'0.17±0.05'17.3±0.8 (15.32-19.19)1.31±0.04'8783±252'9±216.6±0.7 (14.80-18.37)3.2±0.1'10391±330'15±120.9±0.9 (18.46-23.36)3.29±0.05'10130±141'24±3'10.9±0.8' (8.695-13.05)1.09±0.03'10335±294'15±310.9±0.8' (8.695-13.05)1.09±0.03'10335±294'15±310.9±0.8' (8.695-13.05)1.09±0.03'13913±72432±1'44±2' (37.73-49.78)1.63±0.0614851±26844±6'29±3' (22.07-34.96)1.64±0.0812332±5666.6±0.545±2' (40.95-49.15)1.48±0.664024±309'1.4±0.141±2' (35.42-46.55)1.57±0.0310589±477'4.0±0.57.3±0.6' (5.658-8.917)1.9±0.1'14210±54432±3'14±1' (10.85-17.49)1.61±0.04185±33'0.35±0.05⁶33±2' (28.97-36.76)1.52±0.088068±415'2.7±0.46.7±0.5' (5.323-7.990)1.47±0.0614540±79761±4'10.9±0.8' (8.732-13.11)1.27±0.05'8892±1242'3.8±0.623±1 (20.08-26.21)1.46±0.07'9658±986'12±1}

All error estimates are expressed as the mean \pm SEM of 5–13 oocytes. Error estimates for EC₅₀ values are also expressed as the 95% confidence interval. *p < 0.01 and ${}^{6}0.01 compared to response in WT receptor.$

The α -helical character curves are a novel predictor tool that has been used in the analysis of the secondary structure of various lipid-exposed TMDs.³⁶ The α -helical character curves do not display complete α -helical character, suggesting that the δ M3 domain is structurally composed of a combination of different helix types: α -helix and 3₁₀-helix (**Fig. 3C and F**).

Helical net diagrams. Helical net diagrams constructed with the tryptophan periodicity profiles show that the $\delta M3$ loss-offunction and gain-of-function mutant nAChRs are confined to different ranges of rotation angles (180° region) in the open-channel state, thus suggesting they are clustered on opposite sides of the helix (Fig. 4A and B). All gain-of-function mutant nAChRs are oriented toward the same face of the helix in the open-channel state, suggesting a higher degree of packing within this region of the helix. Most of the loss-of-function mutant nAChRs are oriented toward the opposite side of the helix, except for the V302W mutant nAChR that is found in the region where all the gain-offunction nAChRs are clustered. A more disorganized pattern for the loss-of-function and gain-of-function mutants is observed in the helical net diagrams of the closed-channel state. This observation correlates with the more disordered oscillation pattern of the tryptophan periodicity profile in this state.

Discussion

Functional analysis. In this study we extended the FT-TrpScanM approach to the *Torpedo californica* nAChR δ M3 TMD. Eighteen residues (Met-293 to Leu-310) along the core of the nAChR δ M3

TMD were periodically substituted for tryptophan and these mutant receptors were characterized by two-electrode voltage clamp and $^{\rm 125}\mbox{I-labeled}$ $\alpha\mbox{-BgTx}$ binding assays. Tryptophan substitutions at positions Ile-295 and Cys-306 of the nAChR δM3 TMD produced dramatic reductions in nAChR expression levels, suggesting that these positions are critical for proper nAChR assembly and/or oligomerization. The current structure of the nAChR closed-channel state suggests that positions Ile-295 and Cys-306 are oriented toward the interior of the nAChR and previous photolabeling affinity experiments of the Torpedo californica nAChR support this view due to undetected labeling of these residues with the 3-trifluoromethyl-3-(m-[125I]iodophenyl) diazirine (125I-TID) probe.^{2,10} Therefore, the reduction in nAChR expression levels caused by tryptophan substitutions at positions Ile-295 and Cys-306 could be due to a steric clash between this tryptophan and residues in the interior of the protein that could affect the stability of the TMD by disrupting the internal hydrogen bond network and the assembly of the multi-subunit protein.

According to the current structure of the nAChR closedchannel state, mutations Met-293, Phe-294, Ser-297, Thr-300, Gly-301, Val-303, Val-304, Asn-305, Gly-307 and Ile-308 are oriented towards the lipid interface.² Photolabeling affinity experiments using ¹²⁵I-TID only identified positions Met-293, Ser-297, Gly-301, Val-304 and Asn-305 on the δ M3 TMD as being oriented toward the lipid interface.¹⁰ The low number of residues labeled as lipid-exposed by this probe is surprising as the M3 and M4 domains are predicted to have the largest contact with the lipid membrane. Spatial restrictions at a labeling site



Figure 3. Periodicity profiles, Fourier transform power spectra and α -helical character curves for the Torpedo nAChR δ M3 transmembrane domain. (A and D) Tryptophan periodicity profiles for the closed- and open-channel states, respectively. The values inside the boxes indicate the number of residues per helical turn between the adjacent maximum and minimum peaks. (B and E) Fourier transform power spectra of the tryptophan periodicity profiles (A and D). The *gray shading area* displays the region between 85° $\leq \omega \leq 115^\circ$ used to calculate peak ratios. The *asterisk* indicates the peaks corresponding to the average oscillation of the tryptophan periodicity profiles (A and D). (C and F) α -helical character curves at different periodicity intervals. The *red line* and *circle* is the α -helical character curve in the periodicity interval of 3.5–3.7 residues/turn; *yellow line* and *square* is 3.4–3.8 residues/turn; *green line* and *triangle* is 3.3–3.9 residues/turn; and *blue line* and *diamond* is 3.2–4.0 residues/turn.

Table 2. Secondary structure parameters of the Torpedo nAChR δ M3 domain determined by tryptophan periodicity profiles and Fourier transform power spectra

		TrpPPs		FT power spectra	
δ M3 domain state	Length (residues)	Periodicity (residues/turn)	Expected rotation angle (degree)	Rotation angle (degree)	Mean periodicity (residues/turn)
Closed state	18	2.9 ± 0.3	124.14	121.67	2.96
Open state	18	2.6 ± 0.2	138.46	136.87	2.63

Given values correspond to analysis performed on the entire sequence of EC_{s_0} values for ACh activation and nAChR expression levels (fmol/Å³) from the Torpedo nAChR δ M3 domain. Periodicities of the tryptophan periodicity profiles (TrpPPs) are given as the mean ± SEM. Expected rotation angles of the TrpPPs and the mean periodicities of the peaks that correspond to mean periodicities of the TrpPPs were calculated using periodicity (residues/ turn) = 360°/rotation angle.

can lead to poor labeling efficiency and ¹²⁵I-TID labeling may have been hindered due to the bend in the helical structure of the δ M3 TMD, precluding complete or more thorough labeling of the lipid-exposed residues. In addition, ¹²⁵I-TID may have not been able to interact with lipid-exposed residues involved in protein-protein interactions. Tryptophan substitutions at positions Leu-298, Thr-300, Gly-301, Asn-305 and Ile-308 produced statistically significant increases in nAChR expression levels as compared with the WT receptor, suggesting an increase in the efficiency of assembly and/or oligomerization. The increase in nAChR expression levels caused by tryptophan substitution at positions Thr-300, Gly-301, Asn-305 and Ile-308 may be due to a favorable interaction between the large hydrophobic side chain of Trp and the lipid interface, causing an enhanced assembly of the protein. Furthermore, the nAChR has been shown to contain internal sites capable of containing cholesterol whose occupation stabilizes protein structure and regulate function, and molecular dynamics simulations have identified residues Phe-294, Ile-295, Leu-298, Val-299, Val-302 and Ile-303 as being involved in nAChR-cholesterol interactions.³⁷ Therefore, the increase in nAChR expression levels caused by a tryptophan substitution at position Leu-298 may be due to a favorable interaction between Trp and cholesterol. However, since our data was collected from single RNA preparations there is a possibility that the changes in ¹²⁵I-labeled α -BgTx binding levels produced by the mutations might reflect alterations in translation efficiency rather than



Figure 4. Helical net diagrams for the Torpedo nAChR δ M3 transmembrane domain mutants in the open- and closed-channel states. (A–D) Localization and amount of amino acids per helical turn between the adjacent maximum and minimum oscillatory peaks of the periodicity profiles for the Torpedo nAChR δ M3 domain in the open-channel state (A and B) and the closed-channel state (C and D). The *gray box* delimits the area of the helix where the δ M3 mutants that produced loss-of-function (*blue circles*) and gain-of-function (*red circles*) nAChRs are localized based on the data from the tryptophan periodicity profiles.

protein stability. In addition, our conclusions are based on the assumption that the multiple biogenesis steps in between translation and folding are invariant in the oocytes. Overall, our results demonstrate that tryptophan can be accommodated at any position along the $\delta M3$ TMD of the *Torpedo californica* nAChR without inhibiting nAChR assembly.

All 18 tryptophan substitutions produced functional nAChRs. These results are similar to those previously obtained for the Torpedo γ M4 TMD, in which all tryptophan substitutions produced functional nAChRs, and for the Torpedo α M4 and β M3 TMDs, which had only one non-functional nAChR mutant (I417W in α M4 and I296W in β M3).^{15,17,19} In contrast,

tryptophan substitutions along the Torpedo α M3 and β M4 TMDs produced three non-functional nAChRs (M282W, V285W and I289W in α M3; L438W, T551W and F455W in β M4), and eight (T281W, M282W, V285W, S288W, T292W, V293W, V295W and I296W) on the *Mus musculus* α M3 TMD.^{16,20,21} This finding suggests that the Torpedo β M3, δ M3, γ M4 and α M4 TMDs are less tightly packed than the Torpedo α M3, Torpedo β M4, and *Mus musculus* α M3 TMDs.

It has been previously demonstrated that a single tryptophan substitution in the lipid-exposed domains can modulate the ion channel function of the nAChR.^{15-21,38-44} The introduction of a tryptophan residue significantly reduced the maximum macroscopic response of thirteen mutant receptors (M293W, F294W, I295W, M296W, S297W, L298W, V299W, I303W, V304W, C306W, G307W, V309W and L310W). Among all mutations, the C306W mutant nAChR showed the most dramatic reduction (73.3-fold) in macroscopic current, but also exhibited one of the largest reductions in expression levels (29.4-fold). The extremely low nAChR expression levels of this mutant could lead to the observed loss of ion channel function. Moreover, mutant nAChRs M293W, F294W, M296W, S297W, V299W and L310W resulted in a statistically significant reduction of the macroscopic peak current while displaying expression levels that were statistically similar to WT. In these cases, the reduced macroscopic peak currents are likely due to the lock up of nAChRs in a dysfunctional conformation. Several lines of experimental evidence have shown that the M3 TMDs are a key component of the nAChR channel gating mechanism.^{38,45,46} A recent study by De Rosa et al. showed that an aromatic substitution at position 8' (L8'F) of the $\delta M3$ TMD decreases the mean open time (2.3-fold reduction) of the Mus musculus nAChR.47 Since this position is homologous to the Torpedo Leu-298, this result suggests that the decrease in macroscopic current observed for the L298W mutant could be due to a reduction in the mean open time of the mutant nAChR.

Seven mutant receptors (M293W, F294W, G301W, V302W, I303W, V304W and G307W) exhibited statistically significant reductions in potency to ACh relative to WT. In contrast, six mutant receptors (V299W, T300W, N305W, C306W, I308W and V309W) displayed increases in potency relative to the WT nAChR. The gain-of-function mutant nAChRs T300W, N305W and I308W also displayed significantly higher expression values than the WT and increases in maximum macroscopic currents that were not statistically significant. These results suggest that the increased efficiency in channel assembly and/or oligomerization induced by the tryptophan substitutions at these positions may have also led to a stabilization of the open-channel state of the nAChR and a gain-of-function. The normalized macroscopic response for the I295W mutant receptor was drastically increased as compared with WT. This mutant receptor also displayed significantly lower macroscopic current and expression levels than WT, but a similar potency to ACh. The functional data of the δM3 mutant nAChRs has demonstrated that this TMD plays an important role in the mechanism of channel gating of the Torpedo nAChR.

Structural analysis. In this study, we have applied the FT-TrpScanM approach to characterize the secondary structure

and to monitor the conformational changes experienced by the δ M3 TMD of the *Torpedo californica* nAChR. The oscillation patterns in the tryptophan periodicity profiles of the closed- and open-channel states of the $\delta M3$ TMD (Fig. 3A and D) suggest that this domain exhibits an irregular helical secondary structure. The tryptophan periodicity profile of the closed-channel state showed an irregular pattern between positions Met-293 and Val-304 and an ordered oscillation pattern between positions Asn-305 and Leu-310 (Fig. 3A). The tryptophan periodicity profile of the open-channel state displayed a more ordered oscillation along the TMD. Therefore, the periodicity profiles of both the closed- and open-channel states displayed different oscillation patterns that suggest that the $\delta M3$ domain is structurally composed of a combination of different helix types: α -helix and 3_{10} -helix. Short segments of 3_{10} helical structure have been described as part of the TMDs of membrane proteins such as the large conductance mechanosensitive channel,48 sarcoplasmic reticulum Ca2+-ATPase49 and Aquaporin-1,50 among others. However, the periodicity profile of the closed-channel state also exhibits a short segment between positions Val-299 and Val-304 with an oscillation pattern of approximately 4.51 \pm 0.09 residues per helical turn. We suggest the possibility that this short segment could be a wide turn,⁵¹ also known as a π -bulge, in which the backbone dihedral angles indicate a domain with less than perfect π -helical character. Alternatively, this segment could be a π -helix domain, which is rare in transmembrane helices but has been reported in both theoretical⁵² and experimental^{53,54} studies.

Analysis of the amino acid sequence of this TMD shows a Thr-Gly motif at positions 300-301, located at the middle of the TMD (Fig. 1A), suggesting a flexible point that could introduce a distortion in the helical structure. The structural effects caused by the presence of this motif could account for the dramatic changes in the oscillation pattern observed between positions Val-299 and Val-304 during transition between the closed- and open-channel states during channel activation. The changes in periodicity and localization of residues in the helical net diagrams of the closed-and open-channel states could indicate a structural transition between helix types in this segment of the TMD, suggesting that this part of the TMD has a high propensity for helical distortions. This type of helical transition has been reported in molecular dynamics simulations of transmembrane domains.⁵⁵ It is possible that the ability of the protein backbone to undergo helical deformations in this segment allows for interaction of key residues with the lipid bilayer. Indeed, molecular dynamics simulations have identified residues Val-299, Val-302 and Val-303 as being involved in nAChR-cholesterol interactions, which are known to stabilize protein structure and regulate function.³⁷

The tryptophan periodicity profiles and Fourier transform spectra of the $\delta M3$ TMD displayed some structural variations between its closed- and open-channel states, but mostly revealed similar helical structures for both states. The periodicity of the oscillation patterns between the closed- and open-channel states is altered by approximately 0.4 amino acids per helical turn (**Table 2**), suggesting that a discrete and possibly localized conformational change takes place along the $\delta M3$ TMD as a

consequence of channel activation. These results indicate that the overall helix movement during channel activation estimated from the tryptophan periodicity profiles is similar to the ones from the α M4, β M4 and γ M4 TMDs (0.5, 0.4 and 0.3 amino acids per helical turn, respectively).^{15,19,21} Moreover, a recent study used a single-channel proton transfer technique to assess the structure of the nAChR TMDs in the open-channel state.⁵⁶ This study suggested that changes in the structure of the pore domain of the *Mus musculus* nAChR involve only a subtle rearrangement of the δ M1 and δ M3 TMDs. This contrasts with results from the α M3 TMD of the *Mus musculus* nAChR, which displayed a substantial conformational change that resembled a spring-like motion during transition between the closed- and open-channel states.²⁰

In a recent study, mixed elastic network models (MENMs) were developed for the conformational transition between the closed- and open-channel states of two prokaryotic pentameric ligand-gated ion channels (pLGICs).⁵⁷ The MENMs focused on the TMDs, thereby excluding the extracellular domain, but also excluded the M3-M4 loop and the M4 TMD due to gaps in the sequence alignment of the loop connecting M3 and M4 of the two prokaryotic pLGICs. Analysis of the transition trajectories between the closed- and open-channel states identified a substantial tilting of the M3 TMDs, as well as moderate bending of the helix. The Thr-Gly motif at positions 300–301 could account for the bending point in the Torpedo δ M3 TMD. This flexible point could be important as bending of helices has been identified as a major factor in the gating transition of potassium channels.^{58,59}

The localization of several residues in the helical net diagrams suggests a displacement of these residues upon channel activation (Fig. 4A-D). Helical net diagrams constructed with the tryptophan periodicity profiles showed that the $\delta M3$ loss-of-function and gain-of-function mutant nAChRs are confined to different ranges of rotation angles (180° region) in the open-channel state, thus suggesting they are clustered on opposite sides of the helix (Fig. 4A-B). All gain-of-function mutant nAChRs are oriented toward the same face of the helix in the open-channel state helical net diagram, suggesting a higher degree of packing within this region of the helix in this state. Most of the loss-of-function mutant nAChRs are oriented toward the opposite side of the helix, except for the V302W mutant nAChR that is found in the region where all the gain-of-function nAChRs are clustered. According to the current structure of the nAChR closed-channel state, mutations that produced a gain-of-function response (i.e., V299W, T300W, N305W, C306W, I308W and V309W) are clustered at opposite sides of the helix.² Positions Thr-300, Asn-305 and Ile-308 are facing the lipid interface and are confined to the same ranges of rotation angles of the closed-channel state helical net diagram (Fig. 4C); thus, the location of these gain-offunction mutants is consistent with those of previously reported mutant nAChRs such as the Torpedo α M4 C418W and the *Mus* musculus aM3 C447W and L440W. In contrast, positions Val-299, Cys-306 and Val-309 are facing the interior of the protein and, therefore, are confined to another range of rotation angles (opposite side of helix). According to the nAChR structure, these positions are oriented towards a crevice facing the M2 TMD.² This crevice allows for the accommodation of the bulky Trp side chain and the observed gain-of functions of these mutations could be due to interactions of these positions with the neighboring TMD during channel gating, leading to a stabilization of the open-channel state.

Atomic resolution data from the nAChR TMDs in the closedand open-channel states are still unavailable as the structural model of the closed-channel state of the muscle-type nAChR is only known to a 4.0 Å resolution.² Therefore, some uncertainty remains as to the exact positioning of the amino acid side chains along the TMDs and to the finer structural details such as bends and kinks. In this regard, the present study suggests that the closed-channel state of the nAChR exhibits a wide-turn between positions Val-299 and Val-304 of the δ M3 TMD. Furthermore, a Thr-Gly motif at positions 300-301 could cause distortions in this segment of the helix due to the introduction of a bending point along the helical structure of the $\delta M3$ TMD. These two structural features are not present on current models of the nAChR. Further studies are needed to corroborate its potential role in nAChR channel gating. Overall, the FT-TrpScanM approach revealed that the secondary structure of the δ M3 TMD is a mixture of helix types. In addition, this TMD undergoes a discrete and localized conformational change during channel activation that could include a structural transition between helix types around positions Val-299 and Val-304. The present data underscore a functional link between lipid-exposed domains and the gating machinery of the nAChR as the δ M3 TMD seems to play an important role in the mechanism of channel gating of the Torpedo nAChR.

Materials and Methods

Mutagenesis procedures. The coding regions of all Torpedo californica nAChR subunits were sub-cloned into the EcoRI/Hind III site of the pGEM-3Zf(-) vector under the SP6 promoter (Promega, Madison, WI). Eighteen mutations along the core of the Torpedo californica nAChR δM3 transmembrane domain (Met-293 to Leu-310) were engineered with the QuikChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotide primers were generated with the tryptophan codon (TGG) instead of the wild type (WT) codon at the desired position (Invitrogen, Carlsbad, CA). The successful inclusion of mutations was confirmed by DNA sequence analysis performed at the DNA Sequencing Facility in the section of Evolution and Ecology, University of California, Davis, CA. All pGEM-3Zf(-) vectors containing the coding region of the Torpedo nAChR subunits were linearized using the Sma I site, and the digested products were purified with the Wizard® DNA Clean-Up System (Promega, Madison, WI). Torpedo nAChR cRNA transcripts were produced with the SP6 mMessage mMachine Kit (Ambion, Austin, TX). Transcripts were extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 4.5) and once with an equal volume of chloroform:isoamyl alcohol (24:1, pH 4.5), precipitated with 2.5 volumes of 100% ethanol at -20°C, dried, and resuspended in RNase-free H₂O. The integrity and quantity of each cRNA was verified by gel-electrophoresis, weight markers and spectrophotometry, respectively.

nAChR expression in Xenopus laevis oocytes. Stage V-VI oocytes were extracted from Xenopus laevis frogs in accordance with the guidelines of the University of Puerto Rico Institutional Animal Care and Use Committee. The oocytes were incubated in collagenase type IA (Sigma-Aldrich, St. Louis, MO), and this treatment was followed by manual defolliculation to remove follicles. RNA transcripts (55 ng total cRNA/oocyte) of the Torpedo californica nAChR α , β , γ and δ (WT or mutant) subunits were microinjected at a 2:1:1:1 subunit stoichiometry. The cRNA mixtures were pressure injected using a positive displacement injector (Drummond Instruments, Broomhall, PA). The injected oocytes were incubated at 19°C in ND-96 media [96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM N-(2hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 2.5 mM Na-pyruvate] supplemented with 400 µg/mL bovine serum albumin (BSA), 50 µg/mL gentamicin, 50 µg/mL tetracycline and 0.5 mM theophyline. The incubation media was changed daily. Electrophysiology experiments were carried out 3 days after cRNA injection.

Electrophysiological recordings. ACh-induced macroscopic currents were recorded using a GeneClamp 500B amplifier in a two-electrode voltage clamp configuration (Axon Instruments, Foster City, CA) at room temperature. Electrodes were filled with 3 M KCl and had resistances <5 M Ω . Impaled oocytes were automatically perfused with MOR-2 buffer [115 mM NaCl, 2.5 mM KCl, 5 mM MgCl,, 1 mM Na, HPO,, 5 mM HEPES and 0.2 mM CaCl₂ (pH 7.4)] at a rate of 30 mL/min using an eightchannel perfusion valve controller (VC-8; Warner Instruments, Hamden, CT). Membrane potential was held at -70 mV. Membrane currents were filtered at 20 Hz and digitized at 5 kHz using a DigiData 1322A interface (Axon Instruments, Foster City, CA), and data were acquired using Clampex 9.2 from the pClamp 9.2 software package (Axon Instruments, Foster City, CA). Concentration-response curves were generated from macroscopic peak currents (I) obtained from six ACh concentrations (1, 3, 10, 30, 100 and 300 µM). Agonist application was performed for 4 seconds. GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) was used to perform a nonlinear regression fit with a sigmoidal concentration-response equation with variable slope:¹⁹

$$I = I_{\min} + \frac{I_{\max} - I_{\min}}{1 + 10^{((LogEC_{50} - Log[ACh])*nH)}}$$

where I is the macroscopic current at a given ACh concentration; I_{max} and I_{min} are the maximum and the minimum current responses recorded; EC_{50} is the ACh concentration required to achieve half of the maximum response; [ACh] is the concentration of acetylcholine; and nH is the Hill coefficient, which represents the steepness of the concentration-response curve.

¹²⁵I-labeled α -bungarotoxin binding assays. ¹²⁵I-labeled α -bungarotoxin (¹²⁵I-labeled α -BgTx) binding assays (Perkin-Elmer Life and Analytical Sciences, Waltham, MA) were performed immediately after voltage clamp measurements for the same oocytes to determine nAChR expression levels in the plasmatic membrane. Oocytes were incubated in 20 nM ¹²⁵I-labeled α -BgTx, 10 mg/mL BSA, MOR-2 without EGTA, and in the absence of agonist at room temperature for 1.5 h. Non-injected oocytes were incubated under the same conditions to measure non-specific binding. Individual oocytes were washed with 25 mL of MOR-2 without EGTA to remove excess toxin. Calibration curves were used to determine ¹²⁵I-labeled α -BgTx binding sites in the plasmatic membrane (nAChR expression levels) of each oocyte, and they were constructed by plotting radioactivity (counts/min) as a function of ¹²⁵I-labeled α -BgTx concentration (fmol). Radioactivity was measured using a 2,470 Wizard² Automatic Gamma Counter (Perkin Elmer Life and Analytical Sciences, Waltham, MA).

Normalized macroscopic nAChR response. The normalized response (-nA/fmol) for each oocyte was assessed as the ratio of the macroscopic peak current (-nA) induced by a 300 μ M ACh concentration to the ¹²⁵I-labeled α -BgTx binding sites (fmol) in the plasmatic membrane (nAChR expression levels).

Periodicity profiles. The number of residues per helical turn for the open- and closed-channel states was determined by periodicity profiles for the EC₅₀ for ACh activation and ¹²⁵I-labeled α -BgTx binding sites in the plasmatic membrane (nAChR expression levels), respectively. Periodicity profiles were plotted with the EC₅₀ for ACh activation or ¹²⁵I-labeled α -BgTx binding sites as a function of the substituted position along the δ M3 transmembrane domain. The produced oscillation patterns were best fitted by a cubic spline function using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA). The number of residues per helical turn of the periodicity profiles was estimated as the number of amino acids between the adjacent maximum and minimum peaks. nAChR expression levels (fmol) for each mutant were standardized ($X_{Standardized}$) to the change in amino acid volume (Å³) caused by the tryptophan substitution at that position (fmol/Å³):²⁰

$$X_{Standardized} = \frac{X_{Mutant}}{(V_{Trp} - V_{WT})}$$

here $X_{_{Mutant}}$ is the δ M3 mutant nAChR expression level, and $V_{_{Trp}}$ and $V_{_{WT}}$ are the volumes of tryptophan and of the original residue, respectively. Amino acid volumes were taken from crystal-lographic studies.⁶⁰

Generation of fourier transform power spectra. The sequences of EC₅₀ values for ACh activation and of the ¹²⁵I-labeled α -BgTx binding sites were evaluated by a least squares discrete Fourier transform (FT) equation to produce the FT power spectra and estimate the mean periodicities. This analysis was performed as described previously to validate the mean periodicities calculated using tryptophan-scanning mutagenesis.^{21,34,36}

 α -helical character curves. α -Helical character curves were generated as described previously in reference 36.

Helical net diagrams. Helical net diagrams were generated as described previously in reference 20 and 21.

Statistical analysis. Error estimates for EC_{50} values are expressed as the mean \pm SEM and the 95% confidence intervals. All other error estimates are expressed as the mean \pm SEM. Two-sample comparisons were made using an unpaired t test

with Welch's correction. For more than two groups, an ANOVA with a Dunnett's post-test analysis was performed (GraphPad Software Inc., San Diego, CA). A two-tailed p value < 0.05 was considered significant.

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