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Solution NMR Studies of Anesthetic Interactions with Ion Channels

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Abstract

NMR spectroscopy is one of the major tools to provide atomic-resolution protein structural information. It has been used to elucidate the molecular details of interactions between anesthetics and ion channels, to identify anesthetic binding sites, and to characterize channel dynamics and changes introduced by anesthetics. In this chapter, we present solution NMR methods essential for investigating interactions between ion channels and general anesthetics, including both volatile and intravenous anesthetics. Case studies are provided with a focus on pentameric ligand-gated ion channels (pLGICs) and the voltage-gated sodium channel NaChBac.

Keywords

NMR; ion channels; general anesthetics; pLGICs; nAChR; NaChBac; binding sites; dynamics

1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy has a wide range of applications, including the determination of protein structures, characterization of protein-ligand interactions, and detection of changes in protein conformations and dynamics upon ligand binding (Kay, 2016; Kim, Howell, Van Horn, Jeon, & Sanders, 2009; Kitevski-LeBlanc & Prosser, 2012; Liang & Tamm, 2016; Liu, Horst, Katritch, Stevens, & Wuthrich, 2012; Oxenoid & Chou, 2013, 2016; Rosenzweig & Kay, 2016; Ye, Van Eps, Zimmer, Ernst, & Prosser, 2016; Zhuang et al., 2013). For the specific purpose of understanding anesthetic interactions with proteins, NMR has been used to determine the structures of anesthetic targets (Bondarenko et al., 2012; Bondarenko et al., 2014; Bondarenko, Tillman, Xu, & Tang, 2010; Cui et al., 2012; Ma, Brandon, et al., 2008; Ma, Liu, Li, Tang, & Xu, 2005; Ma, Tillman, et al., 2008; Mowrey, Cui, et al., 2013; Mowrey, Kinde, Xu, & Tang, 2015; Tang, Mandal, & Xu, 2002), identify anesthetic binding sites in proteins (Bondarenko, Mowrey, Liu, Xu, & Tang, 2013; Bondarenko et al., 2014; Bondarenko, Yushmanov, Xu, & Tang,

2008; Kinde, Bondarenko, et al., 2016; Kinde, Bu, et al., 2016; Mowrey, Liu, et al., 2013; Tang, Eckenhoff, & Xu, 2000), characterize the direct interactions between proteins and anesthetics over a broad range of binding affinities (Bondarenko et al., 2013; Bondarenko et al., 2008; Canlas, Cui, Li, Xu, & Tang, 2008; Cui et al., 2008; Ma, Brandon, et al., 2008; Tang et al., 2000; Tang, Hu, Liachenko, & Xu, 1999; Xu, Seto, Tang, & Firestone, 2000; Xu, Tang, Firestone, & Zhang, 1996), and determine how anesthetic binding affects protein structures and dynamics (Canlas et al., 2008; Cui et al., 2008; Cui, Canlas, Xu, & Tang, 2010; Mowrey, Liu, et al., 2013).

Pentameric ligand-gated ion channels (pLGICs) are important targets of general anesthetics (Campagna, Miller, & Forman, 2003; Eckenhoff, 1996; Franks & Lieb, 1994; Hemmings et al., 2005). Each pLGIC subunit consists of an extracellular domain (ECD) and a transmembrane domain (TMD) composed of four helices (TM1-TM4) with TM2 forming the pore. Eukaryotic pLGICs also contain an intracellular domain (ICD) composed of a large loop between TM3 and TM4. General anesthetics also interact with tetrameric voltage-gated sodium channels by stabilizing the inactivated state or altering the inactivation kinetics (Barber, Carnevale, Klein, Eckenhoff, & Covarrubias, 2014; Covarrubias, Barber, Carnevale, Treptow, & Eckenhoff, 2015; Herold & Hemmings, 2012; W. Ouyang, Jih, Zhang, Correa, & Hemmings, 2007; Purtell, Gingrich, Ouyang, Herold, & Hemmings, 2015). Although solution NMR has been used to obtain high-resolution TMD structures of the human pLGICs $\alpha 4\beta 2$ nAChR, $\alpha 7$ nAChR, and $\alpha 1$ GlyR (Bondarenko et al., 2012; Bondarenko et al., 2014; Bondarenko et al., 2010; Mowrey, Cui, et al., 2013), its real strength lies in characterizing the interactions between anesthetics and ion channels, revealing anesthetic binding sites with atomic resolution, and measuring the influence of anesthetics on the dynamics of specific regions in a channel. X-ray crystallography has been successfully employed to identify anesthetic binding sites in pLGICs (Chen et al., 2015; Chen et al., 2017; Nury et al., 2011; Pan et al., 2012; Sauguet et al., 2013; Spurny et al., 2013); however, solution NMR provides more flexibility in sample preparation and measurements at more physiologically relevant conditions. Overall, solution NMR is complementary to other tools in providing structural insight into anesthetic interactions with ion channels.

This chapter outlines solution NMR methods used to investigate the interactions of anesthetics with ion channels and the effects of anesthetic binding on the dynamics of these channels. Expression and purification protocols for isotope-labeled ion channels and sample preparation for solution NMR are also presented.

2. Preparation of ion channels for solution NMR

2.1 Design proper constructs

Recent advances in solution NMR spectroscopy have made it possible to determine structures of various ion channels with molecular weights less than 80 kDa (Mowrey et al., 2015; B. OuYang et al., 2013; Oxenoid & Chou, 2016; Schnell & Chou, 2008). With an increased molecular mass, both the sensitivity and resolution of NMR spectra worsen dramatically; NMR signals broaden significantly due to slower rotational tumbling of the larger protein. The use of transverse relaxation-optimized spectroscopy (TROSY) has considerably improved the limit of protein mass in solution NMR (Pervushin, Riek, Wider,

& Wuthrich, 1997). However, a typical full-length eukaryotic pLGIC has a molecular weight of ~280 kDa and also carries extra weight from membrane mimetics used to stabilize the channel. Similarly, voltage-gated sodium channels have a molecular weight of ~125 kDa. Thus, the full-length ion channel challenges the limit of conventional solution NMR for structure determination. One convenient approach to solving the protein-size problem for ion channels is to truncate the large full-length protein into smaller individual domains, which can retain functionality in isolation. For example, the TMDs of nAChRs and GlyRs have been shown to conduct current in the absence of an ECD or ICD (Bondarenko et al., 2012; Bondarenko et al., 2014; Mowrey, Cui, et al., 2013). Conversely, determination of anesthetic binding sites can be conducted using full-length channels, for which isotopic labeling for NMR observation is performed only on selected residues rather than uniformly labeling of the entire protein. Thus, the protein-size issue becomes less problematic, as shown in studies of anesthetic binding to full-length ELIC and NaChBac (Kinde, Bondarenko, et al., 2016; Kinde, Bu, et al., 2016).

2.2. Choose membrane mimetics

The choice of membrane mimetic is an important factor in the stability of ion channel samples for solution NMR. Although a native membrane environment is the most ideal choice, the large size of lipid vesicles is problematic for solution NMR. Alternative choices include detergent micelles, phospholipid bicelles, and nanodiscs (Piai, Fu, Dev, & Chou, 2017; Sanders & Mittendorf, 2011) (Etzkorn et al., 2013; Hagn, Etzkorn, Raschle, & Wagner, 2013; Kucharska, Edrington, Liang, & Tamm, 2015; Laguerre et al., 2016) (Susac, 2014 #693) (Liang & Tamm, 2016). Detergent micelles are often used for solution NMR due to their small size. In most cases, detergent micelles provide better quality of NMR spectra and a greater number of resolved residues due to the faster tumbling compared to bicelles and nanodiscs. However, bicelles and nanodiscs more closely resemble the native membrane environment. The NMR spectra for samples in bicelles and nanodiscs, even with fewer resolved residues, can be used at least as a reference to judge whether the protein in micelles is folded properly.

2.3. Prepare uniformly isotope-labeled ion channels

Ion channel samples for high-resolution solution NMR require a protein concentration of ~200–300 μ M to achieve a good signal to noise ratio of spectra within a reasonable period of time. Ion channel samples must be stable during the period of data acquisition, typically lasting hours to days, depending on the designed experiments. In addition to the selection of membrane mimetic, choices of buffer type, salt concentration, and pH are extremely important for sample stability. Most NMR experiments are required to be performed at pH 7 to prevent exchange-induced signal weakening. Thus, the protein samples must be stable at this pH condition. The ion channel construct for NMR experiments should be designed to fulfill this requirement. For example, in order to stabilize the TMDs of $\alpha 4$, $\beta 2$, and $\alpha 7$ nAChRs at pH 7, a TEV protease recognition site and a polyhistidine tag were placed at the N-terminus of the TMD. The ICD was replaced with a short glycine linker. More importantly, insertion of 2–3 glutamate residues at the N-terminus and mutation of two hydrophobic residues within the TM2–TM3 linker to serine greatly prevented protein

aggregation and extended the lifetime of the samples at low pH (Bondarenko et al., 2012; Bondarenko et al., 2014).

Expression and purification of ion channels in *E. coli* follow the same generalized protocols as described in the chapter “X-ray Crystallographic Studies for Revealing Binding Sites of General Anesthetics in Pentameric Ligand-Gated Ion Channels” by Chen, Xu, and Tang in this volume. For ^{15}N - and ^{13}C -NMR, isotope-labeling is achieved by replacing LB broth with M9 media containing ^{15}N -(NH_4) $_2\text{SO}_4$ and/or ^{13}C -glucose during expression. After purification, the sample is prepared to fit the requirements of NMR, including pH below 7. To make pH adjustment easier, the buffer concentration should be reduced to < 5 mM using a diafiltration or desalting column. The protein should be concentrated to ~200–300 μM and the detergent concentration should be minimized.

2.4. Prepare selectively ^{19}F -labeled ion channels

^{19}F NMR is a popular tool to study protein conformations, dynamics, and interactions with ligands. ^{19}F has the advantage of high natural abundance, large chemical shift dispersion, and the absence of native ^{19}F signals in proteins, offering a clean background for NMR experiments. ^{19}F NMR chemical shifts are sensitive to electrostatic and van der Waals interactions and the linewidth of a ^{19}F resonance signal can be affected predominately by protein dynamics (Kinde et al., 2015; Kitevski-LeBlanc & Prosser, 2012; Liu et al., 2012). The high sensitivity to the chemical and dynamic environment around the ^{19}F probe makes ^{19}F NMR particularly suitable for detecting channel conformational changes and identifying anesthetic binding sites. In principle, a ^{19}F probe can be placed in a designed residue position through either biosynthetic incorporation (Sharaf & Gronenborn, 2015) or postrationally introduced by chemical labeling small ^{19}F -containing molecules to purified proteins with reactive –SH or –NH moieties. However, in practice with ion channels, chemical labeling is preferred to provide a sufficient amount of ^{19}F -labeled channels for NMR. 2,2,2-Trifluoroethanethiol (TET) and 3-bromo-1,1,1-trifluoroacetone (BTFA) have been used as ^{19}F -labeling reagents for ion channels (Kinde, Bondarenko, et al., 2016; Kinde, Bu, et al., 2016; Kinde et al., 2015). 4-(perfluoro-tert-butyl)-phenyliodoacetamide (PFP) and S-ethyl-trifluorothioacetate (SETFA) have also been used for ^{19}F -labeling proteins (Kalbitzer et al., 1992; Mehta, Kulkarni, Mason, Constantinescu, & Antich, 1994). In order to have site-specific labeling, it is ideal to use an ion channel construct that contains a single cysteine residue. Thus, mutations are often required if there are multiple cysteines in the native channel. ^{19}F chemical labeling of ion channels typically begins with a brief treatment of a reducing agent, followed by the addition of the selective labeling reagent. A generalized labeling protocol is provided below:

1. Add a 30-fold molar excess of the reducing agent tris(2-carboxyethyl)phosphine (TCEP) to the purified protein and incubate at 4 °C for 1 h.
2. Remove TCEP using a desalting column.
3. Label protein with a ~70-fold molar excess of ^{19}F -labeling reagents (TET or BTFA) at room temperature for 3 h, and then 4 °C overnight.

4. Remove free ^{19}F -labeling reagent using dialysis (3 changes of 100x volume, 1 hour each), followed by size exclusion chromatography (Superdex 200 10/300GL column (GE Healthcare)).

Note: A few mM of lipids (asolectin or DMPC) can be added to the final protein sample to improve stability.

3. NMR methods to identify anesthetics binding sites and detect anesthetic-induced dynamics changes

3.1. Chemical shift perturbations

The chemical shift is the resonant frequency of a nucleus relative to a standard in NMR spectroscopy. If anesthetics bind to an ion channel, they will alter the chemical environment of contacting residues, causing changes in chemical shifts. Thus, perturbations to ^1H , ^{15}N , ^{13}C , or ^{19}F chemical shifts are an initial indication of anesthetic binding. Anesthetic-induced chemical shift perturbation can be measured in 1D, 2D, or 3D NMR spectra by titrating different concentrations of anesthetics into a sample. In general, only ^1H and ^{19}F are detected in 1D spectra because of their high sensitivity. 1D ^1H spectra of the protein region are usually crowded and are poorly resolved. In contrast, 1D ^{19}F spectra of selectively labeled samples provide distinct signals for characterizing anesthetic binding. 3D ^{15}N - or ^{13}C -filtered NMR spectra provide much better resolution, but data acquisition requires long times. 2D ^1H - ^{15}N or ^1H - ^{13}C NMR spectra, such as HSQC, provide adequate resolution within a reasonable time for data collection. Therefore, they have been commonly used to identify drug-binding sites, including for anesthetics (Bondarenko et al., 2013; Bondarenko et al., 2014; Bondarenko et al., 2008; Mowrey, Liu, et al., 2013). In addition to chemical shift changes, drug binding may also introduce changes in the protein dynamics that can be reflected in the 2D NMR spectra. To prevent artificial chemical shift perturbations, one should use the same buffer for both protein and anesthetics during titration experiments. If any additional chemicals have to be introduced to the sample, a control spectrum must be acquired. The concentration of volatile anesthetics titrated to the protein sample may not be stable due to evaporation. Therefore, the volatile anesthetic concentration after each titration step needs to be calibrated using a reference signal, such as a known concentration of DSS in the 1D ^1H spectrum or trifluoroacetate (TFA) in the ^{19}F spectrum (Xu et al., 2000; Xu et al., 1996).

Chemical shift perturbations can be used to estimate the dissociation constant (K_D) for anesthetics from fitting the data as a function of anesthetic concentration in equation [1] (Fielding, 2003; Morton et al., 1996) below.

$$\Delta\delta_{obs} = \frac{\Delta\delta_{max}}{2[P]_0} \left\{ (K_D + [A]_0 + [P]_0) - \sqrt{(K_D + [A]_0 + [P]_0)^2 - 4[A]_0[P]_0} \right\} \quad [1]$$

δ_{obs} is the observed chemical shift change in the presence and absence of anesthetics at a given anesthetic concentration, δ_{max} is the maximum chemical shift change in the presence

and absence of anesthetics, and $[P]_0$ and $[A]_0$ are the protein and anesthetic concentration, respectively.

Although residues showing large chemical shift changes in the presence of anesthetics are likely directly involved in binding, indirect allosteric effects by anesthetics can also produce changes in the spectra. Additional NMR experiments (see section 3.2) are required to distinguish between the direct and indirect anesthetic effects.

3.2. Saturation transfer difference spectroscopy

Saturation transfer difference (STD) NMR has been used effectively to identify direct binding of small molecules to proteins with a K_D between 10^{-8} and 10^{-3} M (Klein, Meinecke, Mayer, & Meyer, 1999; Mayer & Meyer, 1999). It is particularly suitable for investigations of anesthetic–protein interactions due to the low-affinity nature of the anesthetic binding (Bondarenko et al., 2013; Bondarenko et al., 2014; Cui et al., 2008; Mowrey, Liu, et al., 2013). Recent improvements in the STD NMR pulse sequences have allowed for better information on the most significant molecular contacts and interactions that relate to ligand binding-modes (Megy et al., 2005; Sorge, Wagstaff, Rowe, Williamson, & Howard, 2015; Vogtherr & Peters, 2000; Wagstaff, Vallath, Marshall, Williamson, & Howard, 2010).

STD experiments collect a pair of NMR spectra in an interleaved fashion, with on- and off-resonance frequencies to saturate selected ^1H or ^{19}F of ion channels. Effective spin diffusion saturation can quickly propagate across the protein as well as bound anesthetics. Thus, the signal from bound anesthetics will be attenuated in the on-resonance spectrum. Subtracting the off- and on-resonance saturation spectra provides the STD spectrum, where only signals from the bound anesthetics are observed. Fitting the STD data collected at different saturation times to equation [2] (Mayer & James, 2004) below offers an estimate of several key parameters, including the asymptotic maximum of the STD build-up curve $STD_{\max}$, the observed saturation rate constant k_{sat} , and the cross-relaxation rate constant between the anesthetic and ion channel spins σ ($\sigma = STD_{\max}k_{\text{sat}}$).

$$STD = STD_{\max} \left[1 - e^{(-k_{\text{sat}}t)} \right] \quad [2]$$

where $STD = 100\% \frac{I_{\text{off}} - I_{\text{on}}}{I_{\text{off}}}$, I_{off} and I_{on} are the anesthetic peak intensity from the off- and on-resonance STD spectra, respectively, and t is the saturation time.

If the aforementioned STD NMR experiments are performed over several concentrations of a given anesthetic, the anesthetic binding affinity, K_D , can be measured based on equations [3] and [4] (Mayer & Meyer, 2001; Meyer & Peters, 2003),

$$STD_{Ampl.} = \frac{(I_{off} - I_{on})}{I_{off}} * ligand \ excess \quad [3]$$

$$STD_{Ampl.} = \frac{STD_{max}[L]}{K_D + [L]} \quad [4]$$

where K_D is calculated by fitting the $STD_{Ampl.}$ data as a function of the anesthetic concentration $[L]$.

3.3. Relaxation measurements for anesthetic modulation of protein dynamics

Protein dynamics modulated by anesthetics can be measured both qualitatively and quantitatively. The linewidth and intensity of NMR peaks are related to protein motion. Within the same protein, residues in more flexible regions often experience faster motion and have higher intensities and narrower linewidths in NMR spectra than rigid residues. Anesthetics can cause line broadening and reduced intensity of residues in one region of a protein, but result in narrowed linewidths and higher intensities for residues in another region, indicative of changes in dynamics (Bondarenko et al., 2013; Bondarenko et al., 2014; Canlas et al., 2008; Cui et al., 2008; Mowrey, Liu, et al., 2013). It is not unusual to observe multiple conformations of a given protein in the NMR spectra; the addition of anesthetics can shift the populations of conformations. To quantify changes in protein dynamics introduced by anesthetics, one can perform systematic measurements of spin-lattice (R_1) and spin-spin (R_2) ^{15}N relaxation rate constants and ^1H - ^{15}N heteronuclear *NOE*. In addition, the ^{15}N R_2 relaxation dispersion data are particularly well suited to determine conformational exchange due to anesthetic binding (Canlas et al., 2008; Cui et al., 2008). In regions where conformational exchange is slow (micro- to millisecond time scale), more than one peak per residue is observed. Slow conformational exchange is especially important because of the relevance to protein function, such as channel gating. Anesthetic binding can not only change the populations of different conformations, but also shift the exchange rate between conformations, from the fast to slow regime or vice versa.

Case studies

Two representative studies are presented here to show how solution NMR can be used to identify anesthetic binding sites and characterize the effects of anesthetic binding on the dynamics of ion channels.

4.1. Anesthetic interactions with nAChR transmembrane domains

$\alpha 4\beta 2$ nAChR is inhibited by the volatile anesthetic halothane (Mori et al., 2001) and both $\alpha 4\beta 2$ and $\alpha 7$ nAChRs are inhibited by the intravenous anesthetic ketamine (Coates & Flood, 2001; Flood & Krasowski, 2000; Yamakura, Chavez-Noriega, & Harris, 2000). To identify specific binding sites for these anesthetics in the TMDs of $\alpha 4\beta 2$ and $\alpha 7$ nAChRs, we

performed ^1H - ^{15}N TROSY-HSQC (chemical shift perturbation) and 2D STD NMR experiments (Bondarenko et al., 2013; Bondarenko et al., 2014). nAChR TMD samples for NMR experiments were expressed using plasmids containing N-terminal His-tagged constructs from a T7 promoter, transformed into the *E. coli* strain Rosetta2(DE3)pLysS (Novagen) and plated on a LB-agar plate with ampicillin and chloramphenicol selection. Selected colonies were grown overnight at 37 °C in an environmental shaker at 250 rpm. 5 mL of the overnight culture was added to each liter of LB media with antibiotics and grown at 37 °C to an optical density of 0.7 at 600 nm. Cells were harvested by centrifugation at 5000 x *g* for 20 min. The cell pellet from 2 L LB culture was resuspended in 0.5 L of M9 media containing ^{15}N -(NH_4) $_2$ SO $_4$ and/or ^{13}C -glucose and grown at 15 °C for 1.5 h. Expression was induced by adding 0.2 mM IPTG and cells were grown at 15 °C for 3 days. The harvested cell pellet was resuspended in 30 mL lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl) and cells were digested at 4 °C with lysozyme and benzonase for 1 h. Cells were lysed using a microfluidizer and the membranes were collected by centrifugation at 100,000 x *g* at 4 °C for 1 h. The membranes were homogenized in 10 mL lysis buffer. 2.5% LDAO detergent was subsequently added and the sample was incubated at 4 °C for 1 h with inversion. The solubilized protein was clarified by centrifugation at 13,000 x *g* at 4 °C for 10 min.

To purify nAChR TMDs, the solubilized protein was loaded at 0.5 mL/min into a 1 mL Ni-NTA column that was pre-equilibrated with 20 mM imidazole in 96% Buffer A (50 mM Tris at pH 8.0, 150 mM NaCl, and 0.5% LDAO) and 4% Buffer B (500 mM imidazole, pH 8.0, 500 mM NaCl, and 0.5% LDAO). The Ni-NTA column was washed with 20 mM imidazole until a flat baseline was observed, and then further washed with 10 mL of 50 mM imidazole. The protein was eluted with 250 mM imidazole. The purity of the protein samples was assessed to determine if additional purification was required.

To prepare the nAChR TMD samples for NMR, the concentration of Tris was reduced to < 5 mM using diafiltration or desalting columns. The pH was reduced from 8.0 to 4.7 using 1 M acetic acid. The NaCl concentration was reduced from 100 mM to 10 mM. Finally, the protein samples were concentrated to 250–300 μM . The concentration of LDAO in the samples was ~1% as measured by 1D ^1H NMR. Size exclusion chromatography-multiangle light scattering analysis (SEC-MALS) indicated that the nAChR TMDs spontaneously assembled into pentamers in micelles (Bondarenko et al., 2012; Bondarenko et al., 2014). Furthermore, the purified $\alpha 7$ nAChR TMDs formed ion-conducting channels when injected as reconstituted asolectin vesicles into *Xenopus* oocytes. The TMD does not possess the orthosteric agonist-binding site of full-length $\alpha 7$ nAChR, but channel current can be elicited by ivermectin (Bondarenko et al., 2014), a known positive allosteric modulator that acts through the TMD (Collins & Millar, 2010; Krause et al., 1998). Moreover, ketamine inhibited ivermectin-induced current in the TMD, consistent with the effect of ketamine on native human $\alpha 7$ nAChR (Bondarenko et al., 2014). No ivermectin-elicited current was observed in control oocytes injected with empty asolectin vesicles. These data validate the relevance of using truncated channels for NMR studies.

NMR spectra were acquired on Bruker Avance 600 and 800 MHz spectrometers using triple-resonance inverse-detection cryoprobes (Bruker Instruments, Billerica, MA). For ^1H , ^{15}N ,

and ^{13}C chemical shift assignment and the protein structure determination, a suite of NMR experiments was performed, including HNCA, HN(CO)CA, HNCB, ^{15}N -edited NOESY, ^1H - ^{13}C HSQC, and ^1H - ^{15}N TROSY-HSQC. HSQC spectra were collected at 35, 40, and 45 °C to optimize the temperature condition (Bondarenko et al., 2013; Bondarenko et al., 2012; Bondarenko et al., 2014).

In the $\alpha 4\beta 2$ nAChR TMD, both halothane and ketamine were found in an intra-subunit cavity near the extracellular end of the $\beta 2$ transmembrane helices, homologous to a common anesthetic binding site observed in X-ray structures of GLIC (Nury et al., 2011) and photolabeling of ELIC (Kinde, Bu, et al., 2016). Halothane, but not ketamine, was also found in inter-subunit cavities at the interface of $\alpha 4$ and $\beta 2$. In addition, both anesthetics bound to intra-subunit cavities at the intracellular end of $\alpha 4$ and $\beta 2$ TMDs. In $\alpha 7$ nAChR, ketamine bound to the intracellular end of the TMD and perturbed the channel-gate residue L9'.

Halothane and ketamine binding profoundly altered conformational exchanges of $\alpha 4\beta 2$ nAChR TMDs. A number of residues, close to and remote from the binding sites, showed resonance signal splitting from single to double peaks, indicating a decreased conformation exchange rates in the presence of anesthetics. The populations of two conformations were also shifted by anesthetics. Ketamine also introduced notable changes in peak intensity to residues in the $\alpha 7$ nAChR TMD, particularly in TM4. Perturbations to TM4 are expected to have functional consequences (Lasalde et al., 1996; Lee et al., 1994; Li, Lee, Pappone, Palma, & McNamee, 1992) and the role of TM4 in pLGIC-lipid interactions as well as in nAChR function has been established (daCosta & Baenziger, 2009; Lasalde et al., 1996; Lee et al., 1994; Li et al., 1992). These studies provided compelling NMR evidence of anesthetic-induced changes in protein dynamics.

4.2. Isoflurane interaction with NaChBac

The volatile anesthetic isoflurane inhibits voltage-gated sodium channels, including the bacterial channel NaChBac (W. Ouyang et al., 2007). We used ^{19}F NMR to identify isoflurane binding sites in NaChBac (Kinde, Bondarenko, et al., 2016). NaChBac was expressed in *E. coli* C41 cells (Lucigen) using the in-house expression vector pETHT at 18 °C for 24 h. A six-histidine tag and a TEV protease recognition site were placed in the N terminus of NaChBac. n-dodecyl- β -D-maltoside (DDM, Anatrace) detergent was used for membrane solubilization and purification. Protein expression and purification were otherwise similar to the protocols described above for nAChR TMDs. Single cysteine mutations were performed on selected residues based on docking predictions (Raju, Barber, LeBard, Klein, & Carnevale, 2013). Multiple binding sites were predicted, including sites in the S4–S5 linker, the base of S5 helix, the extracellular interface near the hinge of the P1 helix, near the central pore at the bottom of the selectivity filter, and at the N-terminal hinge of the S6 helix. ^{19}F -labeling with TET to cysteine residues was conducted using the protocol described in section 2.4.

^{19}F NMR STD spectra with on- and off-resonance saturation were acquired at 10 °C using 5 different saturation times (0.1, 0.4, 1, 2, and 4 s) and a 5-s recycle time (Kinde, Bondarenko, et al., 2016). To quantify the isoflurane STD signal in each NaChBac mutant, the $-\text{CF}_2$ and -

CF₃ peaks were integrated in each spectrum and plotted as a function of the saturation time. The data were fit by equation [2](Mayer & James, 2004) in section 3.2.

The ¹⁹F NMR STD spectra showed strong direct interactions between isoflurane and NaChBac at residue positions S129, T189, and S208. Using selective saturation of the –CF₃ and –CF₂ moieties in isoflurane, we observed an orientation preference for isoflurane bound to T189 and S208, but not to S129. The data conclude that isoflurane inhibits NaChBac by two distinct mechanisms: (*i*) as a channel blocker at the base of the selectivity filter, and (*ii*) as a modulator to restrict the pivot motion at the S4–S5 linker and at a critical hinge that controls the gating and inactivation motion of S6 (Kinde, Bondarenko, et al., 2016).

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Abbreviations

NMR	nuclear magnetic resonance
STD NMR	saturation transfer difference NMR
pLGICs	pentameric ligand-gated ion channels
NaChBac	bacterial voltage-gated sodium channel
ECD	extracellular domain
TMD	transmembrane domain
ICD	intracellular domain
nAChR	nicotinic acetylcholine receptors
GluCl	glutamate-gated chloride channel
GlyR	glycine receptor
GABA_AR	type-A gamma-aminobutyric acid receptor
5-HT_{3A}R	5-hydroxytryptamine, or serotonin receptor
LDAO	Lauryldimethylamine-N-oxide
UDM	n-undecyl-β-D-maltopyranoside
DDM	n-dodecyl-β-D-maltopyranoside
TET	2,2,2-Trifluoroethanethiol
DSS	2,2-dimethyl-2-silapentane-5-sulfonic acid

References

- Barber AF, Carnevale V, Klein ML, Eckenhoff RG, & Covarrubias M (2014). Modulation of a voltage-gated Na⁺ channel by sevoflurane involves multiple sites and distinct mechanisms. *Proc Natl Acad Sci U S A*, 111(18), 6726–6731. doi: 10.1073/pnas.1405768111 [PubMed: 24753583]
- Bondarenko V, Mowrey D, Liu LT, Xu Y, & Tang P (2013). NMR resolved multiple anesthetic binding sites in the TM domains of the alpha4beta2 nAChR. *Biochim Biophys Acta*, 1828(2), 398–404. doi: 10.1016/j.bbamem.2012.09.014 [PubMed: 23000369]
- Bondarenko V, Mowrey D, Tillman T, Cui T, Liu LT, Xu Y, & Tang P (2012). NMR structures of the transmembrane domains of the alpha4beta2 nAChR. *Biochim Biophys Acta*, 1818(5), 1261–1268. doi: 10.1016/j.bbamem.2012.02.008 [PubMed: 22361591]
- Bondarenko V, Mowrey DD, Tillman TS, Seyoum E, Xu Y, & Tang P (2014). NMR structures of the human alpha7 nAChR transmembrane domain and associated anesthetic binding sites. *Biochim Biophys Acta*, 1838(5), 1389–1395. doi: 10.1016/j.bbamem.2013.12.018 [PubMed: 24384062]
- Bondarenko V, Tillman T, Xu Y, & Tang P (2010). NMR structure of the transmembrane domain of the n-acetylcholine receptor beta2 subunit. *Biochim Biophys Acta*, 1798(8), 1608–1614. doi: 10.1016/j.bbamem.2010.04.014 [PubMed: 20441771]
- Bondarenko V, Yushmanov VE, Xu Y, & Tang P (2008). NMR study of general anesthetic interaction with nAChR beta2 subunit. *Biophys J*, 94(5), 1681–1688. doi: 10.1529/biophysj.107.116772 [PubMed: 17993502]
- Campagna JA, Miller KW, & Forman SA (2003). Mechanisms of actions of inhaled anesthetics. *N Engl J Med*, 348(21), 2110–2124. doi: 10.1056/NEJMra021261 [PubMed: 12761368]
- Canlas CG, Cui T, Li L, Xu Y, & Tang P (2008). Anesthetic modulation of protein dynamics: insight from an NMR study. *J Phys Chem B*, 112(45), 14312–14318. doi: 10.1021/jp805952w [PubMed: 18821786]
- Chen Q, Kinde MN, Arjunan P, Wells MM, Cohen AE, Xu Y, & Tang P (2015). Direct Pore Binding as a Mechanism for Isoflurane Inhibition of the Pentameric Ligand-gated Ion Channel ELIC. *Sci Rep*, 5, 13833. doi: 10.1038/srep13833 [PubMed: 26346220]
- Chen Q, Wells MM, Tillman TS, Kinde MN, Cohen A, Xu Y, & Tang P (2017). Structural Basis of Alcohol Inhibition of the Pentameric Ligand-Gated Ion Channel ELIC. *Structure*, 25(1), 180–187. doi: 10.1016/j.str.2016.11.007 [PubMed: 27916519]
- Coates KM, & Flood P (2001). Ketamine and its preservative, benzethonium chloride, both inhibit human recombinant alpha7 and alpha4beta2 neuronal nicotinic acetylcholine receptors in *Xenopus* oocytes. *Br J Pharmacol*, 134(4), 871–879. doi: 10.1038/sj.bjp.0704315 [PubMed: 11606328]
- Collins T, & Millar NS (2010). Nicotinic acetylcholine receptor transmembrane mutations convert ivermectin from a positive to a negative allosteric modulator. *Mol Pharmacol*, 78(2), 198–204. doi: 10.1124/mol.110.064295 [PubMed: 20463059]
- Covarrubias M, Barber AF, Carnevale V, Treptow W, & Eckenhoff RG (2015). Mechanistic Insights into the Modulation of Voltage-Gated Ion Channels by Inhalational Anesthetics. *Biophysical Journal*, 109(10), 2003–2011. doi: 10.1016/j.bpj.2015.09.032 [PubMed: 26588560]
- Cui T, Bondarenko V, Ma D, Canlas C, Brandon NR, Johansson JS, ... Tang P. (2008). Four-alpha-helix bundle with designed anesthetic binding pockets. Part II: halothane effects on structure and dynamics. *Biophys J*, 94(11), 4464–4472. doi: 10.1529/biophysj.107.117853 [PubMed: 18310239]
- Cui T, Canlas CG, Xu Y, & Tang P (2010). Anesthetic effects on the structure and dynamics of the second transmembrane domains of nAChR alpha4beta2. *Biochim Biophys Acta*, 1798(2), 161–166. doi: 10.1016/j.bbamem.2009.08.009 [PubMed: 19715664]
- Cui T, Mowrey D, Bondarenko V, Tillman T, Ma D, Landrum E, ... Xu Y. (2012). NMR structure and dynamics of a designed water-soluble transmembrane domain of nicotinic acetylcholine receptor. *Biochim Biophys Acta*, 1818(3), 617–626. doi: 10.1016/j.bbamem.2011.11.021 [PubMed: 22155685]
- daCosta CJ, & Baenziger JE (2009). A lipid-dependent uncoupled conformation of the acetylcholine receptor. *J Biol Chem*, 284(26), 17819–17825. doi: 10.1074/jbc.M900030200 [PubMed: 19357079]

- Eckenhoff RG (1996). An inhalational anesthetic binding domain in the nicotinic acetylcholine receptor. *Proc Natl Acad Sci U S A*, 93(7), 2807–2810. [PubMed: 8610122]
- Etzkorn M, Raschle T, Hagn F, Gelev V, Rice AJ, Walz T, & Wagner G (2013). Cell-free Expressed Bacteriorhodopsin in Different Soluble Membrane Mimetics: Biophysical Properties and NMR Accessibility. *Structure*, 21(3), 394–401. doi: 10.1016/j.str.2013.01.005 [PubMed: 23415558]
- Fielding L (2003). NMR methods for the determination of protein-ligand dissociation constants. *Current Topics in Medicinal Chemistry*, 3(1), 39–53. doi: 10.2174/1568026033392705 [PubMed: 12577990]
- Flood P, & Krasowski MD (2000). Intravenous anesthetics differentially modulate ligand-gated ion channels. *Anesthesiology*, 92(5), 1418–1425. [PubMed: 10781289]
- Franks NP, & Lieb WR (1994). Molecular and Cellular Mechanisms of General-Anesthesia. *Nature*, 367(6464), 607–614. doi: 10.1038/367607a0 [PubMed: 7509043]
- Hagn F, Etzkorn M, Raschle T, & Wagner G (2013). Optimized Phospholipid Bilayer Nanodiscs Facilitate High-Resolution Structure Determination of Membrane Proteins. *Journal of the American Chemical Society*, 135(5), 1919–1925. doi: 10.1021/ja310901f [PubMed: 23294159]
- Hemmings HC Jr., Akabas MH, Goldstein PA, Trudell JR, Orser BA, & Harrison NL (2005). Emerging molecular mechanisms of general anesthetic action. *Trends Pharmacol Sci*, 26(10), 503–510. doi: 10.1016/j.tips.2005.08.006 [PubMed: 16126282]
- Herold KF, & Hemmings HC Jr. (2012). Sodium channels as targets for volatile anesthetics. *Front Pharmacol*, 3, 50. doi: 10.3389/fphar.2012.00050 [PubMed: 22479247]
- Kalbitzer HR, Rohr G, Nowak E, Goody RS, Kuhn W, & Zimmermann H (1992). A new high sensitivity 19F probe for labeling cysteine groups of proteins. *NMR Biomed*, 5(6), 347–350. [PubMed: 1489670]
- Kay LE (2016). New Views of Functionally Dynamic Proteins by Solution NMR Spectroscopy. *J Mol Biol*, 428(2 Pt A), 323–331. doi: 10.1016/j.jmb.2015.11.028 [PubMed: 26707200]
- Kim HJ, Howell SC, Van Horn WD, Jeon YH, & Sanders CR (2009). Recent Advances in the Application of Solution NMR Spectroscopy to Multi-Span Integral Membrane Proteins. *Prog Nucl Magn Reson Spectrosc*, 55(4), 335–360. doi: 10.1016/j.pnmrs.2009.07.002 [PubMed: 20161395]
- Kinde MN, Bondarenko V, Granata D, Bu W, Grasty KC, Loll PJ, ... Xu Y. (2016). Fluorine-19 NMR and computational quantification of isoflurane binding to the voltage-gated sodium channel NaChBac. *Proc Natl Acad Sci U S A*, 113(48), 13762–13767. doi: 10.1073/pnas.1609939113 [PubMed: 27856739]
- Kinde MN, Bu W, Chen Q, Xu Y, Eckenhoff RG, & Tang P (2016). Common Anesthetic-binding Site for Inhibition of Pentameric Ligand-gated Ion Channels. *Anesthesiology*, 124(3), 664–673. doi: 10.1097/ALN.0000000000001005 [PubMed: 26756520]
- Kinde MN, Chen Q, Lawless MJ, Mowrey DD, Xu J, Saxena S, ... Tang P. (2015). Conformational Changes Underlying Desensitization of the Pentameric Ligand-Gated Ion Channel ELIC. *Structure*, 23(6), 995–1004. doi: 10.1016/j.str.2015.03.017 [PubMed: 25960405]
- Kitevski-LeBlanc JL, & Prosser RS (2012). Current applications of 19F NMR to studies of protein structure and dynamics. *Prog Nucl Magn Reson Spectrosc*, 62, 1–33. doi: 10.1016/j.pnmrs.2011.06.003 [PubMed: 22364614]
- Klein J, Meinecke R, Mayer M, & Meyer B (1999). Detecting binding affinity to immobilized receptor proteins in compound libraries by HR-MAS STD NMR. *Journal of the American Chemical Society*, 121(22), 5336–5337. doi: 10.1021/Ja990706x
- Krause RM, Buisson B, Bertrand S, Corringer PJ, Galzi JL, Changeux JP, & Bertrand D (1998). Ivermectin: a positive allosteric effector of the alpha7 neuronal nicotinic acetylcholine receptor. *Mol Pharmacol*, 53(2), 283–294. [PubMed: 9463487]
- Kucharska I, Edrington TC, Liang BY, & Tamm LK (2015). Optimizing nanodiscs and bicelles for solution NMR studies of two beta-barrel membrane proteins. *Journal of Biomolecular Nmr*, 61(3–4), 261–274. doi: 10.1007/s10858-015-9905-z [PubMed: 25869397]
- Laguette A, Lohr F, Henrich E, Hoffmann B, Abdul-Manan N, Connolly PJ, ... Dotsch V. (2016). From Nanodiscs to Isotropic Bicelles: A Procedure for Solution Nuclear Magnetic Resonance Studies of Detergent-Sensitive Integral Membrane Proteins. *Structure*, 24(10), 1830–1841. doi: 10.1016/j.str.2016.07.017 [PubMed: 27618661]

- Mowrey DD, Kinde MN, Xu Y, & Tang P (2015). Atomistic insights into human Cys-loop receptors by solution NMR. *Biochimica Et Biophysica Acta-Biomembranes*, 1848(1), 307–314. doi: 10.1016/j.bbamem.2014.03.014
- Mowrey DD, Liu Q, Bondarenko V, Chen Q, Seyoum E, Xu Y, ... Tang P. (2013). Insights into distinct modulation of $\alpha 7$ and $\alpha 7\beta 2$ nicotinic acetylcholine receptors by the volatile anesthetic isoflurane. *J Biol Chem*, 288(50), 35793–35800. doi: 10.1074/jbc.M113.508333 [PubMed: 24194515]
- Nury H, Van Renterghem C, Weng Y, Tran A, Baaden M, Dufresne V, ... Corringer PJ. (2011). X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel. *Nature*, 469(7330), 428–431. doi: 10.1038/nature09647 [PubMed: 21248852]
- OuYang B, Xie S, Berardi MJ, Zhao X, Dev J, Yu W, ... Chou JJ. (2013). Unusual architecture of the p7 channel from hepatitis C virus. *Nature*, 498(7455), 521–525. doi: 10.1038/nature12283 [PubMed: 23739335]
- Ouyang W, Jih TY, Zhang TT, Correa AM, & Hemmings HC Jr. (2007). Isoflurane inhibits NaChBac, a prokaryotic voltage-gated sodium channel. *J Pharmacol Exp Ther*, 322(3), 1076–1083. doi: 10.1124/jpet.107.122929 [PubMed: 17569823]
- Oxenoid K, & Chou JJ (2013). The present and future of solution NMR in investigating the structure and dynamics of channels and transporters. *Curr Opin Struct Biol*, 23(4), 547–554. doi: 10.1016/j.sbi.2013.03.010 [PubMed: 23628285]
- Oxenoid K, & Chou JJ (2016). A functional NMR for membrane proteins: dynamics, ligand binding, and allosteric modulation. *Protein Sci*, 25(5), 959–973. doi: 10.1002/pro.2910 [PubMed: 26928605]
- Pan J, Chen Q, Willenbring D, Mowrey D, Kong X-P, Cohen A, ... Tang P. (2012). Structure of the pentameric ligand-gated ion channel GLIC bound with anesthetic ketamine. *Structure*, 20(9), 1463–1469. doi: 10.1016/j.str.2012.08.009 [PubMed: 22958642]
- Pervushin K, Riek R, Wider G, & Wuthrich K (1997). Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. *Proc Natl Acad Sci U S A*, 94(23), 12366–12371. [PubMed: 9356455]
- Piai A, Fu Q, Dev J, & Chou JJ (2017). Optimal Bicelle Size q for Solution NMR Studies of the Protein Transmembrane Partition. *Chemistry*, 23(6), 1361–1367. doi: 10.1002/chem.201604206 [PubMed: 27747952]
- Purcell K, Gingrich KJ, Ouyang W, Herold KF, & Hemmings HC (2015). Activity-dependent depression of neuronal sodium channels by the general anaesthetic isoflurane. *British Journal of Anaesthesia*, 115(1), 112–121. doi: 10.1093/bja/aev203 [PubMed: 26089447]
- Raju SG, Barber AF, LeBard DN, Klein ML, & Carnevale V (2013). Exploring volatile general anesthetic binding to a closed membrane-bound bacterial voltage-gated sodium channel via computation. *PLoS Comput Biol*, 9(6), e1003090. doi: 10.1371/journal.pcbi.1003090 [PubMed: 23785267]
- Rosenzweig R, & Kay LE (2016). Solution NMR Spectroscopy Provides an Avenue for the Study of Functionally Dynamic Molecular Machines: The Example of Protein Disaggregation. *J Am Chem Soc*, 138(5), 1466–1477. doi: 10.1021/jacs.5b11346 [PubMed: 26651836]
- Sanders CR, & Mittagdorff KF (2011). Tolerance to changes in membrane lipid composition as a selected trait of membrane proteins. *Biochemistry*, 50(37), 7858–7867. doi: 10.1021/bi2011527 [PubMed: 21848311]
- Sauguet L, Poitevin F, Murail S, Van Renterghem C, Moraga-Cid G, Malherbe L, ... Delarue M. (2013). Structural basis for ion permeation mechanism in pentameric ligand-gated ion channels. *EMBO J*, 32(5), 728–741. doi: 10.1038/emboj.2013.17 [PubMed: 23403925]
- Schnell JR, & Chou JJ (2008). Structure and mechanism of the M2 proton channel of influenza A virus. *Nature*, 451(7178), 591–595. doi: 10.1038/nature06531 [PubMed: 18235503]
- Sharaf NG, & Gronenborn AM (2015). (19)F-modified proteins and (19)F-containing ligands as tools in solution NMR studies of protein interactions. *Methods Enzymol*, 565, 67–95. doi: 10.1016/bs.mie.2015.05.014 [PubMed: 26577728]

- Sorge JL, Wagstaff JL, Rowe ML, Williamson RA, & Howard MJ (2015). Q2DSTD NMR deciphers epitope-mapping variability for peptide recognition of integrin α v β 6. *Org Biomol Chem*, 13(29), 8001–8007. doi: 10.1039/c5ob01237f [PubMed: 26119198]
- Spurny R, Billen B, Howard RJ, Brams M, Debaveye S, Price KL, ... Ulens C. (2013). Multisite binding of a general anesthetic to the prokaryotic pentameric *Erwinia chrysanthemi* ligand-gated ion channel (ELIC). *J Biol Chem*, 288(12), 8355–8364. doi: 10.1074/jbc.M112.424507 [PubMed: 23364792]
- Tang P, Eckenhoﬀ RG, & Xu Y (2000). General anesthetic binding to gramicidin A: the structural requirements. *Biophys J*, 78(4), 1804–1809. doi: 10.1016/S0006-3495(00)76730-0 [PubMed: 10733961]
- Tang P, Hu J, Liachenko S, & Xu Y (1999). Distinctly different interactions of anesthetic and nonimmobilizer with transmembrane channel peptides. *Biophys J*, 77(2), 739–746. doi: 10.1016/S0006-3495(99)76928-6 [PubMed: 10423422]
- Tang P, Mandal PK, & Xu Y (2002). NMR structures of the second transmembrane domain of the human glycine receptor α (1) subunit: model of pore architecture and channel gating. *Biophys J*, 83(1), 252–262. doi: 10.1016/S0006-3495(02)75166-7 [PubMed: 12080117]
- Vogtherr M, & Peters T (2000). Application of NMR based binding assays to identify key hydroxy groups for intermolecular recognition. *Journal of the American Chemical Society*, 122(25), 6093–6099. doi: 10.1021/ja0001916
- Wagstaff JL, Vallath S, Marshall JF, Williamson RA, & Howard MJ (2010). Two-dimensional heteronuclear saturation transfer diﬀerence NMR reveals detailed integrin α v β 6 protein-peptide interactions. *Chem Commun (Camb)*, 46(40), 7533–7535. doi: 10.1039/c0cc01846e [PubMed: 20838674]
- Xu Y, Seto T, Tang P, & Firestone L (2000). NMR study of volatile anesthetic binding to nicotinic acetylcholine receptors. *Biophys J*, 78(2), 746–751. doi: 10.1016/S0006-3495(00)76632-X [PubMed: 10653787]
- Xu Y, Tang P, Firestone L, & Zhang TT (1996). ¹⁹F nuclear magnetic resonance investigation of stereoselective binding of isoflurane to bovine serum albumin. *Biophys J*, 70(1), 532–538. doi: 10.1016/S0006-3495(96)79599-1 [PubMed: 8770230]
- Yamakura T, Chavez-Noriega LE, & Harris RA (2000). Subunit-dependent inhibition of human neuronal nicotinic acetylcholine receptors and other ligand-gated ion channels by dissociative anesthetics ketamine and dizocilpine. *Anesthesiology*, 92(4), 1144–1153. [PubMed: 10754635]
- Ye L, Van Eps N, Zimmer M, Ernst OP, & Prosser RS (2016). Activation of the A2A adenosine G-protein-coupled receptor by conformational selection. *Nature*, 533(7602), 265–268. doi: 10.1038/nature17668 [PubMed: 27144352]
- Zhuang T, Chen Q, Cho MK, Vishnivetskiy SA, Iverson TM, Gurevich VV, & Sanders CR (2013). Involvement of distinct arrestin-1 elements in binding to different functional forms of rhodopsin. *Proc Natl Acad Sci U S A*, 110(3), 942–947. doi: 10.1073/pnas.1215176110 [PubMed: 23277586]