



NMR structures of the human $\alpha 7$ nAChR transmembrane domain and associated anesthetic binding sites

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ABSTRACT

The $\alpha 7$ nicotinic acetylcholine receptor (nAChR), assembled as homomeric pentameric ligand-gated ion channels, is one of the most abundant nAChR subtypes in the brain. Despite its importance in memory, learning and cognition, no structure has been determined for the $\alpha 7$ nAChR TM domain, a target for allosteric modulators. Using solution state NMR, we determined the structure of the human $\alpha 7$ nAChR TM domain (PDB ID: 2MAW) and demonstrated that the $\alpha 7$ TM domain formed functional channels in *Xenopus* oocytes. We identified the associated binding sites for the anesthetics halothane and ketamine; the former cannot sensitively inhibit $\alpha 7$ function, but the latter can. The $\alpha 7$ TM domain folds into the expected four-helical bundle motif, but the intra-subunit cavity at the extracellular end of the $\alpha 7$ TM domain is smaller than the equivalent cavity in the $\alpha 4\beta 2$ nAChRs (PDB IDs: 2LLY; 2LM2). Neither drug binds to the extracellular end of the $\alpha 7$ TM domain, but two halothane molecules or one ketamine molecule binds to the intracellular end of the $\alpha 7$ TM domain. Halothane and ketamine binding sites are partially overlapped. Ketamine, but not halothane, perturbed the $\alpha 7$ channel-gate residue L9'. Furthermore, halothane did not induce profound dynamics changes in the $\alpha 7$ channel as observed in $\alpha 4\beta 2$. The study offers a novel high-resolution structure for the human $\alpha 7$ nAChR TM domain that is invaluable for developing $\alpha 7$ -specific therapeutics. It also provides evidence to support the hypothesis: only when anesthetic binding perturbs the channel pore or alters the channel motion, can binding generate functional consequences.

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1. Introduction

Nicotinic acetylcholine receptors (nAChRs) belong to a superfamily of pentameric ligand-gated ion channels (pLGICs), including 5HT₃, GABA_A, and glycine receptors, that mediate fast synaptic transmission in the central and peripheral nervous systems. The $\alpha 7$ nAChR is one of the most abundant nAChR subtypes in the brain and assembles as homomeric functional pentamers [1]. High expression levels of the $\alpha 7$ nAChR have been observed in brain regions involved in learning, memory, and cognition [2,3]. Therefore, the $\alpha 7$ nAChR is a viable target for therapeutics to regulate processes impaired in schizophrenia, Alzheimer's disease, and other neurological disorders [4,5]. $\alpha 7$ nAChR is also a target for therapeutic modulation of angiogenesis and inflammation [6,7].

In order to rationally design therapeutics specifically targeting the $\alpha 7$ nAChR, a high-resolution structure of $\alpha 7$ is highly desired. However, no experimental structure for the full-length $\alpha 7$ nAChR currently exists. The highest degree of structural information for $\alpha 7$ nAChR has been

achieved for the extracellular (EC) domain, which contains the orthosteric ligand-binding site. X-ray structures of chimeras that have systematically modified the sequence of acetylcholine binding proteins [8–10] toward the human $\alpha 7$ nAChR provide invaluable atomic details for the $\alpha 7$ EC domain [11,12]. The overall topology and structural information for the transmembrane (TM) domain and the intracellular (IC) domain of $\alpha 7$ nAChR have relied on the 4-Å resolution model of the *Torpedo marmorata* nAChR determined by cryo-electron microscopy [13]. Recent crystal structures of homologous bacterial pLGICs from *Erwinia chrysanthemi* (ELIC) [14] and *Gloeobacter violaceus* (GLIC) [15,16] as well as the *Caenorhabditis elegans* glutamate-gated chloride channel (GluCl) [17], have also added valuable structural templates for modeling pLGICs.

Previous molecular models for the $\alpha 7$ nAChR [18,19] were based on structures of the *Torpedo marmorata* nAChR [13]. Homology modeling can capture overall structural features that are likely sufficient for many purposes, but it may miss specific structural details that can differentiate functions and pharmacology of different nAChR subtypes. For example, the $\alpha 7$ and $\alpha 4\beta 2$ nAChRs would have similar structural models, which cannot provide sufficient insights for reasoning why $\alpha 7$ is insensitive but $\alpha 4\beta 2$ is hypersensitive to functional modulation by volatile anesthetics [20,21]. Reliable structures for individual subtypes of nAChRs, especially their TM domains, are also important for

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the development of positive allosteric modulators with therapeutic potential, such as PNU-120596 [22–24] and TQS [25,26]. They are specific modulators for $\alpha 7$ nAChRs and have virtually no effect on other nAChR subtypes.

In the study reported here, we determined the structure of the human $\alpha 7$ nAChR TM domain using high-resolution solution state NMR. The structures newly determined for $\alpha 7$ and previously determined for $\alpha 4\beta 2$ nAChRs (PDB codes: 2LLY; 2LM2) [27] offer an opportunity to make structural comparisons and to reveal a structural basis that differentiates function and pharmacology of different nAChR subtypes. In addition to the new structure for $\alpha 7$, we also determined binding sites in $\alpha 7$ for the volatile anesthetic halothane and the intravenous anesthetic ketamine. The identified structural and dynamics determinants from the study have general implication for anesthetic action in pLGICs.

2. Materials and methods

2.1. Sample preparations

The human $\alpha 7$ nAChR TM domain for the NMR study contained 137 residues (Fig. S1). In order to reduce complexity of the NMR spectra, the cytoplasmic loop between TM3 and TM4 was replaced with GGGE, a sequence designed to avoid imposing structural constraints on interactions of the TM helices while providing a hydrophilic surface to enhance stability of the isolated TM domain. The TM3–4 loop of $\alpha 7$ nAChR is involved in receptor assembly and trafficking to the cell surface in eukaryotes [28,29], but studies with related pLGICs have established that the TM3–4 loop is not essential for channel function [30]. Glutamate mutations at the N- and C-termini (Fig. S1), designed to lower the pI of the construct, were necessary to secure protein stability for NMR measurements. Additional mutation of three hydrophobic residues to serine within the TM2–TM3 linker (Fig. S1) was also instrumental to prevent protein destabilization, similar to the previous observation on $\alpha 4\beta 2$ TM domains [27]. Without these mutations, the isolated $\alpha 7$ TM domain had a tendency to aggregate on purification, most likely because hydrophobic residues normally shielded by the EC domain were exposed to solvent.

The same protocol as reported previously [27] was used for the $\alpha 7$ expression and purification. The protein was expressed in *Escherichia coli* Rosetta 2(DE3) pLysS (Novagen) at 15 °C for three days using the Marley protocol [31]. The protein was purified in LDAO using his-tag affinity column before and after cleavage of the his-tagged region. Each NMR sample contained 0.25–0.3 mM $\alpha 7$, 1–2% (40–80 mM) LDAO detergent, 5 mM sodium acetate at pH 4.7, 10 mM NaCl, and 20 mM 2-mercaptoethanol to prevent disulfide bond formation. 5% D₂O was added for deuterium lock in NMR experiments. The anesthetics ketamine (80–240 μ M) or halothane (0.7–5.5 mM) were titrated into the samples using a micropipette or a gas-tight microsyringe, respectively. The concentration of the volatile anesthetic halothane was quantified based on ¹⁹F NMR using the method reported previously [32].

2.2. NMR spectroscopy

NMR spectra were acquired on Bruker Avance 600 and 800 MHz spectrometers at 45 °C using triple-resonance inverse-detection cryoprobes (Bruker Instruments, Billerica, MA). For ¹H, ¹⁵N, and ¹³C chemical shift assignment and the protein structure determination, a suite of NMR experiments were performed: HNCA (1024 × 28 × 72) and HN(CO)CA (1024 × 28 × 54), both with spectral windows of ¹H-12 ppm, ¹⁵N-20.5 ppm, ¹³C-19 ppm; HNCO (1024 × 32 × 40) with spectral widths of ¹H-11 ppm, ¹⁵N-22 ppm, and ¹³C-10 ppm; ¹⁵N-edited NOESY (1024 × 36 × 104) with spectral windows of ¹H-11 ppm and ¹⁵N-22 ppm, and a mixing time of 120 ms; ¹H-¹³C HSQC (1024 × 256) with spectral windows of ¹H-11 ppm and ¹³C-64 ppm; and ¹H-¹⁵N TROSY-HSQC (1024 × 128) with spectral windows of ¹H-11 ppm and ¹⁵N-22 ppm. HSQC spectra showing temperature dependence of amide

proton chemical shifts were collected at 35, 40, and 45 °C. Residues of temperature coefficients below 4.5 ppb/K were considered to be in helical structure and involved in hydrogen bonds [33].

¹H-¹⁵N TROSY-HSQC spectra were acquired at 600 MHz in the absence and presence of the anesthetics halothane or ketamine. Direct contacts of halothane with the $\alpha 7$ TM domain were determined by saturation transfer difference (STD) spectra [34]. A series of 1D STD spectra with different saturation times were collected in an interleaved fashion with on- and off-resonance frequencies of 0.4 ppm and 25 ppm, respectively. A recycle delay of 12 s and 64 scans were used for each STD spectrum. 2D saturation transfer spectra [35] were acquired in the presence of 3.2 mM halothane in an interleaved fashion with on- and off-¹H resonance frequencies of 6.48 ppm (the halothane proton frequency) and 25 ppm (blank), respectively. The selective saturation was achieved using an IBURP2 pulse train (50 ms Gauss1.1000-shaped with an interpulse delay of 4 μ s). The total saturation time was 2 s and a recycle delay was 3 s. The ¹H chemical shifts were referenced to the DSS resonance at 0 ppm and the ¹⁵N and ¹³C chemical shifts were referenced indirectly [36].

2.3. Structure calculation and analysis

NMR data were processed using NMRPipe 4.1 and NMRDraw 1.8 [37] and analyzed using Sparky 3.10 [38]. ¹H, ¹⁵N, and ¹³C chemical shift assignments were performed manually. NOE cross-peak assignment was initially carried out manually and more cross-peaks were assigned later by CYANA 2.1 [39]. CYANA 3.0 was used for structural calculations. A total of 100 structures were calculated based on NOE and hydrogen-bonding restraints as well as TALOS dihedral angle restraints derived from the chemical shifts [40]. Of the 100 structures, 25 structures with the lowest target function were used for further refinement in CYANA 3.0. The 20 structures with the lowest target function after refinement were analyzed using VMD [41] and Molmol [42].

Contact map analysis (CMA) [43] was used for comparison of the $\alpha 7$ TM tertiary structures with structures of other homologous proteins. Internal cavities in the $\alpha 7$ TM domain were determined for each of the 20 NMR structures using the POVME algorithm [44]. Grids for cavities at the EC and IC ends of the nAChR TM domains were generated with 0.5 Å grid spacing. The mean \pm standard error calculated based on cavity volumes for the 20 NMR structures is reported.

2.4. Visualization and molecular docking of anesthetics in the $\alpha 7$ nAChR

To assist with visualizing halothane- and ketamine-binding sites identified by NMR experiments, we performed targeted anesthetic docking to the $\alpha 7$ NMR structures. The targeted docking kept only those sites consistent with the NMR results. Docking was performed with Autodock4 [45] using a Lamarckian genetic algorithm with a grid spacing of 0.375 Å. For each binding site suggested by NMR, 250 independent anesthetic dockings were performed within a cube covering ~6600 Å³ located at the IC end of the TM domain. Each docking calculation used an initial population size of 500.

2.5. Size exclusion chromatography–multi-angle light scattering (SEC–MALS) analysis

Oligomerization states of the $\alpha 7$ TM domain in the NMR samples were determined using size exclusion chromatography (Superdex 200 10/300, GE Healthcare) coupled with multi-angle light scattering (HELEOS, Wyatt Technology), UV (Agilent 1100 Series; Agilent Technology), and differential refractive index (Optilab rEX; Wyatt Technology) detection. The molar mass of the protein-detergent complex was determined using ASTRA software (Wyatt Technology) [46]. The conjugate analysis module of ASTRA was used to differentiate contributions of the protein and detergent to the molecular weight. The specific refractive index (dn/dc) values of 0.185 and 0.148 were used for the protein and

LDAO detergent, respectively [47]. The UV extinction coefficient of $\alpha 7$ was calculated based on the $\alpha 7$ sequence. A measured UV extinction coefficient of 0.06 for a 1% solution at 280 nm was used for LDAO.

2.6. Functional measurements in *Xenopus* oocytes

Purified $\alpha 7$ TM domain in LDAO detergent was reconstituted into asolectin vesicles by adsorption of detergent using Bio-Beads SM-2 non-polar polystyrene adsorbent (Biorad) in the presence of a 100:1 molar ratio of asolectin to protein following the manufacturer's instructions. The prepared vesicles (50 nl) containing 100 ng of $\alpha 7$ TM domain were injected into *Xenopus laevis* oocytes (stages 5–6). Oocytes were maintained in modified Barth's solution containing 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 15 mM HEPES, 0.3 mM $\text{Ca}(\text{NO}_3)_2$, 0.41 mM CaCl_2 , 0.82 mM MgSO_4 , 10 $\mu\text{g}/\text{ml}$ sodium penicillin, 10 $\mu\text{g}/\text{ml}$ streptomycin sulfate, and 100 $\mu\text{g}/\text{ml}$ gentamycin sulfate, pH 6.7 at 18 °C. After 1–3 days, channel function was measured by two-electrode voltage clamp experiments [48]. Oocytes in a 20- μl oocyte recording chamber (Automate Scientific) were clamped at -60 mV with an OC-725C Amplifier (Warner Instruments) and currents were elicited using ivermectin as an agonist. The recording solutions contained 130 mM NaCl, 0.1 mM CaCl_2 , 10 mM HEPES, pH 7.0 with the indicated concentrations of ivermectin and ketamine. Data were collected and processed using Clampex 10 software (Molecular Devices).

3. Results

3.1. NMR structures of the human $\alpha 7$ nAChR TM domain

The $\alpha 7$ TM domain spontaneously assembled into pentamers in LDAO (Fig. S2) and formed ion-conducting channels when the purified $\alpha 7$ TM domain was injected into *Xenopus* oocytes as reconstituted asolectin vesicles (Fig. 1). Although the $\alpha 7$ TM domain does not possess the orthosteric agonist-binding site of native human $\alpha 7$ nAChR, the channel current could be elicited by ivermectin, a known positive allosteric modulator acting through the TM domain [49,50]. Ketamine inhibited ivermectin-induced current (Fig. 1B), consistent with the effect of ketamine on native human $\alpha 7$ nAChR [51–53]. No ivermectin-elicited current was observed in control oocytes injected with the asolectin vesicles not containing the $\alpha 7$ TM domain. These data demonstrate that the $\alpha 7$ TM domain retains pharmacological responses observed for the full-length $\alpha 7$ nAChR.

NMR spectra of the $\alpha 7$ TM domain permitted assignment of ~95% of its residues (Fig. S3). A bundle of the 20 lowest target function

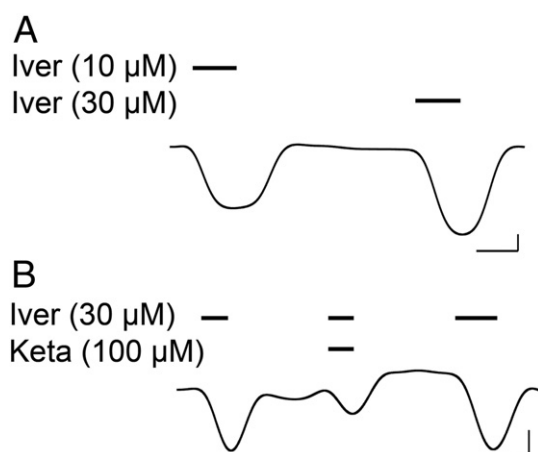


Fig. 1. Representative traces for *Xenopus* oocytes injected with vesicles containing the purified human $\alpha 7$ nAChR TM domain. (A) Current response at 10 and 30 μM ivermectin. (B) Inhibition of ivermectin (30 μM)-elicited current by 100- μM ketamine. Bars over the trace indicate length of application of the indicated compounds. Scale bars indicate 0.5 min and 0.1 μA .

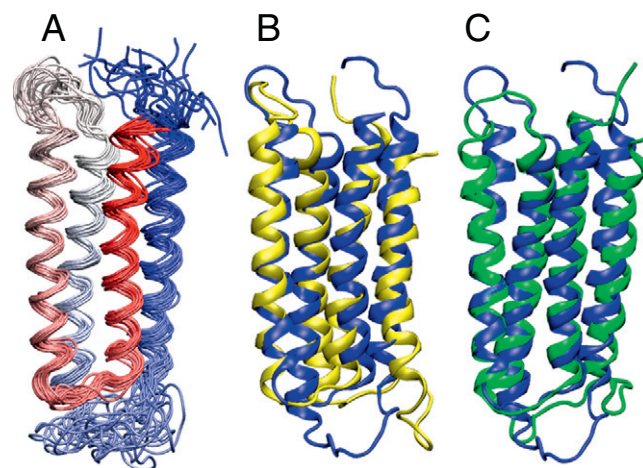


Fig. 2. NMR structures of the $\alpha 7$ TM domain. (A) A bundle of the 20 lowest-energy structures of the $\alpha 7$ TM domain (PDB ID: 2MAW). The structures are colored from red for TM1 to blue for TM4. The backbone atom RMSD for the helical regions is 1.24 ± 0.32 Å. Full statistics for the $\alpha 7$ structure calculations are summarized in Table S1. (B) Overlay of representative structures of $\alpha 7$ (blue) and $\alpha 4$ (yellow; PDB ID: 2LLY). The backbone atom RMSD for the helical regions of $\alpha 7$ and $\alpha 4$ is 2.9 Å. (C) Overlay of representative structures of $\alpha 7$ (blue) and $\beta 2$ (green; PDB ID: 2LM2). The backbone atom RMSD between the helical regions of $\alpha 7$ and $\beta 2$ is 2.1 Å.

structures of the $\alpha 7$ TM domain (PDB code: 2MAW), as shown in Fig. 2A, were determined based on short-, medium-, and long-range NOEs, dihedral angle constraints, and hydrogen bonding constraints (Fig. S4). The average pair-wise root mean square deviations (RMSD) in the helical regions are 1.24 ± 0.32 Å for the backbone and 1.64 ± 0.30 Å for all heavy atoms. Detailed statistics of structural calculations are provided in Table S1.

The tertiary structure of the $\alpha 7$ TM domain resembles those determined previously for the $\alpha 4\beta 2$ (PDB IDs: 2LLY; 2LM2) nAChR [27] and several other homologous pLGICs [14–17]. However, small structural differences can be observed among the nAChR TM domains in the contact map analysis (Fig. S5). The angles between TM2 and TM4 helices are $3.9 \pm 0.5^\circ$ in $\alpha 7$, but $8.8 \pm 0.9^\circ$ and $10.5 \pm 1.1^\circ$ in $\alpha 4$ and $\beta 2$, respectively. The angles between TM1 and TM3 helices are $3.8 \pm 0.7^\circ$ in $\alpha 7$, but $5.3 \pm 0.6^\circ$ and $5.7 \pm 0.7^\circ$ in $\alpha 4$ and $\beta 2$, respectively. Structural alignment of $\alpha 7$ onto $\alpha 4$ or $\beta 2$ (Fig. 2B and C) shows that the $\alpha 7$ structure is more compact at the EC end of the TM domain, where $\alpha 7$ has an intra-subunit cavity with a volume of 122 ± 10 Å³. In contrast, $\alpha 4$ and $\beta 2$ have larger cavities in the same region with volumes of 232 ± 6 Å³ and 179 ± 12 Å³, respectively. The structural differences at the IC end of the TM domain seem to be reversed. The intra-subunit cavities at the IC end of the TM domains have volumes of 209 ± 8 Å³, 139 ± 11 Å³, and 131 ± 10 Å³ for $\alpha 7$, $\alpha 4$, and $\beta 2$, respectively.

3.2. Halothane binding site in the human $\alpha 7$ nAChR TM domain

The anesthetic halothane directly interacts with the $\alpha 7$ TM domain. As shown in the STD spectra of $\alpha 7$ acquired in the presence of halothane (Fig. 3), intensity of the halothane signal is modulated by different saturation times for the $\alpha 7$ signals. A longer saturation time for the selected $\alpha 7$ protons (0.4 ppm) resulted in greater attenuation of the halothane signal due to effective saturation transfer from protein to ligand. Consequently, the net halothane signal in the STD spectra increased, because a STD spectrum resulted from subtraction of a pair of spectra acquired in an interleaved fashion with saturation off-resonance (25 ppm) and on-resonance (0.4 ppm). In the absence of $\alpha 7$, no halothane signal was detected in the STD spectra under the same NMR experimental condition (Fig. S6), confirming that halothane signals in Fig. 3A result predominately from direct halothane interactions with $\alpha 7$.

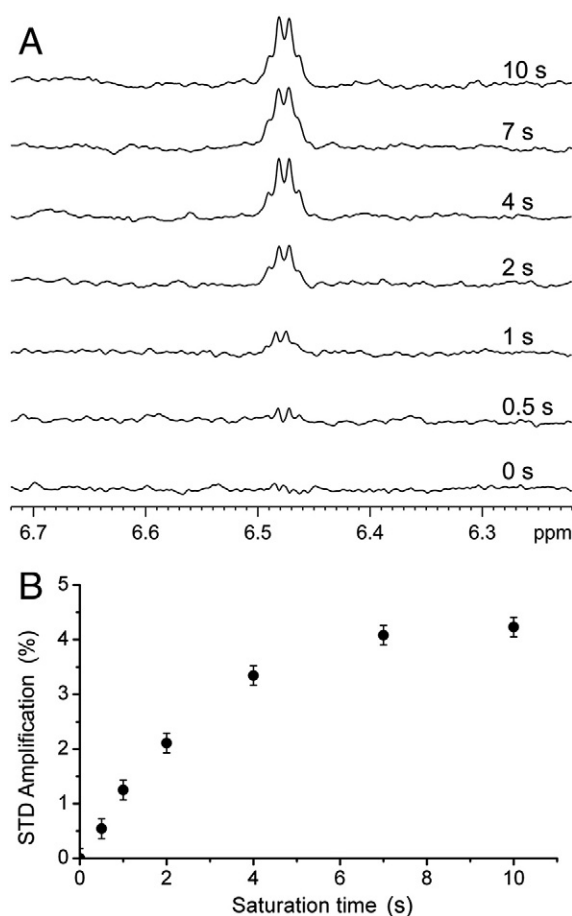


Fig. 3. Saturation transfer difference (STD) spectra of the $\alpha 7$ TM domain for haloethane binding. (A) Prolonged saturation time increased haloethane (3.2 mM) signal in the STD spectra in the presence of $\alpha 7$. The STD spectra resulted from the subtraction of the off- (25 ppm; blank region) from the on-resonance (0.4 ppm; protein methyl group) spectra. (B) STD amplification (%) as a function of the saturation time. STD amplification is defined as $(V_{\text{off}} - V_{\text{on}})/V_{\text{off}}$. V_{off} and V_{on} are the integrals of haloethane peak in the spectra with off- and on-resonance saturation, respectively.

In order to reveal residues directly contacting haloethane, we performed 2D saturation transfer NMR experiments, in which the $\alpha 7$ spectra in the presence of haloethane were acquired in an interleaved fashion with the ^1H saturation frequencies at 6.48 ppm (the haloethane proton frequency) and 25 ppm (blank), respectively. When haloethane was saturated, residues showing substantial decrease in their peak intensities should be in close contact with haloethane (Fig. 4A). These residues include F230 in TM1, K239 in TM2, and F453 and C449 in TM4. The full spectra of the 2D saturation transfer experiments are provided in Fig. S7.

^1H - ^{15}N TROSY HSQC spectra of $\alpha 7$ were acquired in the absence and presence of haloethane (Figs. 4B and S8). Several residues, including C219, V220, S223 of TM1, K239 of TM2, and T289 of TM3, show notable changes after the addition of haloethane. When these residues along with those identified in saturation transfer experiments are mapped onto the $\alpha 7$ structure, haloethane binding to an intra-subunit cavity becomes clear (Fig. 4C). The upper part of the cavity is located at the middle of the TM domain and lined by residues from TM1, TM3, and TM4. The lower part of the cavity is located at the IC end of the TM domain and lined with residues from TM1, TM2, and TM3. The cavity size is large enough for hosting two haloethane molecules. This site is similar to one of the sites observed in the $\alpha 4\beta 2$ nAChR [54]. However, unlike $\alpha 4\beta 2$, $\alpha 7$ does not have haloethane bound to the EC end of the TM domain. The different binding sites may account for high functional sensitivity of the $\alpha 4\beta 2$ nAChR and low functional sensitivity of $\alpha 7$ nAChR to haloethane [20,21].

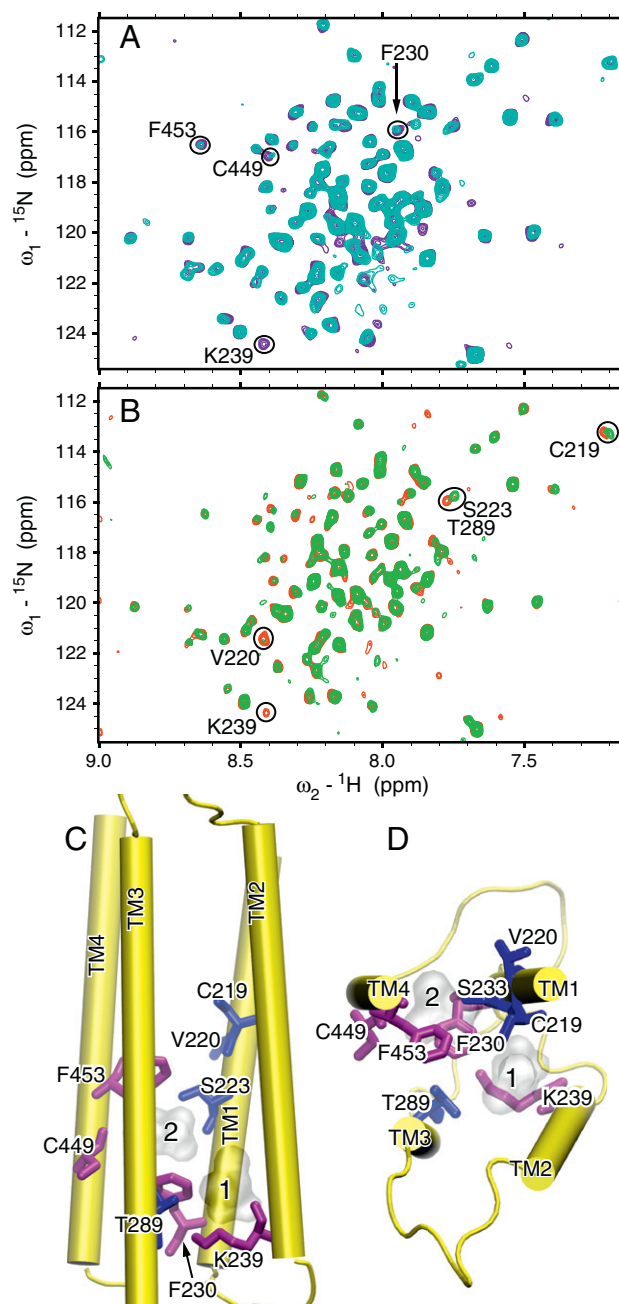


Fig. 4. Haloethane binding sites in the TM domain of the human $\alpha 7$ nAChR. (A) Overlay of 2D saturation transfer NMR spectra of $\alpha 7$ acquired with ^1H saturation frequency on (cyan) and off (purple) the proton resonance of haloethane (3.2 mM). Residues showing considerable decreases in their peak intensities upon saturation of the haloethane signal are labeled with the one-letter amino acid code and the sequence number. (B) Overlay of ^1H - ^{15}N TROSY-HSQC spectra of $\alpha 7$ in the absence (red) and the presence (green) of haloethane (1.7 mM). Residues showing significant changes in chemical shift or relative peak intensity are labeled. (C) Side and (D) top views of the $\alpha 7$ structure highlighting the residues affected by haloethane in (A) and (B) using purple and blue sticks, respectively. Two haloethane molecules are shown in silver surface.

3.3. Ketamine binding sites in the human $\alpha 7$ nAChR TM domain

Because of severe signal overlap between ketamine and $\alpha 7$ in the ^1H spectra, the saturation transfer experiments cannot be performed on ketamine in $\alpha 7$. Thus, we compared the $\alpha 7$ ^1H - ^{15}N TROSY HSQC spectra in the absence and presence of ketamine to identify ketamine binding sites. Residues showing significant changes in the spectra upon ketamine binding are highlighted (Figs. 5A and S9) and mapped onto the

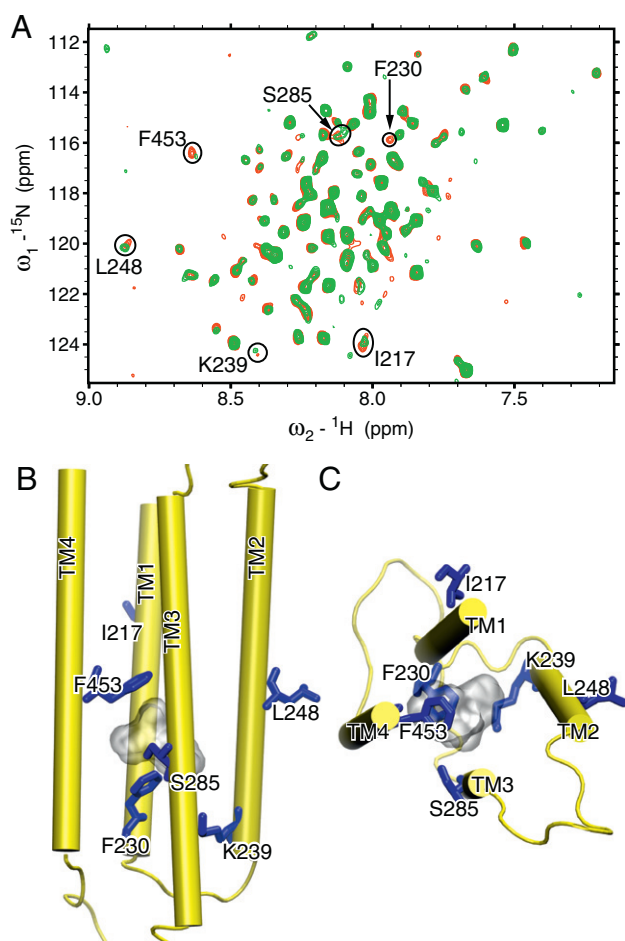


Fig. 5. Ketamine binding site in the TM domain of the human $\alpha 7$ nAChR. (A) Overlay of ${}^1\text{H}$ - ${}^{15}\text{N}$ TROSY-HSQC spectra of $\alpha 7$ in the absence (red) and the presence (green) of 80 μM ketamine. Residues involved in ketamine binding demonstrated significant changes in chemical shift or peak intensity. They are highlighted in circles and labeled with the one-letter amino acid code and the sequence number. (B) Side and (C) top views of the $\alpha 7$ structure highlighting the residues (blue sticks) perturbed by ketamine (gray surface) binding.

$\alpha 7$ structure (Fig. 5B). Most residues affected by ketamine are located in the lower half of the TM domain, similar to the case for halothane (Fig. 4C). It is noteworthy that the binding sites for ketamine and halothane largely overlap, but these two drugs perturbed different residues in this relatively large cavity near the IC end of the $\alpha 7$ TM domain. For example, F453, S285, I217, and L248 had obvious changes in their chemical shifts upon adding 80- μM ketamine, but the same change was not observed when 3.2-mM halothane was added to the sample (Fig. 4B). Conversely, C219, S223, and T289 showed changes in their chemical shifts only upon the addition of halothane, but not ketamine. Only a single ketamine molecule can fit into the cavity because of the larger molecular volume of ketamine. In contrast, the cavity can host two halothane molecules simultaneously. One of the most notable differences between ketamine and halothane binding is that ketamine, but not halothane, introduced changes to the pore-lining residue L248 (L9' using the conventional prime numbering system). L9' is a key residue in the channel gate. Its chemical shift change signifies perturbation to the channel gate, which will most likely generate a functional consequence.

The half maximal inhibitory concentration (IC_{50}) of halothane is $\sim 1200 \mu\text{M}$ for the $\alpha 7$ nAChR [21], but only $\sim 27 \mu\text{M}$ for the $\alpha 4\beta 2$ nAChR [55]. The IC_{50} values of ketamine for the $\alpha 7$ and $\alpha 4\beta 2$ nAChRs are $\sim 20 \mu\text{M}$ and $50\text{--}72 \mu\text{M}$ [52,53], respectively. Anesthetic concentrations used for functional measurements are typically referred to the

concentrations measured in saline. If one considers a membrane/saline partition coefficient of ~ 100 for anesthetics [56,57], halothane and ketamine concentrations used for our NMR experiments, in the presence of the LDAO micelles, are reasonably close to those used for anesthetic inhibition of nAChRs. Thus, the observed anesthetic-induced changes in the NMR experiments are likely relevant to functional modulation by the anesthetics.

3.4. Anesthetics modulation of the $\alpha 7$ dynamics

Upon adding anesthetics to $\alpha 7$, relative amide peak intensities of some residues increased or decreased in the $\alpha 7$ NMR spectra (Fig. S10), indicating changes in protein dynamics [54,58,59]. Residues lining the binding site for halothane or ketamine tended to experience intensity decrease, while residues distant from the binding sites had intensity decrease or increase (Fig. 6). Among residues whose intensity changed more than 25%, halothane binding decreased intensity for 7 out of 10 residues; those in TM2 and TM3 decreased exclusively. In contrast, six out of 11 residues having more than 25% intensity changes in response to ketamine binding experienced peak intensity increase. Another notable difference between halothane and ketamine binding is the number of residues in TM4 affected by ketamine (R447, F453, S447, I458, and T461) and halothane (C449). The profound perturbation to TM4 is expected to introduce functional consequences [60–62]. The role of TM4 in Cys-loop receptor-lipid interactions as well as in nAChR function has been established [60–63].

Peak splitting was observed previously in NMR spectra of the $\alpha 4\beta 2$ nAChR TM domain in the presence of anesthetics [54]. The splitting likely indicates a shift of conformational exchange from intermediate (or fast) to slow time scale. It is noteworthy that the splitting observed on $\alpha 4\beta 2$ did not occur on $\alpha 7$ (Fig. 6C). Neither halothane nor ketamine was able to drive $\alpha 7$ into slow conformational exchange mode as they did on $\alpha 4\beta 2$.

4. Discussion

4.1. Small structural differences can make profound functional impact

The NMR structure reported here offers valuable spatial details specifically for the human $\alpha 7$ nAChR TM domain that may not have been accurately captured by computer modeling. Although pLGICs share a common scaffold, variations in the TM helical lengths and orientations among various pLGICs exist. Even for the TM domains of the $\alpha 7$, $\alpha 4$, and $\beta 2$ nAChRs that share sequence identities up to $\sim 50\%$, structural deviations in their TM domains are observable. Differences in helical tilting in the range of 5 to 7° were observed that could account for differences in intra-subunit cavities and helical packing (Fig. 2, Fig. S5). Can these seemingly subtle structural variances generate impact to drug binding and channel functions? Unwin and Fujiyoshi recently reported gating movement of *Torpedo* nAChR caught by plunge-freezing [64]. The EM images show only a small magnitude structural displacement for the closed- and open-channel nAChRs, in which no more than a 2° tilt and a 2 \AA shift were found in the pore-lining helices [64]. Furthermore, open and locally closed crystal structures of GLIC revealed only a $\sim 6^\circ$ difference in the TM2 tilting angles [65]. Hence, a subtle structural change is not unexpected for a profound functional difference. Following the same principle, a subtle structural difference may be sufficient for defining pharmacological characteristics of individual receptors. Indeed, our recent study on $\alpha 7\beta 2$ demonstrated that the subtle structure difference at the EC end of the TM domain produced a profound impact to isoflurane binding and inhibition [66]. Furthermore, certain positive allosteric modulators interacting at an intra-subunit TM site, such as PNU-120596 [22–24] and TQS [25,26], are known to have direct modulatory effects only on $\alpha 7$ nAChRs, but virtually no effect on other subtypes of nAChRs.

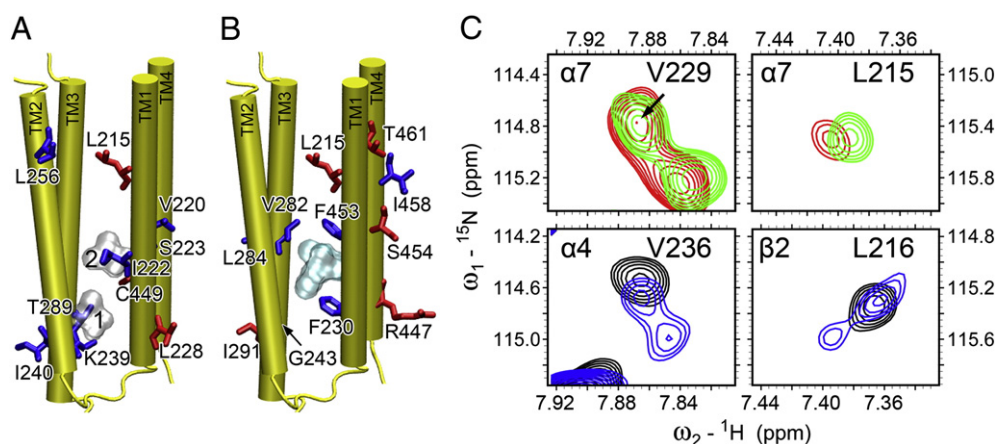


Fig. 6. Anesthetic effects on backbone dynamics of the TM domain of the human $\alpha 7$ nAChR. Residues, whose relative peak intensity increased (red) or decreased (blue) upon the addition of (A) halothane (silver surface) and (B) ketamine (cyan surface) binding, are highlighted in the $\alpha 7$ structure. (C) Representative regions of ^1H - ^{15}N TROSY-HSQC spectra in the absence (red or black) and presence (green or blue) of halothane. $\alpha 7$ -V229 (top, left) is equivalent to $\alpha 4$ -V236 (bottom, left); $\alpha 7$ -L215 (top, right) is equivalent to $\beta 2$ -L216 (bottom, right). Note the halothane-induced peak splitting in $\alpha 4$ -V236 and $\beta 2$ -L216, a sign of decrease of conformational exchange rates by halothane. Such changes were not observed in $\alpha 7$.

4.2. Anesthetic binding is necessary but not sufficient for altering channel functions

The $\alpha 7$ and $\alpha 4\beta 2$ nAChRs are the two most abundant nAChR subtypes in the brain. Previous investigations indicate that the $\alpha 7$ nAChR, unlike the $\alpha 4\beta 2$ nAChR, has distinct low functional sensitivity to volatile anesthetics, such as halothane [20,21]. The reason why volatile anesthetics are ineffective on $\alpha 7$ but effective on $\alpha 4\beta 2$ has been a mystery in the past. Here, we have determined the $\alpha 7$ NMR structures (Fig. 2) and a halothane-binding site in $\alpha 7$ (Fig. 4). We showed that $\alpha 7$ does not have a binding site for halothane at the EC end of the TM domain as revealed previously for $\alpha 4\beta 2$ [54]. Furthermore, we have disclosed an association of anesthetic modulation on channel dynamics and channel function (Fig. 6). Comparisons of structural, dynamics, and anesthetic binding information between $\alpha 7$ and $\alpha 4\beta 2$ offer a clue for reasoning why $\alpha 7$ is insensitive to halothane and other volatile anesthetics. Halothane binds to $\alpha 7$ (Figs. 3, 4), but the binding to the IC end of the TM domain did not effectively modulate dynamics of channel residues as it did in $\alpha 4\beta 2$ (Fig. 6), where more profound dynamics changes were observed. These results suggest a plausible association between dynamics modulation and functional modulation by anesthetics. Anesthetic binding would not produce functional impact unless the binding can significantly alter channel motions coupled with functions.

4.3. Ketamine action site in the $\alpha 7$ nAChR

The functional insensitivity of $\alpha 7$ to halothane or other volatile anesthetics may result from an inability to effectively modulate channel dynamics due to anesthetic binding to the IC end of the TM domain and/or lack of anesthetic binding to the EC end of the $\alpha 7$ TM domain. However, the intravenous anesthetic ketamine binds to the $\alpha 7$ TM domain site; yet ketamine inhibits the $\alpha 7$ nAChR with a similar inhibition efficacy as it acts on the $\alpha 4\beta 2$ nAChRs [51–53]. It is possible that with its larger molecular size, ketamine can accomplish what halothane and other volatile anesthetics cannot. Supporting evidence for such a possibility includes that ketamine, but not halothane, changed the chemical shift of the pore-lining residue L9' (Fig. 5) and ketamine affected the motions of the $\alpha 7$ TM domain with a pattern different from that of halothane, particularly in TM4 (Fig. 6).

It is worth mentioning that ketamine has been found to inhibit functions of GLIC [67], a homologue of the $\alpha 7$ nAChR. Allosteric inhibition was via ketamine binding to an inter-subunit cavity in the EC domain of GLIC. It was shown (Fig. S5 [67]) that the $\alpha 7$ nAChR has a homologous cavity in the EC domain that mimics the ketamine-binding site in GLIC [67]. Although the structural study for the $\alpha 7$ nAChR reported here

includes only the TM domain, ketamine binding to the TM site as identified in Fig. 5 and to the EC site as suggested previously [67] can both contribute to functional inhibition of the $\alpha 7$ nAChR [51–53].

5. Conclusions

The high-resolution NMR structure for the $\alpha 7$ nAChR TM domain determined in this study offers an invaluable structural framework for designing new therapeutic modulators and for rationalizing extensive biochemical and functional data collected previously on nAChRs. The discovery of halothane binding to the $\alpha 7$ nAChR TM domain provides convincing evidence that insensitivity of a pLGIC to anesthetics, such as in the case of the $\alpha 7$ nAChR to volatile anesthetics [20,21], is not necessarily due to a lack of anesthetic binding. Comparisons of halothane sites in $\alpha 7$ with those in the $\alpha 4\beta 2$ nAChR [54] and distinct dynamic responses of these receptors to halothane binding convey an important message, that is, effective functional modulation occurs only when the binding of anesthetics, or any modulators, induces dynamics or conformational changes in the channel pore.

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Appendix A. Supplementary data

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