Supporting Information

Fluorine-19 NMR and computational quantification of isoflurane binding to the voltage-gated sodium channel NaChBac

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SI Methods

Protein and NMR Sample Preparation. The protein samples were purified using Ni-NTA columns (GE Healthcare) followed by size exclusion chromatography (Superdex 75 10/300GL, GE Healthcare) to ensure the tetrameric assembly of NaChBac and the complete removal of free 2,2,2-trifluoroethanethiol (TET). The samples prepared for NMR experiments typically contained 40-100 μM NaChBac, 1-2% DDM, 20 mM Tris at pH 7.7, 250 mM NaCl, and 5% D_2O for deuterium lock in NMR measurements. The anesthetic isoflurane in a pre-saturated solution was added to each NMR sample up to 10-fold of the protein concentration. The isoflurane concentrations of up to 0.5 mM and 1 mM were used in the NaChBac samples for the ^{19}F and ^{1}H STD NMR experiments, respectively. These concentrations are comparable to the anesthetizing concentration of isoflurane in the blood. The isoflurane concentration in each NMR sample was determined experimentally by the ^{19}F NMR signal intensity calibrated against a 100-μM trifluoroacetic acid standard.

NMR Data Acquisition and Analysis. All NMR spectra were acquired at 10ºC on Bruker Avance spectrometers (Bruker Instruments, Billerica, MA) equipped with either a triple-resonance inverse-detection cryoprobe (^{1}H frequency: 600.23 MHz) or a triple-resonance, ^{19}F-detection cryoprobe (^{1}H frequency: 600.13 MHz and ^{19}F frequency: 564.68 MHz). ^{1}H STD spectra had a spectral window of 16 ppm, 16384 data points, a constant recycle delay of 12 s, and 64 averages. ^{19}F STD spectra had 8192 data points, a constant recycle delay of 5 s compensating for different saturation times, and 1600 averages. The NMR spectra were processed and analyzed using TOPSPIN™ (Bruker Instruments, Billerica, MA) and MestReNova v8.0.1-10878 (Mestrelab Research S.L.). For STD data, each NMR spectrum was zero-filled to 32768 data points and processed with an exponential function. A line-broadening factor of 3 Hz or 30 Hz was applied to the ^{1}H or ^{19}F STD spectra, respectively.

Molecular Dynamics Simulations. The simulation systems contained a total of ~122,000 atoms, including a NaChBac tetramer, 434 lipid molecules (POPC), 25,310 water molecules, 236 ions (Cl and Na), and 12 isoflurane molecules. The systems were equilibrated through three consecutive stages of 500 ps each, in which position restraints on different groups were progressively released. CHARMM36 force field was used for the phospholipids and CHARMM27 for the protein (1, 2). The parameters for the ^{19}F probe were obtained using CGenFF webserver (3). Isoflurane parameters were obtained from a previous publication (4). Periodic boundary conditions were employed and the electrostatic potential was evaluated using the particle-mesh Ewald method. The lengths of all bonds containing hydrogen were constrained with the SHAKE/RATTLE algorithm. Each system was maintained at a temperature of 300 K and pressure of 1 atm using the Langevin thermostat and barostat (NPT) methods as implemented in NAMD2.10. The rRESPA multiple time step method was employed, with a high frequency time step of 2 fs and a low frequency time step of 4 fs. Four independent MD simulations (two for each system) were performed, and each simulation lasted for ~250 ns.

Functional Studies with NaChBac Mutants. Oocytes were maintained at 15°C in modified Barth's solution containing (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 15 HEPES, 0.3 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, and 10 µg/ml sodium penicillin, 10 µg/ml streptomycin sulfate, 100 µg/ml gentamycin sulfate, pH 6.7. Two-electrode voltage clamp electrophysiology experiments were performed at room temperature 24–72 hrs after injection. The running buffer contained (in mM): 130 NaCl, 10 $CaCl_2$, 5 KCl, 20 HEPES, 10 glucose, pH 7.4. An OC-725C Amplifier (Warner Instruments) was used to determine the voltage-dependent channel activation and the inhibition by isoflurane. Oocytes were held at −100 mV and stepped in increments of 10 mV to a maximum of 40 mV. Isoflurane in the running buffer was prepared from a saturated isoflurane solution (~13.5 mM). Whole-cell currents were recorded and processed using Clampex 10 software (Molecular Devices).

References

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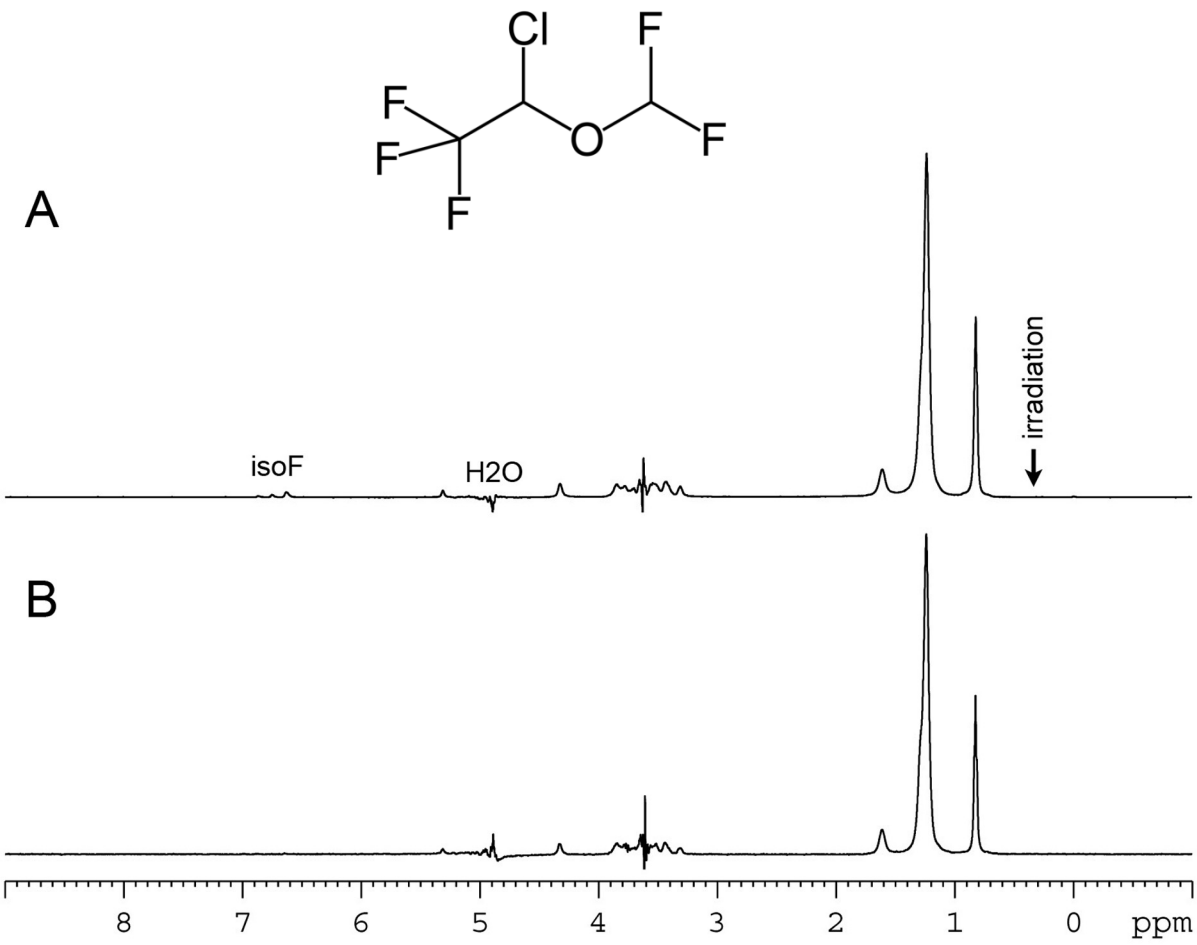

Fig. S1. ^{1}H Saturation Transfer Difference (STD) Spectra. STD spectra from (*A*) the sample containing 50 μM TET-labeled NaChBac S129C and 1.1 mM isoflurane; and (*B***)** the control sample, identical to the sample in (A) but without protein. A 10 s saturation time and a 12 s relaxation delay were used. The isoflurane peaks (isoF) appeared only in (A) but not in (B). The results confirm that the observed isoflurane signals in (A) result from direct anesthetic interactions with the protein.

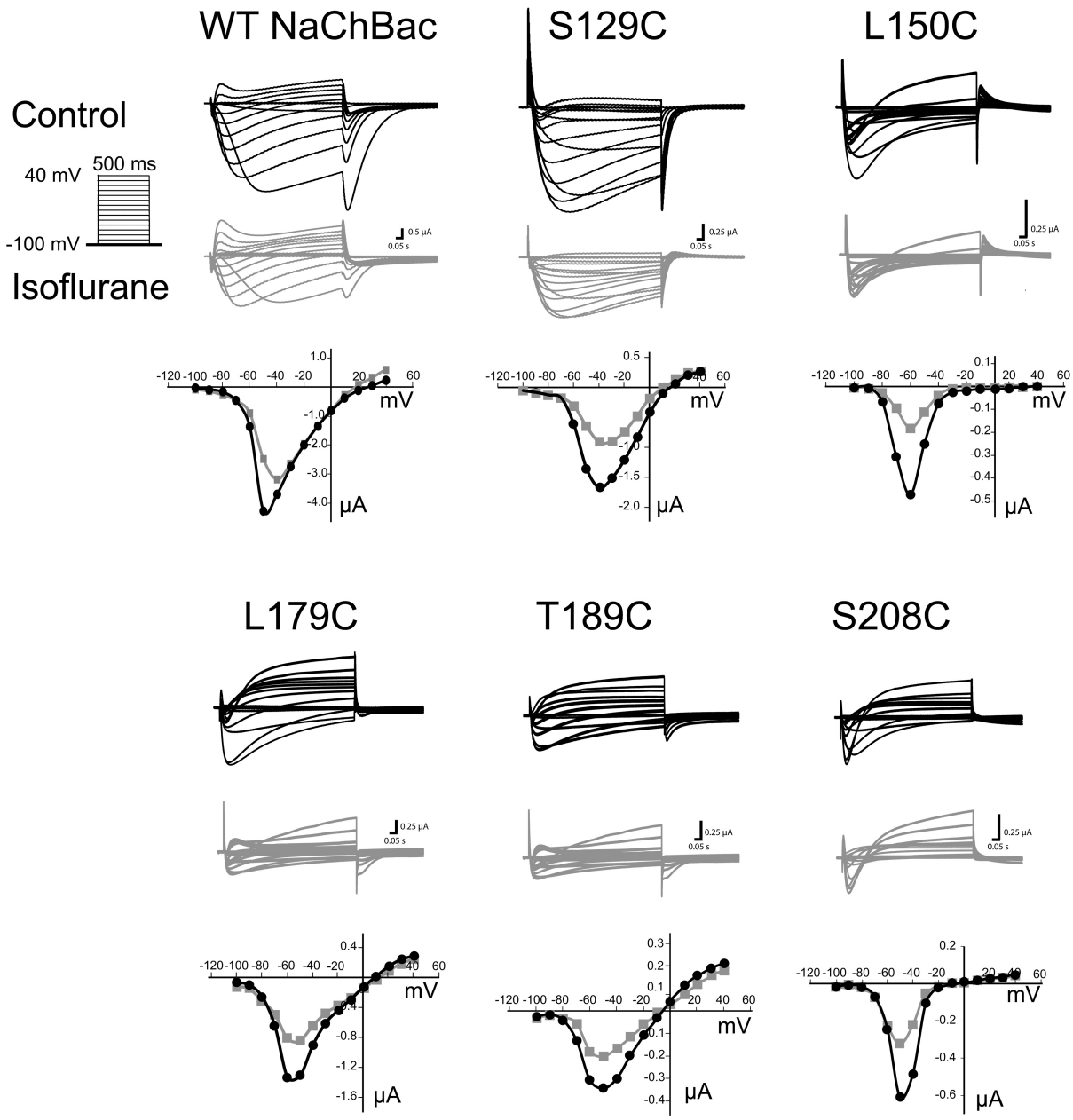

Fig. S2. Electrophysiology characterization of the wild-type NaChBac and various cysteine mutants expressed in *Xenopus* oocytes. Representative current traces of two-electrode voltage-clamp measurements of oocytes expressing the wild type (WT) NaChBac or the cysteine mutants used in the NMR studies, in the absence (black traces) and presence (gray traces) of isoflurane (400 µM, ~IC_{50}) using the protocol in *inset*. Oocytes were held at −100 mV and stepped in increments of 10 mV to a maximum of 40 mV. Also plotted are the peak current-voltage (*I-V*) relations for the respective currents measured at different voltages. Similar to the WT NaChBac, the mutants begin to activate at approximately −70 mV and reach a peak at approximately −50 mV or −40 mV. Isoflurane inhibits peak currents of the WT NaChBac as well as the mutants.

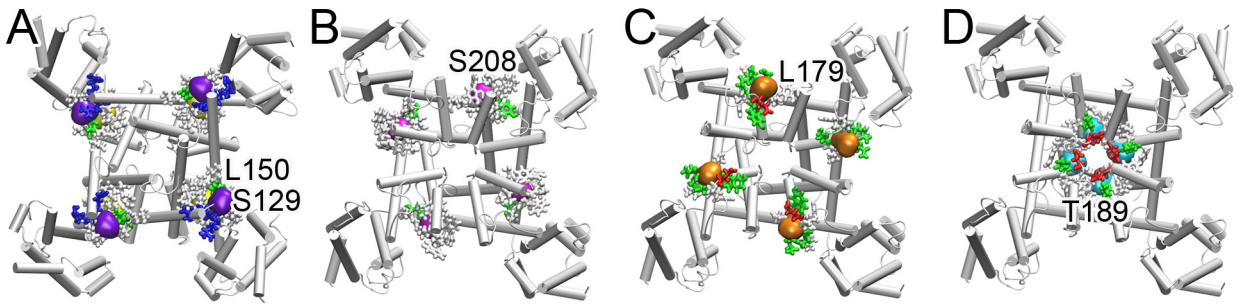

Fig. S3. Isoflurane binding environment at various sites in NaChBac. Residues within 6 Å of C_β of ^{19}F-labeled residues are presented in licorice and colored by the residue type: white-hydrophobic; green-hydrophilic; red-negatively charged; blue-positively charged. The ^{19}F-labeled residues are shown in colored surfaces. (*A*) The linker region, including S129 (purple) and L150 (yellow), viewed from the intracellular side. (*B*) S208 (magenta) in the extracellular surface. (*C*) L179 (orange) in the extracellular surface. (*D*) T189 (cyan) in the selective filter region. Hydrophobic residues dominate the majority of these sides: 69% in (A), 83% in (B), 72% in (D), but only 47% in (C).

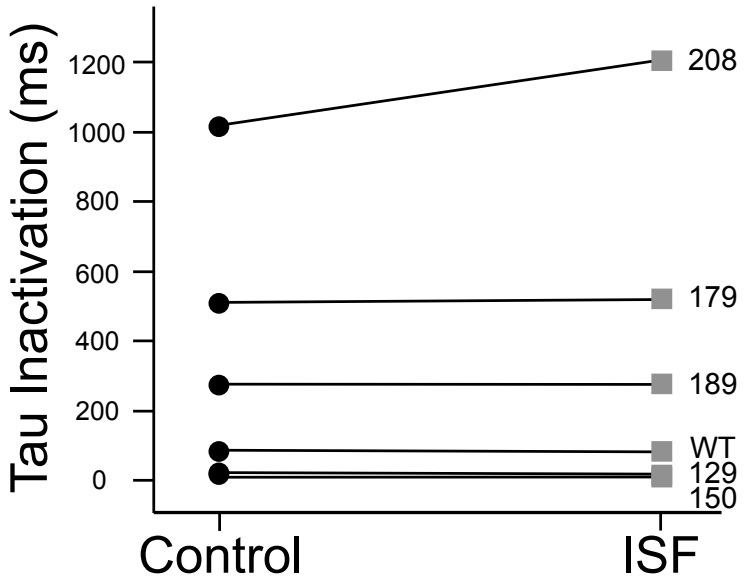

Fig. S4. Isoflurane effects on NaChBac channel inactivation kinetics. Plotted here are the inactivation time constants (Tau) of the wild-type (WT) NaChBac and various mutants used this study in the absence (●) and presence (■) of 400 μM isoflurane (ISF). Unlike NaChBac channels expressed in the human embryonic kidney 293 cells, the inactivation kinetics of NaChBac expressed in oocytes is relatively insensitive to isoflurane except S208C.

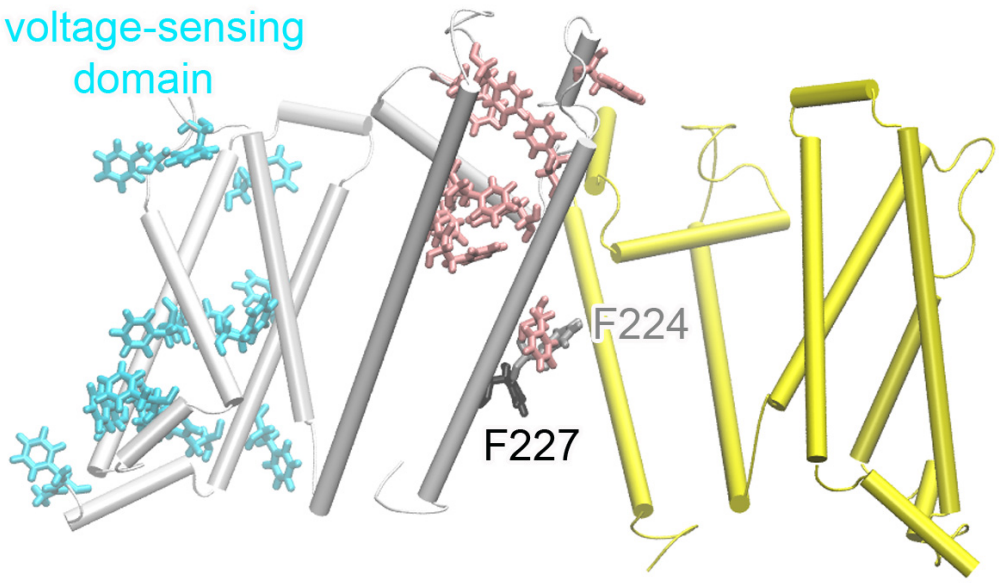

Fig. S5. Locations of phenylalanine residues in each subunit of NaChBac. The voltage-sensing domain is a phenylalanine-rich region and contains 13 phenylalanine residues (shown in cyan color). Two phenylalanine residues in the pore, F227 and F224, are labeled. Others are colored in pink. There are a total of 25 phenylalanine residues in each subunit. For clarity, only two subunits are shown.