Mechanistic insights into volatile anesthetic modulation of K2P channels

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Abstract

K2P potassium channels are known to be modulated by volatile anesthetic (VA) drugs and play important roles in clinically relevant effects that accompany general anesthesia. Here, we utilize a photoaﬃnity analog of the VA isoflurane to identify a VA binding site in the TREK1 K2P channel. The functional importance of the identified site was validated by mutagenesis and biochemical modification. Molecular dynamic simulations of TREK1 in the presence of VA found multiple neighboring residues on TREK1 TM2, TM3 and TM4 that contribute to anesthetic binding. The identified VA binding region contains residues that play roles in the mechanisms by which heat, mechanical stretch, and pharmacological modulators alter TREK1 channel activity and overlaps with positions found to modulate TASK K2P channel VA sensitivity. Our findings define molecular contacts that mediate VA binding to TREK1 channels and suggest a mechanistic basis to explain how K2P channels are modulated by VAs.

Introduction

Tandem pore (K2P) potassium channels are a group of physiologically important K⁺ leak channels that modulate cellular resting membrane potential to control excitability (Enyedi and Czirjak 2010). Human K2P channel abnormalities cause a wide range of physiological syndromes, including cardiac conduction abnormalities that lead to ventricular tachycardia and fibrillation (Gurney and Manoury 2009, Friedrich et al. 2014, Decher et al. 2017), familial pulmonary arterial hypertension (Ma et al. 2013), salt-sensitive hyperaldosteronism (Davies et al. 2008), and familial migraines (Lafreniere et al. 2010, Royal et al. 2019), underscoring roles for K2P channels in human cardiac, vascular, renal, and pain physiology. K2Ps are proven contributors to the potency of volatile anesthetics (VAs), with K2P knockout animals exhibiting a measurable resistance to VA induced loss of consciousness (Heurteaux et al. 2004, Pang et al. 2009, Steinberg et al. 2015). Systemic effects of VA administration have been directly linked to K2P channel activity, including a role for carotid body TASK channels in mediating VA induced respiratory depression (Cotten 2013, Chokshi et al. 2015) and for TREK1 channels in the vasodilatory and neuroprotective effects of both polyunsaturated fatty acids and VAs (Blondeau et al. 2007, Tong et al. 2014).

Initial evidence for a VA induced potassium leak current was found in molluscan pacemaker neurons, an activity subsequently determined to be mediated by a member of the TASK K2P subfamily (Franks and Lieb 1988, Andres-Enguix et al. 2007). Follow-up studies of both snail and human TASK channels identified specific amino acids that alter TASK responsiveness to VAs or eliminate stereoselective discrimination between enantiomeric VA agents (Andres-Enguix et al. 2007, Conway and Cotten 2012, Luethy et al. 2017). These findings enabled predictions of structural determinants of anesthetic binding within the TASK channel family (Bertaccini et al. 2014). However, significant differences in the gating and regulation of divergent K2P subfamilies make it difficult to generalize molecular details of the interactions between VAs and TASK K2P channels to other anesthetic sensitive K2Ps (Patel et al. 1999), most notably the TREK1 channel.

TREK1 is a member of a subgroup of K2Ps (including TREK1, TREK2, and TRAAK) regulated by the biophysical properties of their surroundings, including membrane stretch or deformation, temperature, and bilayer lipid composition (Honore 2007). This pronounced sensitivity of TREK1 to its surrounding environment is unique amongst known biologically

relevant VA targets. VAs are small hydrophobic drugs that readily partition into lipid bilayers and *in-vivo* anesthetic potency has long been known to correlate with lipid solubility, a relationship that holds true over a wide range of structurally diverse anesthetic agents (Sonner and Cantor 2013). While the majority of studies exploring the interaction between anesthetics and ion channels have identified discrete binding sites thought to underlie the modulatory effects of these drugs (Nury et al. 2011, Sauguet et al. 2013, Hemmings et al. 2019), modulation of TREK1 by VAs could be posited to occur via indirect effects on the properties of the surrounding bilayer. In fact, a recent study has proposed that *in vitro* administration of VA agents disrupt membrane lipid rafts, altering phospholipase C activity and modifying the milieu of lipids surrounding TREK1 to modulate its activity (Pavel et al. 2019).

The question of how VA drugs modulate TREK1 is the key issue addressed in this study. By utilizing an unbiased photolabeling approach, we show that isoflurane, a volatile anesthetic, binds to a TREK1 TM2 residue in close proximity to the previously proposed VA modulatory site in TASK K2P channels. MD simulation studies based on our photolabeling findings show that isoflurane, a known low affinity ligand for TREK1, is mobile within this newly identified TREK1 binding site and interacts with residues from TM2, TM3, and TM4. These interactions include key molecular contacts with residues known to influence the conformational rearrangement of the TM4 helix that drive K2P modulation by heat, mechanical stretch, and other pharmacological channel modulators (Dong et al. 2015, Lolicato et al. 2014, Brohawn, Campbell and MacKinnon 2014a, Pope et al. 2018). Our photolabeling and MD results enabled us to design multiple point mutations within the putative VA binding pocket that specifically diminished TREK1 VA responsiveness without otherwise perturbing channel function. Our data support the notion of a shared VA modulatory site across the TREK and TASK K2P subfamilies and suggest a mechanism by which binding of VA within this site promotes conformational rearrangements known to gate K2P channels.

Experimental Methods

Purification of drTREK1 or hTRAAK

TREK1 and TRAAK proteins were expressed in *Pichia pastoris* using previously described pPICZ vectors bearing residues 1–300 of the human TRAAK gene (K2P4.1, UniProt Q9NYG8-2) or residues 1–322 of the *D. rerio* TREK1 gene (K2P2.1, UniProt X1WC65), followed by a PreScission protease-cleavage site (LEVLFQ/GP) and C-terminal GFP and His₁₀ tags (Brohawn, Su and MacKinnon 2014b). Mutations to eliminate N-linked glycosylation sites were

inserted into both the human TRAAK (N104Q and N108Q) and zebrafish TREK1 (N95Q, N122Q) gene constructs. Expression plasmids were linearized with the PmeI restriction enzyme and were subsequently transformed by electroporation into *P. pastoris* strain SMD1168H. Screening for successful recombinant integration was performed by plating transformants on yeast extract peptone dextrose sorbitol (YPDS) plates containing increasing concentrations of zeocin from 0.5 to 3 mg/ml.

Screening, expression, and purification of K2Ps was performed as previously described (Lolicato et al. 2014). Briefly, yeast transformants were grown in buffered minimal medium (2 × YNB, 1% glycerol, 0.4 mg L⁻¹ biotin, 100 mM potassium phosphate [pH 6.0]) for 2 days at 30°C in a shaker at 225 rpm. Cells were then pelleted by centrifugation (4,000 × g at 20°C, 5 min) and resuspended in methanol minimal medium (2 × YNB, 0.5% methanol, 0.4 mg L⁻¹ biotin, 100 mM potassium phosphate [pH 6.0]) to induce protein expression. Cells were then shaken for 2 additional days at 22°C in a shaker at 225 rpm, with additional methanol added (final concentration 0.5% [v/v]) to the culture after 24 hours of protein expression. After 48 hours of protein expression, cells were pelleted by centrifugation (6,000 × g at 4°C for 10 min), flash frozen in liquid nitrogen, and then subjected to three rounds of cryo-milling (Retsch model MM301) in liquid N₂ for 3 min at 25 Hz to disrupt yeast cell walls and membranes. Frozen yeast cell powder was then stored at -80°C.

Cell powder was added to breaking buffer (150 mM KCl, 50 mM Tris pH 8.0, 1 mM phenylmethanesulfonyl fluoride, 0.1 mg/mL DNase 1, and 1 tablet/50 ml of EDTA-free complete inhibitor cocktail [Roche]) at a ratio of 1 g cell pellet/4 mL lysis buffer. Solubilized cell powder was centrifuged at 4,000 × g at 4°C for 5 min to pellet large debris and the supernatant was then centrifuged at 100,000 × g at 4°C for 1.5 hours to pellet cell membranes. The pellet was re-suspended in 50 ml breaking buffer containing 60mM n-Dodecyl-B-D-Maltoside (DDM) and incubated for 3 hours with gentle stirring to solubilize the membranes, followed by centrifugation at 35,000 × g for 45 min. Talon cobalt resin (Takara Bio USA) was added to the supernatant at a ratio of 1 ml of resin per 10 g of cell powder and incubated in an orbital rotor overnight at 4°C. Resin was then collected on a column and washed with 10 column volumes of Buffer A (150 mM KCl, 50 mM Tris pH 8.0, 6 mM DDM, 30 mM imidazole) and bound protein was subsequently eluted from the resin by washing with Buffer B (150 mM KCl, 50 mM Tris pH 8.0, 6 mM DDM, 300 mM imidazole). PreScission protease (~1:25 wt:wt) was added to the eluate and the cleavage reaction was allowed to proceed overnight at 4°C under gentle rocking. Cleaved TREK1

or TRAAK protein was concentrated in 50 kDa molecular weight cutoff (MWCO) Amicon Ultra Centrifugal Filters (Millipore) and applied to a Superdex200 10/300 gel filtration column (GE Healthcare) equilibrated in size exclusion chromatography (SEC) buffer (150 mM KCl, 20 mM Tris pH 8.0, 1mM DDM). Purified *h*TRAAK or *dr*TREK1 protein was concentrated (50 kDa MWCO) to 8 mg/mL prior to reconstitution and analyzed for purity by SDS-PAGE [12% (wt/vol) gels; Bio-Rad] followed by staining with coomassie blue. All protein purification steps were carried out at 4 °C.

Reconstitution of K2P channels into liposomes

Immediately following purification, *dr*TREK1 or *h*TRAAK channel protein was reconstituted into liposomes (Heginbotham, Kolmakova-Partensky and Miller 1998). 5mg of a 3:1 (wt/wt) ratio of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho- (1'-rac-glycerol) (POPG) in chloroform was used for reconstitution. The lipids were dried in a borosilicate glass vial under nitrogen flow and then solubilized in reconstitution buffer (400 mM KCl, 10 mM HEPES, 5 mM NMDG [N-methylglucamine D-gluconate], pH 7.6) containing 34 mM CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate). The lipid containing solution was bath sonicated until clear. *dr*TREK1 or *h*TRAAK protein was then added to the lipid solution at a concentration of 100 µg protein per mg of solubilized lipids. Proteoliposomes were formed by applying the lipid/protein containing sample (500 µl total volume) to an 18 ml detergent-removal column (Sephadex G-50 fine beads, GE Healthcare Life Sciences). Turbid fractions containing proteoliposomes were pooled, aliquoted, flash frozen in liquid nitrogen, and stored at -80°C.

Photoaffinity labeling of *dr*TREK1 or *h*TRAAK potassium channels for protein microsequencing.

5-7 µg of *dr*TREK or *h*TRAAK in proteoliposomes was added to a 30 µM solution of azidoisoflurane ± 3 mM isoflurane. Each sample was equilibrated on ice in the dark for 5 min and then transferred to a 1 mm path length quartz cuvette and exposed for 25 min to 300 nm ultraviolet light produced by an RPR-3000 Rayonet lamp filtered by a WG295 295nm glass filter (Newport Corporation).

In-Solution Protein Digestion.

Photolabeled samples underwent dialysis and buffer exchange using 10 kDa MWCO Amicon Ultra Centrifugal Filters (Millipore). ProteaseMAX™ Surfactant (Promega) was added to a concentration of 0.2% and the samples were vortexed vigorously for 30 sec. Samples were

then diluted with NH_4HCO_3 to a final concentration of 50 mM NH_4HCO_3 in a 93.5 μL volume. 1 μL of 0.5 M dithiothreitol (DTT) was then added and samples were incubated at 56 °C for 30 min. 2.7 μL of 0.55 M iodoacetamide (IAA) was subsequently added and protein samples were incubated at room temperature in the dark for 45 min. An additional 1 μL of 1% (w/v%) ProteaseMax™ Surfactant was added to the sample, followed by sequencing grade-modified trypsin (Promega) to a 1:20 protease:protein final ratio (w:w). Proteins were digested overnight at 37°C. Trypsin digested peptides were again diluted with NH_4HCO_3 to a final concentration of 100 mM NH_4HCO_3 and 0.02% ProteaseMAX Surfactant in a 200 μL total volume, prior to addition of sequencing grade chymotrypsin (Promega) to a final 1:20 protease:protein ratio (w:w). Proteins were again digested overnight at 37°C. Acetic acid was added to reach a pH < 2 and the peptide digests were then incubated at room temperature for 10 min, prior to centrifugation at 16, 000 x g for 20 min to remove insoluble debris. Samples were then desalted using C18 stage tips prepared in house, dried by speed-vac, and resuspended in 0.1% formic acid immediately prior to mass spectrometry analysis.

In-Gel Protein Digestion.

Photolabeled samples underwent dialysis and buffer exchange using 10 kDa MWCO Amicon Ultra Centrifugal Filters (Millipore). Samples were then mixed with SDS loading buffer containing DTT to a final concentration of 100 mM DTT, vortexed vigorously and incubated at room temperature for 45 min before separation by SDS-PAGE. The resulting gels were stained with Coomassie Blue G250 (BioRad), destained, and washed with ddH₂O. Protein bands between ~30-40kDa (corresponding to *drTREK* or *hTRAAK*) were identified and excised. Excised bands were destained, dehydrated and dried by speed vac. Proteins were then reduced by incubation at 56° C for 30 min in 5 mM DTT and 50 mM NH_4HCO_3 . The DTT solution was removed and proteins were then alkylated by the addition of 55 mM IAA in 50 mM NH_4HCO_3 and incubation at room temperature for 45 min in the dark. Bands were dehydrated and dried by speed vac before resuspension in 100 μL 0.2 % ProteaseMAX™ surfactant and 50 mM NH_4HCO_3 solution containing trypsin at a 1:20 protease:protein ratio (w:w). Proteins were digested overnight at 37°C. To enhance sequence coverage, a second protease digestion was performed with chymotrypsin. Samples were diluted to a final volume of 200 μL with final concentrations of 100 mM NH_4HCO_3 and approximately 0.02% ProteaseMAX™ Surfactant. Sequencing grade chymotrypsin (Promega) to a 1:20 protease:protein ratio (w:w) was then added and proteins were digested overnight at 37 °C. Multiple extraction steps were performed to remove peptides embedded in the gel. The digest solution was removed and the remaining gel was suspended in

100 μ L 30% acetonitrile and 5% acetic acid in ddH₂O (v/v%) and sonicated for 20 min. The solution was removed and the gel was then resuspended in 100 μ L 70% acetonitrile and 5% acetic acid in ddH₂O (v/v%) and sonicated for 20min. The digest and two extraction solutions were pooled and dried by speed vac before resuspension in 0.5% acetic acid (pH < 2). Samples were sonicated for 10 min prior to centrifugation to remove insoluble debris. Samples were desalted using C18 stage tips prepared in house. Samples were dried by speed vac and resuspended in 0.1% formic acid immediately before mass spectrometry analysis.

Mass spectrometry.

Desalted peptides were analyzed employing either an Orbitrap Elite™ Hybrid Ion Trap-Orbitrap mass spectrometer (MS) or Q Exactive™ Hybrid Quadrupole-Orbitrap MS coupled to an Easy-nanoLC 1000 system. In both instances the same liquid chromatography procedure and data dependent acquisition mode was applied. Peptides were eluted over 100 min with linear gradients of ACN in 0.1% formic acid in water (v/v%) starting from 2% to 40% (85 min), then 40% to 85% (5 min) and finally 85% (10 min) using a flow rate of 300mL/min. For Orbitrap Elite™, in every 3 s cycle, one full MS scan was collected at a scan range of 350 to 1500 m/z, a resolution of 60K, and a maximum injection time of 50 ms with automatic gain (AG) control of 500000. The MS2 scans were followed from the most intense parent ions. Ions were filtered with charge 2-5 with an isolation window of 1.5 m/z in quadrupole isolation mode. Ions were fragmented using collision induced dissociation (CID) with collision energy of 35%. Iontrap detection was used with normal scan range mode and rapid iontrap scan rate. For Q Exactive™, one full MS was performed with 70K resolution, maximum injection time of 100 ms and scan range of 350-1200 m/z. The MS2 scans were performed in Higher-energy Collisional Dissociation (HCD) with normalized collision energy (NCE) of 30, and isolation window of 3 m/z. The AG control was set to be 10000 with a maximal injection time of 100 ms.

Mass spectrometry analysis.

Analysis was performed as previously reported (Eckenhoff et al., 2010; Woll et al., 2017). Spectral analysis was conducted using Thermo Proteome Discoverer 2.0 (Thermo Scientific) and the Mascot Daemon search engine with a customized database containing *drTREK* or *hTRAAK* protein sequences. All analyses included dynamic oxidation of methionine (+15.9949 m/z) as well as static alkylation of cysteine (+57.0215 m/z; iodoacetamide alkylation). Photolabeled samples were run with the additional dynamic azi-isoflurane (+195.97143 m/z) modification. A mass variation tolerance of 10 ppm and 20 ppm for MS and 0.8 and 0.02 Da for MS/MS were

used for the for Orbitrap Elite™ and Q Exactive™ respectively. Both the in-solution and in-gel sequential trypsin/chymotrypsin digests were searched without enzyme specification with a false discovery rate of 0.01. All MS experiments were conducted in triplicate and samples containing no photoaffinity ligand were processed and analyzed equivalently to those containing the photolabel, to control for false positive detection of photoaffinity ligand modifications.

Molecular Dynamics Simulation

The crystallographically derived structural model of *Mus musculus* TREK1 (PDBID 6CQ6) was used for MD simulation. Missing nonterminal loops were predicted using MODELLER (Webb and Sali 2017) with side chain conformations predicted using SCRWL4 (Krivov, Shapovalov and Dunbrack 2009). Molecular mechanics parameters for isoflurane were used, as previously published (Henin et al. 2010). In the ligand-bound simulation, one isoflurane molecule was placed manually, adjacent to G182. The receptor was embedded in a POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine):cholesterol 70:30 lipid bilayer and the system was solvated with TIP3P water with 0.15 M NaCl using the CHARMM-GUI service (Jo et al. 2008, Jo et al. 2009). The CHARMM36 force field (Klauda et al. 2010, Best et al. 2012) and NAMD 2.12 simulation software (Phillips et al. 2005) were used. Periodic boundary conditions with particle mesh Ewald summation of long-range electrostatics was used. The system was equilibrated according to the CHARMM-GUI protocol and production equilibrium MD simulation was run for 10 nanoseconds using the isothermic-isobaric ensemble with Langevin thermostat and barostat at 303.15 K. Further equilibrium MD simulations were continued from that point.

Two electrode voltage clamp electrophysiology

For electrophysiological studies, *Xenopus laevis* oocytes were microinjected with capped RNA translated from full length mouse TREK1 (K2P2.1, UniProt P97438-2) or mouse TRAAK (K2P4.1, UniProt O88454) genes. TREK1 mutations were introduced using a Phusion site directed mutagenesis kit (ThermoFisher), with the TREK1 C93S C159S C219S C365S C399S mutant channel referred to as TREK1 cys- for brevity. cRNA from WT or mutant K2P genes was synthesized using an mMessage mMachine Kit (T7 promoter, Ambion, Life Technologies) and purified using an RNeasy RNA cleanup kit (Qiagen). Defolliculated stage V–VI *Xenopus laevis* oocytes were purchased commercially from Xenocyte (Dexter, MI) and were stored in antibiotic supplemented ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 2 mM MgCl₂, 100 units mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin) until use. Oocytes were microinjected with 2.5 ng cRNA (unless

otherwise noted) and two-electrode voltage clamp (TEVC) recordings were performed 24 to 48 hours after microinjection.

For recordings, oocytes were impaled with borosilicate recording microelectrodes (0.3–2.0 MΩ resistance) backfilled with 3 M KCl. Oocytes were perfused with ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, and 2.0 mM MgCl₂, 5 mM HEPES [pH 7.4]) at a rate of 2.5 ml/min. Currents were evoked from a –90 mV holding potential by a 2000 ms ramp from –150 mV to +50 mV. Data were acquired using an OC-725C oocyte clamp amplifier (Warner Instruments) controlled by pClamp software (Molecular Devices), and digitized at 2 kHz using a Digidata 1332A digitizer (MDS Analytical Technologies).

For temperature experiments, recording solutions were heated by an SC-20 in-line heater/cooler combined with an LCS-1 liquid cooling system operated by the CL-100 bipolar temperature controller (Warner Instruments). Temperature was monitored using a CL-100-controlled thermistor placed in the bath solution immediately downstream of the oocyte. The perfusate was warmed from 15°C to 35°C in 5°C increments, with recordings performed once temperature readings stabilized at the desired values. For external pH experiments (pH_o), each oocyte was initially perfused with ND96 solution at pH 7.4, switched to ND96 at pH 9.0 and then subsequently assayed at the experimental pH, with recordings performed 15 s after each new solution was applied. This allowed for all pH_o dependent changes to be normalized against the current density at pH 9.0. The buffer present in ND96 solutions was altered as appropriate, with one of the following: 10 mM TRIS (pH 9.0 - 8.1), 5 mM HEPES (pH 8.1-6.5) or 5 mM MES (pH 6.5 - 5.9). Aggregate pH_o data were fitted with a modified Hill equation: $I = I_{\min} + (I_{\max} - I_{\min}) / (1 + 10^{((\text{pH}_o \text{IC}_{50} / \text{pH}_o) * H))}$, where $I_{\max}$ and $I_{\min}$ are maximal and minimal current values, respectively, pH_oIC₅₀ is a half-maximal effective pH_o value, and H is the Hill coefficient.

For volatile anesthetic experiments, isoflurane saturated ND96 was prepared by adding commercially available isoflurane (Baxter Healthcare Corporation) to a 100ml volume of ND96 until phase separation was clearly observable. This mixture was stirred vigorously overnight in a closed glass bottle prior to use. The established maximal solubility of isoflurane in salt solution at room temperature is 15 mM (Scheller et al. 1997) and working isoflurane concentrations used for electrophysiological experiments were prepared by dilutions of the 15mM saturated stock solution. Azi-isoflurane was diluted into DMSO prior to use and the concentration of this stock solution was determined using the optical extinction coefficient of 126 M⁻¹ cm⁻¹ at 300nm Abs.

Anesthetic solutions were stored in closed perfusion bags and were perfused onto oocytes through Teflon perfusion tubing, to prevent loss of isoflurane to the environment or electrophysiology rig.

Results

Azi-isoflurane photolabeling of TREK1 identifies VA binding sites

In order to identify TREK1 volatile anesthetic (VA) binding sites, we utilized a previously validated photo-reactive analog of the clinically important VA isoflurane (Eckenhoff et al. 2010). This biochemical adduct, azi-isoflurane, features a diazirine moiety capable of generating a highly reactive and chemically non-selective carbene adduct when irradiated with UV light. Azi-isoflurane has been shown to retain the anesthetic effects of isoflurane in animals and we first sought to ensure that the chemical modifications present in the azi-isoflurane compound would not alter the effect of this drug on the TREK1 channel. Two electrode voltage clamp studies of *Xenopus laevis* oocytes expressing TREK1 showed that application of azi-isoflurane causes a dose dependent potentiation of TREK1, with an EC_{50} of $735 \pm 192 \mu M$. We found no statistically significant difference between the effect of 2 mM isoflurane or 3 mM azi-isoflurane on TREK1 channel function (Fig. 1C).

For photolabeling studies, recombinantly expressed, purified and liposome reconstituted TREK1 protein (Supplemental Figure 1) was reacted with 30 μM azi-isoflurane, a concentration well below the EC_{50} of azi-isoflurane for TREK1, chosen to minimize non-specific modification. Following photolabeling, mass spectrometry (MS) of the TREK1 protein showed evidence of adduction of azi-isoflurane at two residues, G182 and K194, both located on the second transmembrane domain (TM2) of the channel (Figure 2). To examine whether the clinically relevant parent VA isoflurane also binds at these two sites, we performed a parallel azi-isoflurane photolabeling study of TREK1 in the presence of 3mM isoflurane as a competitive inhibitor. The 100-fold excess concentration of isoflurane protected the G182 site from azi-isoflurane photolabeling but did not prevent labeling at K194, suggesting that only the G182 site is specifically occupied by the parent VA isoflurane (Supplemental Figure 2).

While our MS results cover >91% of the TREK1 protein sequence with high confidence, there are notable regions of the TM3 and TM4 helices that are not identified by MS (Supplemental Figure 2). We attributed lack of coverage in these regions to the difficulty in ionizing and resolving hydrophobic transmembrane protein regions with this technique. Alternatively, binding of the

hydrophobic azi-isoflurane photolabel could reduce the MS signal from bound protein peptides, causing gaps in MS coverage that correspond to regions where photolabeling has occurred. To exclude this possibility, we performed MS on non-photolabeled TREK1 protein, finding essentially the same lack of high confidence coverage within the TM3 and TM4 region. This suggests that the poor MS coverage of TREK1 TM3 and TM4 is not the result of photolabeling within these regions. However, we cannot rule out the possibility that azi-isoflurane might label positions within the TM3 or TM4 domains that we are simply unable to detect with our MS approach.

Functional validation of TREK1 volatile anesthetic binding sites

To determine the functional importance of the VA binding sites identified by azi-isoflurane photolabeling, we introduced mutations at TREK1 positions 182 and 194 and used two-electrode voltage clamp recordings to assay for changes in TREK1 channel properties. We first substituted the endogenous amino acids at positions 182 and 194 with tryptophan, to mimic the size and hydrophobic nature of the azi-isoflurane photolabel. Tryptophan mutagenesis at position 194 (as well as other more conservative modifications) had no significant effect on the functional properties of the resultant mutant TREK1 channels (Figure 3). The amino acid at position 194 is poorly conserved across species, a lysine in the zebrafish TREK1 gene used for azi-isoflurane photolabeling but an aspartic acid in the mouse TREK1 construct used for our functional studies. To account for this difference, we introduced a D194K mutant into the mouse TREK1 background and found that this mutation also had no effect on TREK1 basal current or temperature dependence (Figure 3D, 3E). The absence of any observable functional effect of mutation at TREK1 194, along with the inability of excess isoflurane to protect position 194 from photolabeling by azi-isoflurane, suggest that despite being modified by azi-isoflurane, this site is unlikely to be relevant to the mechanism by which isoflurane and other clinically relevant VAs modulate TREK1. This notion is supported by the location of position 194 at the far end of TM2, facing toward the bulk solution and away from the core of the TREK1 protein.

TREK1 G182 is located within the center of TM2, one helical turn away from a glycine at position 178 previously shown to be a hinge point for a buckling motion of the TM2 helix that occurs during K2P gating (Lolicato et al. 2014). Tryptophan mutagenesis at TREK1 G182 demonstrated a large increase in basal current density and a near complete loss of modulation by heat or isoflurane, findings suggestive of TREK1 channel activation (Figure 3A-C, note that in panel 3B and 3C the concentration of injected cRNA was adjusted to normalize basal current density between mutant and WT TREK1 channels). Given the unique ability of glycine residues

to impart helical flexibility and the known conformational movements in the region of TREK1 around G182 (Lolicato et al. 2014, Brohawn et al. 2014a, Dong et al. 2015), we sought to determine whether loss of flexibility at position 182 was responsible for the major functional effects observed in the TREK1 G182W mutant. By introducing more conservative mutations at G182 we discovered that the effect of mutagenesis best correlated with the size of the introduced amino acid. TREK1 G182A showed only a small potentiating effect on current density and no significant effect on gating by heat and TREK1 G182C showed an intermediate phenotype (Figure 3D-F).

To further demonstrate the potentiating effect of increased side chain size at TREK1 G182, we treated TREK1 G182C channels with MMTS, a membrane permeant cysteine-modifying reagent (Figure 4). In wild type TREK1 channels, MMTS treatment caused an increase in channel activity that washed out within 2-3 minutes of removal of MMTS, a seemingly non-specific effect that occurred even in a TREK1 construct with all five endogenous cysteines removed (TREK1 cys-). By contrast, modification of the TREK1 G182C mutant with MMTS caused potentiation of channel activity that persisted after washout of the MMTS reagent (Fig. 4A). An equivalent effect was observed in a TREK1 cys- G182C mutant, specifically confirming that it is modification of the G182 cysteine that leads to the observed persistent potentiation of TREK1 currents following MMTS application (Fig. 4B). These findings support the notion that steric crowding is a significant determinant of the potentiation observed after perturbation of the G182 residue, whether by mutagenesis, MTS reagent modification, or by occupancy with a VA agent or photolabel.

To explore whether disruption of isoflurane binding contributes to the VA insensitivity of the G182W mutant channel, we performed azi-isoflurane photolabeling studies on purified TREK1 G182W protein. We found no evidence of azi-isoflurane labeling at position 182 or at any neighboring residues, suggesting that the G182W mutation either eliminates azi-isoflurane binding or reduces the affinity of VA for this site. We did observe azi-isoflurane photolabeling at two positions far from the G182 site (Supplemental Figure 2), one near the top of TM1 (A67) and one in the C-terminal domain (T303). These residues were not photolabeled in the VA sensitive TREK1 WT background and as such they are unlikely to contribute to the mechanism by which VAs modulate TREK1. However, enhanced accessibility of these residues in only the G182W background could be explained by global changes in protein tertiary structure induced by the G182W mutation, consistent with the dramatic functional effects observed in this mutant.

404

405 While the G182 residue is located at the cytosolic face of the TREK1 channel, activation
 406 gating in K2P channels is thought to occur via a “C-type” gating mechanism involving a
 407 rearrangement of the extracellular selectivity filter region (Bagriantsev et al. 2011, Piechotta et
 408 al. 2011, Zilberberg, Ilan and Goldstein 2001). Given that the G182 residue is located far from
 409 the selectivity filter (Figure 2B), a significant allosteric effect would be required for steric crowding
 410 at G182 to exert an effect on this distant gate. To explore whether the G182W mutation
 411 modulates the TREK1 “C-type” gate, we examined the stability of the selectivity filter against
 412 closure by application of extracellular acidosis. Acidification of the extracellular face of TREK1
 413 has been shown to inhibit TREK1 channel activity and to simultaneously diminish potassium
 414 selectivity, a hallmark of selectivity filter based gating (Cohen et al. 2008, Sandoz et al. 2009).
 415 While the TREK1 WT and TREK1 D194W mutants were both strongly inhibited by application of
 416 extracellular acidosis, the TREK1 G182W channel was resistant to closure by acid (Figure 3G,
 417 $\text{IpH}_{5.5}/\text{pH}_{9.0}$ 0.15 ± 0.05 for TREK1 WT, 0.17 ± 0.1 for TREK1 D194W, 0.51 ± 0.08 for TREK1
 418 G182W), though the IC_{50} of the effect was similar for all three channels tested (pH 7.88 ± 0.07
 419 for TREK1 WT, 7.63 ± 0.08 for TREK1 D194W, and 7.59 ± 0.07 for TREK1 G182W). These data
 420 support a mechanistic model in which isoflurane modulation of the TREK1 channel occurs via
 421 anesthetic binding to the G182 site causing allosteric stabilization of the selectivity filter “C-type”
 422 gate, which leads to increased TREK1 channel activity.

423

424 Many of the biophysical modalities known to modulate TREK1 channels, including heat,
 425 mechanical stretch, intracellular acidosis, and bioactive lipids, are believed to alter channel
 426 activity by affecting the selectivity filter “C-type” gate (Bagriantsev et al. 2011, Bagriantsev, Clark
 427 and Minor 2012, Piechotta et al. 2011), though the input sensor for these biophysical gating cues
 428 was proposed to be the intracellular C-terminal domain (Honore et al. 2002, Chemin et al. 2005,
 429 Bagriantsev et al. 2012). Structurally defined “TM4 up” and “TM4 down” conformational states
 430 (Brohawn et al. 2014a, Lolicato et al. 2014, Dong et al. 2015) have been identified as the key
 431 rearrangements that allow these C-terminal domain inputs to traverse the protein and converge
 432 at the selectivity filter to modulate gating (Zhuo et al. 2016). Within this gating model, the location
 433 of the G182 anesthetic binding site along the TM2 helix in direct opposition to TM4 (Figure 4C)
 434 is intriguing. It suggests that VA occupancy at the G182 site could modulate TREK1 activity by
 435 influencing TM4 intra-molecular rearrangements known to play a key role in K2P gating. To
 436 further explore the isoflurane binding site and gain insight into the effect of anesthetic binding on
 437 TREK1 channel gating, we utilized molecular dynamics (MD) simulation.

MD simulation identifies residues important for VA modulation to TREK1 channels

In order to establish a suitable starting point for MD simulations, we evaluated potential ligand binding configurations compatible with our photoaffinity results, using Autodock Vina docking software (Trott and Olson 2010). A pocket formed by G182 and the nearby TM3 and TM4 helices had the highest ranked score of -5.1, and was the only predicted binding site near the G182 residue. This binding site is located entirely within the transmembrane region of TREK1 and isoflurane is a relatively low-affinity ligand with minimal electrostatic interactions. Our docking results confirmed that the binding site near G182 was a sterically favorable starting point to place isoflurane for subsequent equilibrium MD simulation. Simulations of TREK1 embedded in a POPC:cholesterol lipid bilayer were conducted of TREK1 wildtype (440 ns) and TREK1 G182W in the absence of isoflurane (960 ns), and of TREK1 wildtype (WT) with a single isoflurane molecule placed at the G182 site in one of the two K2P subunits (700 ns). A representative snapshot of the isoflurane binding site is depicted in Figure 5A.

Simulation of WT TREK1 in the presence of VA demonstrated isoflurane to be highly mobile within the binding pocket, adopting a range of positions (figure 5B) and ultimately escaping the binding site after approximately 360 ns of simulation. This observed high degree of isoflurane mobility is consistent with both the low binding affinity predicted by docking analysis and the high micromolar concentrations of isoflurane required to activate TREK1 in functional assays. Despite the mobility of the isoflurane ligand, we were able to identify a number of residues that show greater than 20% occupancy by isoflurane (Table 1, Figure 5A), with occupancy defined as the percentage of MD trajectory snapshots (prior to isoflurane escaping the binding site) in which isoflurane was located within 7 Å of a given residue. High occupancy positions (Figure 5A) include the G182 residue and additional amino acids on TREK1 TM2, TM3, and TM4, as listed in Table 1. In agreement with our photolabeling findings, MD simulations demonstrated that isoflurane remained in close proximity to the G182 residue for >94% of the time VA occupied the binding site. This finding supports the notion that isoflurane imposes a consistent steric crowding at the G182 position, akin to the activity enhancing modifications we introduced through either mutagenesis or biochemical modification.

The proximity of the VA binding site to the neighboring TM4 helix (Figure 4C) suggested a mechanism by which anesthetic binding could influence K2P activity by influencing TM4 position, which is bolstered by our MD simulation data. We found a number of TM4 residues that

directly interact with the bound isoflurane molecule, most notably Y284 and M291. TREK1 Y284, the only polar residue in the otherwise hydrophobic TM4 helix, has been proposed to form stabilizing hydrogen bonds with one of two alternative TM3 backbone carbonyls in the “up” versus “down” conformations of TM4 (Dong et al. 2015), but is highly occupied by isoflurane when the drug is bound in the VA site. Similarly, the M291 position has been proposed to form TM4 conformation dependent interactions with neighboring TM3 and TM4 residues and in our simulation M291 is 55% occupied by the bound isoflurane molecule.

To assess for structural changes in the TREK1 protein induced by the presence of bound isoflurane, we compared the MD simulations of isoflurane-free versus isoflurane-bound TREK1 and calculated the per residue root mean square deviation (RMSD) of the TREK1 protein during the two simulations. The isoflurane molecule did not induce any major conformational changes in TREK1 during our simulations and we did not observe any alteration in the position of the TM4 helix attributable to the presence of isoflurane. However, within the VA binding pocket, we found an increase in RMSD along a short span of the TM2 helix (Figure 5C) that contains residues exhibiting the highest measured isoflurane occupancy. This localized structural change induced by the presence of isoflurane alters the positioning of the F185 residue, a residue previously shown to impact K2P responsiveness to heat, volatile anesthetics, mechanical stretch, and the pharmacological efficacy of BL1249, a K2P activator (Lolicato et al. 2014, Dong et al. 2015, Luethy et al. 2017, Pope et al. 2018). The aromatic group of the F185 residue is known to form a pi stacking interaction with the TM3 F214 residue, an interaction proposed to stabilize the “TM4-down” gating conformation in TREK2 channels (Dong et al. 2015).

To explore the effect of isoflurane binding on this structurally important interaction, we quantified pi-stacking between F185 and F214 in our simulations, such that the two aromatic rings were considered to be pi-stacked if their centroids were separated by 4.4 Å or less and the angles between the ring planes were less than 30 degrees (McGaughey, Gagne and Rappe 1998). In the TREK1 WT and TREK1 G182W simulations, pi-stacking between these residues occurred in 37% and 38% of trajectory frames, respectively, but pi-stacking occurred in only 2% of the trajectory frames in the isoflurane-bound wildtype TREK1 simulation. Disruption of this interaction was caused in large part by the presence of the isoflurane molecule itself distorting the binding pocket (Figure 5C, 5D), though we found that pi stacking between F185 and F214 remained disrupted for an additional ~300 ns of MD simulation time even after the isoflurane molecule had left the VA binding site. We surmise that this persistent disruption of the F185/F214

interaction is due to the observed local deformation of the TM2/TM3/TM4 interface induced by the presence of isoflurane, as shown in Figure 5D.

Based on these findings, we hypothesize that a combination of VA induced local structural perturbations, disruption of the F185/F214 pi stacking interaction, and direct steric effects upon TM4 movement all combine to alter the dynamics of the TM4 movements known to influence K2P gating. While our MD simulations did not directly reveal major conformational rearrangements of TM4, the observed isoflurane occupancy at numerous positions known to govern the energetics of TM4 translocation from the “down” to “up” position suggest a mechanism by which VA binding ultimately alters K2P activity by modulating TM4 movements.

Key residues transfer VA insensitivity from TRAAK to TREK1

While both TREK and TASK channels are potentiated by VA agents, the TRAAK K2P channel is anesthetic insensitive (Patel et al. 1999). TRAAK is 44% identical and 69% homologous to TREK1 and the two channels share many functional properties, including modulation by heat, mechanical stretch, pH, and arachidonic acid (Maingret et al. 1999, Kang, Choe and Kim 2005). The relative selectivity of VA agents for TREK1 over TRAAK despite the many similarities between these two channels allowed us to utilize the TRAAK channel as a negative control to further characterize the K2P VA binding site.

VA insensitivity of TRAAK could arise from one of two possibilities; failure of VAs to bind to TRAAK or inability of bound VA to modulate TRAAK activity. To distinguish between these possibilities and ascertain whether VAs bind to the TRAAK channel, we recombinantly expressed, purified and reconstituted TRAAK into liposomes (Supplemental Figure 1) and performed azi-isoflurane photolabeling studies identical to those used for TREK1 channels. The G182 residue identified by azi-isoflurane photolabeling of TREK1 is conserved in the TRAAK sequence, but we found no evidence of azi-isoflurane photolabeling at this glycine or at any other position in the TRAAK channel. MS of TRAAK protein after photolabeling showed a relatively lower total coverage of the TRAAK sequence compared to our results for TREK1 (84% for TRAAK versus 91% for TREK1), but all TRAAK residues homologous to the TREK1 azi-isoflurane binding region identified by MD simulations were positively identified, precluding the possibility that azi-isoflurane binds within this region of TRAAK. Our results suggest that the inability of VA agents to activate TRAAK channels is due to either an absence of binding or a significantly reduced affinity of anesthetic for this VA binding region.

540

541 We next explored the sequence similarity between TREK1 and TRAAK at the residues
542 that showed the highest isoflurane occupancy during MD simulations. Amongst the identified
543 amino acids, we found four residues that differ significantly between the TREK1 and TRAAK
544 sequences (Figure 6A-B), and created a series of mutant TREK1 channels that contain one or
545 two of the corresponding TRAAK residues at these positions (Fig. 6C-D). When examined by
546 TEVC, the “TRAAK-like” mutant TREK1 channels all exhibit diminished responsiveness to the
547 potentiating effect of isoflurane (Figure 6C), supporting a role for these residues in either forming
548 the VA binding domain or transducing the effects of binding to channel opening. While the extent
549 of the effect of these mutants was less dramatic than the near complete absence of isoflurane
550 responsiveness in the G182W mutant, the effect of combining two TRAAK-like mutations was
551 additive, as shown for the double mutant TREK1 F185L G186R (Figure 6C).

552

553 Whereas the G182W mutant exhibited diminished responsiveness to both VA activation
554 and heat, the “TRAAK-like” mutants maintain a preserved sensitivity to activation by heat (Figure
555 6D). The fact that the “TRAAK-like” mutants selectively alter VA activation without perturbing
556 gating by heat argues for their specificity to the mechanism of action of VAs. Activation of TREK1
557 by heat is known to be critically related to the phosphorylation state of serine 333 in the
558 intracellular C-terminal domain of TREK1 (Maingret et al. 2000) and the persistence of TREK1
559 heat activation in these “TRAAK-like” mutants suggests that these mutations do not eliminate VA
560 modulation by simply causing a global activation of the TREK1 “C-type” gate (as was the case
561 for the G182W mutant). Rather, the “TRAAK-like” mutations appear to reduce VA sensitivity by
562 acting specifically at the VA site of action, consistent with predictions made by our MD simulation
563 data and TREK1 vs TRAAK sequence homology.

564

565 Discussion

566 Effort to determine regions within the K2P channel structure susceptible to small molecule
567 modulators has become a topic of significant recent interest (Dong et al. 2015, Lolicato et al.
568 2017, Pope et al. 2018, Rinné et al. 2019), motivated by the lack of specific and high affinity
569 pharmacology targeting K2P channels. Here we focus on K2P modulation by VAs, drugs heavily
570 utilized in common clinical practice. We identify an interface formed by the TM2, TM3, and TM4
571 helices of TREK1 as the binding site for the VA isoflurane and identify site-specific interactions
572 between TREK1 and isoflurane that define this binding region. The identified VA modulatory site
573 features TREK1 residues previously identified to play key roles in K2P modulation by mechanical

stretch, heat, and the pharmacological activator BL1249, suggesting a common mechanistic pathway shared by VA and other K2P gating cues.

Prior to our study, the strongest evidence for a VA binding site in K2P channels was found in the TASK subfamily, where a single point mutation in the *lymneal* TASK TM3 residue M159 (homologous to TREK1 position T211) caused loss of stereoselective discrimination between R and S optical isomers of isoflurane (Andres-Enguix et al. 2007). TREK1 T211 lies within a span of the TM3 helix we were unable to resolve with MS but through MD simulation we identified T211 as a determinant of TREK1 VA binding (Table 1). Two additional residues identified by MD simulation, TREK1 F185 and M291, also align to positions implicated in TASK VA sensitivity (Andres-Enguix et al. 2007, Conway and Cotten 2012). All three of these residues (F185, T211, M291) differ significantly in the TRAAK protein sequence and “TRAAK-like” mutations at these positions decreased TREK1 VA sensitivity (Figure 6B). The overlapping molecular determinants of TREK1 and TASK1 VA sensitivity (and of TRAAK VA insensitivity) suggest a shared VA binding site across the K2P family. While the gating mechanisms of TASK channels have not been as extensively studied as those of the mechanosensitive K2Ps, recent work exploring the inhibitory effect of the local anesthetic agent bupivacaine on TASK1 channels demonstrates that bupivacaine binding constrains TM4 helix movement and ultimately alters selectivity filter behavior (Rinne et al. 2019). The bupivacaine site of action in TASK channels differs from the VA modulatory site identified in our study, but the effect of drug binding on TM4 conformation may be a shared feature in K2P gating utilized by both local and general anesthetics.

Our data suggest that VAs modulate K2P channel activity by influencing the position of the TM4 helix. The proximity of the azi-isoflurane labeled TREK1 G182 residue to the TM4 helix initially suggested such a mechanism and the potentiating effect of increased G182 side chain size supported this hypothesis. MD simulation studies identified direct contacts between isoflurane and the TM4 helix. However, the MD simulation data do not show major TM4 conformational rearrangements in the presence of isoflurane and thus whether VAs induced TM4 movements. Similarly, we have no evidence of whether the VA bound TREK1 channel more closely resembles the “TM4 up” or “TM4 down” states defined by crystallography (Dong et al. 2015, Brohawn et al. 2014a). As there is evidence to suggest that both the “TM4 up” and “TM4 down” conformations can promote active K2P channel states (McClenaghan et al. 2016), each of the two conformations could be consistent with a VA activated channel. However, both the direct steric constraints imposed by the presence of VA near TM4 and the disruption of the “TM4

down” state stabilizing F185/F214 pi-stack interaction would be predicted to favor a “TM4 up” conformation. The starting point for our MD simulations is a crystallographically-determined structural model of TREK1 already in the “TM4 up” state (Lolicato et al. 2017) and may explain the lack of any additional TM4 rearrangement in the presence of isoflurane. Further MD simulation utilizing a homology model of TREK1 based on K2P structures determined in the “TM4 down” state might more definitively answer this question. However, it is also possible that VA binding induces a conformation distinct from either of these two structurally defined states. The molecular mechanisms governing the connectivity between TM4 conformation and the flux gating behavior of the selectivity filter (Schewe et al. 2016) remains poorly defined and is an area of significant ongoing interest.

Having identified a VA modulatory site that appears to be shared across the TREK and TASK K2P subfamilies, we are cognizant of some of the inconsistent VA responses observed across these K2P subfamilies. For instance, while the obsolete VAs chloroform and diethyl-ether activate TREK1 and TASK3, they paradoxically inhibit the TASK1 channel (Patel et al. 1999, Andres-Enguix et al. 2007). Nitrous oxide and xenon have been reported to activate TREK1 but have no effect on TASK channels (Gruss et al. 2004), and TRAAK channels are unresponsive to VAs despite their close similarity to other mechanosensitive and VA sensitive K2Ps. Meanwhile, little is known about the gating of THIK channels but they are inhibited by the VAs halothane and isoflurane, an effect that appears to play an important role in the clinically relevant central respiratory depressant effect of VA administration (Rajan et al. 2001, Lazarenko et al. 2010). How can we explain the paradoxical effects of VA agents at structurally similar targets? One explanation would be that the VA binding site identified in our study represents only one of a subset of VA modulatory domains in the K2P family. Within the anesthetic mechanisms literature there are numerous examples of VA responsive ion channels that feature multiple low affinity anesthetic modulatory sites acting in a combinatorial fashion to produce a given output (Fourati et al. 2018, Heusser et al. 2018, Woll et al. 2018, Hemmings et al. 2019). However, the location of the identified K2P VA binding site at a position central to TM4 gating offers an alternative explanation. We understand relatively little about the mechanism by which TM4 movements govern K2P channel activity and it is possible that VA binding to this single site may produce opposing effects in the context of subtle differences in the connectivity between TM4 movements and the channel gate. Recent studies utilizing the state dependent binding of the mechanosensitive K2P inhibitor fluoxetine demonstrate that the resting and activated conformation of the TM4 helix in TREK1 versus TRAAK channels are inverted, underscoring this

point (Soussia et al. 2018) and perhaps contributing to the absence of VA binding and responsiveness in TRAAK channels.

While our results support the presence of a defined modulatory site within the TREK1 channel structure, these findings do not preclude a contribution from indirect effects of VAs on membrane lipid composition, as has been recently proposed (Pavel et al. 2019). Such a mechanism is in fact a particularly appealing explanation for the effects of the structurally simple and TREK1 specific VA agents chloroform, nitrous oxide, and xenon (Gruss et al. 2004). In this context, the location of the TREK1 VA binding site described in our study at a region key to the mechanism by which C-terminal domain dependent signals (including lipid modulation) are translated to TM4 (Zhuo et al. 2016) and ultimately to the selectivity filter gate would allow both an indirect and direct modulatory effect on channel behavior to feed into a shared common pathway.

Figure 1: Functional validation of azi-isoflurane activity on TREK1 channels. Representative two electrode voltage clamp recordings demonstrate the potentiating effects of saturating doses of isoflurane (A) and azi-isoflurane (B). Chemical structures of isoflurane and azi-isoflurane are shown. (C) Fold effect of administration of either isoflurane or azi-isoflurane on TREK1 outward current, as determined by the ratio of the recorded current at a voltage of 0mV, immediately prior to and following administration of VA agent. No significant difference was found between the responses of TREK1 to isoflurane versus azi-isoflurane, unpaired two tailed t-test P value of 0.11 (D) Dose response curve for azi-isoflurane activation of TREK1. Data derived from n>6, N>2 experimental observations. Error bars in panel C and D are mean $\pm$ SEM

695 Figure 2

Figure 2: Azi-isoflurane photolabeling of TREK1: (A) Mass spectra of TREK1 photoaffinity labeled peptides labeled at Glycine182 (top) and Lysine 194 (bottom). Colored intensities denote the identified peptide a, b, z, and y ion fragments for the sequence assignment, as shown in the inset boxes. See Supp. Fig. 4 for corresponding peptide tables. (B) A structural model of mouse TREK1 (PDBID 6CQ6), showing the positions of residues G182 and K194 (labeled red spheres) along the TM2 helix. (C) Alignment of the TREK1 TM2 and TM3 helices from human (hTREK1), mouse (mTREK1), and zebrafish (drTREK1).

Figure 3

Figure 3: Function assessment of TREK1 residues identified by azi-isoflurane photolabeling: Representative two electrode voltage clamp recordings of TREK1 wildtype and mutant channels TREK1 G182W and D194W. (A) Basal currents measured 24 hours after microinjection of 2.4 ng cRNA. (B) Temperature dependence of TREK1 currents, measured at temperatures of 20°C to 35°C, in 5°C increments. (C) Response to administration of 2mM Isoflurane, followed by washout. For temperature and isoflurane experiments performed on TREK1 channels bearing mutations that alter basal current density, the concentration of microinjected cRNA was titrated to achieve 1 μA of current at 20°C, to approximate wildtype channel current density. (D) Quantification of TREK1 channel activity on basal current level, (E) temperature dependence as measured by Q10 (30°C/20°C), (F) response to isoflurane administration, or (G) changes in external pH, as measured by TREK1 current at 0 mV. Number of replicate experiments indicated. Error bars are mean $\pm$ SEM. Statistically significant results indicated, * $p < 0.5$, ** $p < 0.05$, **** $p < 0.0005$.

Figure 4

Figure 4: Cysteine modification at the G182 position leads to TREK1 activation: (A) Representative time courses of the response of TREK1 WT or TREK1 G182C channels to treatment with 2mM MMTS. Shown is the recorded current at 0mV holding potential, measured every 5 seconds for 5 minutes, with current values normalized to the initial value recorded at the beginning of the time course (B) Quantification of effect size at the end of the time course (300 seconds), for TREK1 WT and TREK1 Cys-, a mutant TREK1 channel lacking all 5 of the endogenous TREK1 cysteine residues, and for G182 mutants in both of the above backgrounds. Number of replicate experiments indicated. Error bars are mean $\pm$ SEM. Statistically significant results indicated, **** $p < 0.0005$. (C) Crystallographically defined structural models of TREK2 in the TM4 up (PDB ID 4xd1, tan) and TM4 down (PDB ID 4bw5, maroon) conformations (Dong et al. 2015), highlighting the TM2, TM3, and TM4 helices from only a single subunit. The position of G182 (black sphere) is noted.

Figure 5

Figure 5: A) Isoflurane binding pocket predicted by MD simulation. B) Position of the oxygen atom at the center of the isoflurane molecule during the first 300ns of equilibrium MD simulation of TREK1 WT in the presence of isoflurane C) Density map of the position of the bound isoflurane. Isosurfaces represent 10%, 30%, and 50% isoflurane occupancy. D) Mean RMSD of residues within the TREK1 TM2/TM3 loop during the ~360 ns when isoflurane was bound, as compared with equilibrated free wild-type TREK1. Relative isoflurane occupancy for each residue (see Table 1) is shown as quartiles, described on right. Regions demonstrating the largest RMSD deviations are highlighted. E) Representative snapshot of equilibrium MD simulations of TREK1 WT or F) G182W show pi-stacking of the F185 and F214 residues. Equilibrium MD simulations of TREK1 in the presence of isoflurane reveal disruption of F185/F214 pi-stacking, both during the period that isoflurane remains in the binding pocket (panel G, t=300ns) as well as after isoflurane has left the binding pocket (panel H, t=700ns)

873

874 Table 1

Mouse TREK1 Residue Number	Percent Occupancy During MD Simulation	Transmembrane Domain
GLY178	47.65%	TM2
ASP179	35.19%	TM2
LEU181	68.40%	TM2
GLY182	94.01%	TM2
THR183	69.07%	TM2
PHE185*	92.17%	TM2
GLY186	89.16%	TM2
ILE189	52.96%	TM2
THR211*	56.20%	TM3
PHE214	44.39%	TM3
ILE215	25.65%	TM3
ALA283	28.07%	TM4
TYR284	67.95%	TM4
PHE285	27.41%	TM4
ALA287	88.05%	TM4
VAL288	78.20%	TM4
MET291*	56.11%	TM4

875

876 **Table 1.** Occupancy of residues in G182 isoflurane binding pocket, defined as the percentage of
877 snapshots where isoflurane within 7 Å of the given residue during MD simulation. Residues
878 with occupancy less than 20% are omitted. Positions previously shown to mediate VA
879 sensitivity in TASK K2P channels are annotated (*)

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889 Supplemental Figure 1

908 **Supplemental Figure 1: Purification of TREK1 and TRAAK proteins.** (A) Zebrafish TREK1 or
 909 human TRAAK DNA sequences were purified by metal chromatography via a C-terminal HIS tag,
 910 and expressed as fusion proteins with GFP, cleaved off via a 3C protease cleavage site prior to
 911 (B,E) final size exclusion chromatography. (C, F) After SDS PAGE electrophoresis, purified
 912 protein ran at a molecular weight of approximately 65 kDa, consistent with a K2P dimer. Prior
 913 to photolabeling experiments, purified TREK1 protein was assessed for functional integrity by
 914 reconstitution into planar lipid bilayers to measure single channel activity at the indicated
 915 holding potentials. Recordings were performed in symmetrical 150 mM KCl solution (D).
 916 hTRAAK protein was similarly active when reconstituted into bilayers (not shown).

917 Supplemental Figure 2

935 **Supplemental Figure 2:** Mass spectrometry analysis of purified zebrafish TREK1. Results of MS
936 analysis of TREK1 WT in the (top) absence of reaction with Azi-isoflurane, (second) following
937 reaction with 30 μM Azi-isoflurane, (third) following reaction with 30 μM Azi-isoflurane in the
938 presence of 3mM Isoflurane, or (bottom) TREK1 G182W following reaction with 30 μM Azi-
939 isoflurane. Regions positively identified by mass spectrometry analysis are shown in red in the
940 TREK1 structural model (PDB ID 6CQ6) and in black font in the sequence data. Regions absent
941 from MS data occurred in five distinct regions, all of which are displayed in matching color in
942 both the structural model and the sequence data. The G182 and K194 residues found to be
943 modified by azi-isoflurane in TREK1 WT are shown as pink spheres in the structural model, and
944 positive photolabeling is denoted in the sequence data by enlarged font and pink color. The
945 A67 and T303 residues modified by azi-isoflurane in TREK1 G182W are similarly denoted in
946 blue. The initial and final residues in the TREK1 protein were not identified in the majority of
947 the MS results and are shown in grey to denote absence from positive MS identification. These
948 residues are not present in the TREK1 structural model.

Supplemental Figure 3

Supplemental Figure 3: Mass spectrometry analysis of purified human TRAAK. Results of MS analysis of TRAAK in the absence of reaction with Azi-isoflurane (top) or following reaction with 30 μM Azi-isoflurane (bottom). Regions positively identified by mass spectrometry analysis are shown in blue in the TRAAK structural model (PDB ID 4WFE) and in black font in the sequence data. Regions absent from MS data are displayed in matching color in both the structural model and the sequence data. TRAAK residues homologous to TREK1 positions that exhibit high isoflurane occupancy in MD simulation are displayed as spheres in the structural model of TRAAK and are denoted in the sequence data by an enlarged font. All of these residues are identified by MS, but none show evidence of Azi-isoflurane labeling. A group of residues in the C-terminal region of TRAAK (marked in grey in the sequence data) were absent from our MS analysis but are not present in the TRAAK structural model.

964 Supplemental Figure 4

Protein fragment containing Azi-isoflurane modified Gly182

#1	Immonium	a ⁺	a ²⁺	b ⁺	b ²⁺	Seq.	y ⁺	y ²⁺	z ⁺	z ²⁺	#2
1	30.03383	30.03383	15.52055	58.02875	29.51801	G					9
2	72.08078	129.10225	65.05476	157.09717	79.05222	V	998.40199	499.70463	982.38326	491.69527	8
3	30.03383	186.12372	93.56550	214.11864	107.56296	G	899.33357	450.17042	883.31484	442.16106	7
4	88.03931	301.15067	151.07897	329.14559	165.07643	D	842.31210	421.65969	826.29337	413.65032	6
5	101.07094	429.20925	215.10826	457.20417	229.10572	Q	727.28515	364.14621	711.26642	356.13685	5
6	86.09643	542.29332	271.65030	570.28824	285.64776	L	599.22657	300.11692	583.20784	292.10756	4
7	30.03383	795.28623	398.14675	823.28114	412.14421	G-AziISO	486.14250	243.57489	470.12377	235.56552	3
8	74.06004	896.33391	448.67059	924.32882	462.66805	T	233.14959	117.07843	217.13087	109.06907	2
9						I	132.10191	66.55459	116.08319	58.54523	1

Protein fragment containing Azi-isoflurane modified Lys194

#1	Immonium	a ⁺	a ²⁺	a ³⁺	b ⁺	b ²⁺	b ³⁺	Seq.	y ⁺	y ²⁺	y ³⁺	z ⁺	z ²⁺	z ³⁺	#2
1	102.05496	102.05496	51.53112	34.68984	130.04988	65.52858	44.02148	E							17
2	101.10733	426.12137	213.56432	142.71197	454.11628	227.56178	152.04361	K-AziISO	2177.07669	1089.04198	726.36375	2161.05797	1081.03262	721.02417	16
3	104.05286	573.15678	287.08203	191.72378	601.15170	301.07949	201.05542	M-Oxidation	1853.01029	927.00878	618.34161	1836.99156	918.99942	613.00204	15
4	120.08078	720.22520	360.61624	240.74658	748.22012	374.61370	250.07822	F	1705.97487	853.49107	569.32981	1689.95615	845.48171	563.99023	14
5	72.08078	819.29362	410.15045	273.76939	847.28854	424.14791	283.10103	V	1558.90645	779.95686	520.30700	1542.88773	771.94750	514.96743	13
6	101.10733	947.38859	474.19793	316.46771	975.38351	488.19539	325.79935	K	1459.83803	730.42265	487.28419	1443.81931	722.41329	481.94462	12
7	159.09168	1133.46791	567.23759	378.49415	1161.46283	581.23505	387.82579	W	1331.74306	666.37517	444.58587	1315.72434	658.36581	439.24630	11
8	87.05529	1247.51084	624.25906	416.50846	1275.50576	638.25652	425.84010	N	1145.66374	573.33551	382.55943	1129.64502	565.32615	377.21986	10
9	72.08078	1346.57926	673.79327	449.53127	1374.57418	687.79073	458.86291	V	1031.62081	516.31404	344.54512	1015.60209	508.30468	339.20555	9
10	60.04439	1433.61129	717.30928	478.54195	1461.60621	731.30674	487.87359	S	932.55239	466.77983	311.52231	916.53367	458.77047	306.18274	8
11	101.07094	1561.66987	781.33857	521.22814	1589.66479	795.33603	530.55978	Q	845.52036	423.26382	282.51164	829.50164	415.25446	277.17206	7
12	74.06004	1662.71755	831.86241	554.91070	1690.71247	845.85987	564.24234	T	717.46178	359.23453	239.82544	701.44306	351.22517	234.48587	6
13	101.10733	1790.81252	895.90990	597.60902	1818.80744	909.90736	606.94066	K	616.41410	308.71069	206.14288	600.39538	300.70133	200.80331	5
14	86.09643	1903.89659	952.45193	635.30371	1931.89151	966.44939	644.63535	I	488.31913	244.66320	163.44456	472.30041	236.65384	158.10499	4
15	129.11348	2059.99771	1030.50249	687.33742	2087.99263	1044.49995	696.66906	R	375.23506	188.12117	125.74987	359.21634	180.11181	120.41030	3
16	72.08078	2159.06613	1080.03670	720.36023	2187.06105	1094.03416	729.69187	V	219.13394	110.07061	73.71616	203.11522	102.06125	68.37659	2
17								T	120.06552	60.53640	40.69336	104.04680	52.52704	35.35378	1

Protein fragment containing unmodified Lys194 after incubation with 30 μM Azi-isoflurane and 3mM Isoflurane

#1	Immonium	a ⁺	a ²⁺	a ³⁺	b ⁺	b ²⁺	b ³⁺	Seq.	y ⁺	y ²⁺	y ³⁺	z ⁺	z ²⁺	z ³⁺	#2
1	102.05496	102.05496	51.53112	34.68984	130.04988	65.52858	44.02148	E							17
2	101.10733	426.12137	213.56432	142.71197	454.11628	227.56178	152.04361	K-AziISO	2177.07669	1089.04198	726.36375	2161.05797	1081.03262	721.02417	16
3	104.05286	573.15678	287.08203	191.72378	601.15170	301.07949	201.05542	M-Oxidation	1853.01029	927.00878	618.34161	1836.99156	918.99942	613.00204	15
4	120.08078	720.22520	360.61624	240.74658	748.22012	374.61370	250.07822	F	1705.97487	853.49107	569.32981	1689.95615	845.48171	563.99023	14
5	72.08078	819.29362	410.15045	273.76939	847.28854	424.14791	283.10103	V	1558.90645	779.95686	520.30700	1542.88773	771.94750	514.96743	13
6	101.10733	947.38859	474.19793	316.46771	975.38351	488.19539	325.79935	K	1459.83803	730.42265	487.28419	1443.81931	722.41329	481.94462	12
7	159.09168	1133.46791	567.23759	378.49415	1161.46283	581.23505	387.82579	W	1331.74306	666.37517	444.58587	1315.72434	658.36581	439.24630	11
8	87.05529	1247.51084	624.25906	416.50846	1275.50576	638.25652	425.84010	N	1145.66374	573.33551	382.55943	1129.64502	565.32615	377.21986	10
9	72.08078	1346.57926	673.79327	449.53127	1374.57418	687.79073	458.86291	V	1031.62081	516.31404	344.54512	1015.60209	508.30468	339.20555	9
10	60.04439	1433.61129	717.30928	478.54195	1461.60621	731.30674	487.87359	S	932.55239	466.77983	311.52231	916.53367	458.77047	306.18274	8
11	101.07094	1561.66987	781.33857	521.22814	1589.66479	795.33603	530.55978	Q	845.52036	423.26382	282.51164	829.50164	415.25446	277.17206	7
12	74.06004	1662.71755	831.86241	554.91070	1690.71247	845.85987	564.24234	T	717.46178	359.23453	239.82544	701.44306	351.22517	234.48587	6
13	101.10733	1790.81252	895.90990	597.60902	1818.80744	909.90736	606.94066	K	616.41410	308.71069	206.14288	600.39538	300.70133	200.80331	5
14	86.09643	1903.89659	952.45193	635.30371	1931.89151	966.44939	644.63535	I	488.31913	244.66320	163.44456	472.30041	236.65384	158.10499	4
15	129.11348	2059.99771	1030.50249	687.33742	2087.99263	1044.49995	696.66906	R	375.23506	188.12117	125.74987	359.21634	180.11181	120.41030	3
16	72.08078	2159.06613	1080.03670	720.36023	2187.06105	1094.03416	729.69187	V	219.13394	110.07061	73.71616	203.11522	102.06125	68.37659	2
17								T	120.06552	60.53640	40.69336	104.04680	52.52704	35.35378	1

965

966 **Supplemental Figure 4:** Mass spectrometry protein fragment tables. Shown are the
967 fragmentation tables of the (top) 176-GVGDQLGTI-184 photolabeled peptide in the presence
968 of 30 μM Aziisoflurane (middle) the 193-EKMFVKWNVSQTKIRVT-209 photolabeled peptide in
969 the presence of 30 μM Aziisoflurane, and (bottom) the 193-EKMFVKWNVSQTKIRVT-209
970 photolabeled peptide in the presence of 30 μM Aziisoflurane (AziISO) and 3 mM isoflurane.
971 Detected identified a, b (red) and z, y (blue) ions are colored red and blue, respectively.
972 Residues detected with a modification are noted, and those modified by Aziisoflurane are
973 additionally noted in bold and underlined.

References

- Andres-Enguix, I., A. Caley, R. Yustos, M. A. Schumacher, P. D. Spanu, R. Dickinson, M. Maze & N. P. Franks (2007) Determinants of the anesthetic sensitivity of two-pore domain acid-sensitive potassium channels: molecular cloning of an anesthetic-activated potassium channel from *Lymnaea stagnalis*. *J Biol Chem*, 282, 20977-90.
- Bagriantsev, S. N., K. A. Clark & D. L. Minor, Jr. (2012) Metabolic and thermal stimuli control K(2P)2.1 (TREK-1) through modular sensory and gating domains. *Embo j*, 31, 3297-308.
- Bagriantsev, S. N., R. Peyronnet, K. A. Clark, E. Honore & D. L. Minor, Jr. (2011) Multiple modalities converge on a common gate to control K2P channel function. *Embo j*, 30, 3594-606.
- Bertaccini, E. J., R. Dickinson, J. R. Trudell & N. P. Franks (2014) Molecular modeling of a tandem two pore domain potassium channel reveals a putative binding site for general anesthetics. *ACS Chem Neurosci*, 5, 1246-52.
- Best, R. B., X. Zhu, J. Shim, P. E. Lopes, J. Mittal, M. Feig & A. D. Mackerell, Jr. (2012) Optimization of the additive CHARMM all-atom protein force field targeting improved sampling of the backbone phi, psi and side-chain chi(1) and chi(2) dihedral angles. *J Chem Theory Comput*, 8, 3257-3273.
- Blondeau, N., O. Petrault, S. Manta, V. Giordanengo, P. Gounon, R. Bordet, M. Lazdunski & C. Heurteaux (2007) Polyunsaturated fatty acids are cerebral vasodilators via the TREK-1 potassium channel. *Circ Res*, 101, 176-84.
- Brohawn, S. G., E. B. Campbell & R. MacKinnon (2014a) Physical mechanism for gating and mechanosensitivity of the human TRAAK K⁺ channel. *Nature*, 516, 126-30.
- Brohawn, S. G., Z. Su & R. MacKinnon (2014b) Mechanosensitivity is mediated directly by the lipid membrane in TRAAK and TREK1 K⁺ channels. *Proc Natl Acad Sci U S A*, 111, 3614-9.
- Chemin, J., A. J. Patel, F. Duprat, I. Lauritzen, M. Lazdunski & E. Honore (2005) A phospholipid sensor controls mechanogating of the K⁺ channel TREK-1. *Embo j*, 24, 44-53.
- Chokshi, R. H., A. T. Larsen, B. Bhayana & J. F. Cotten (2015) Breathing Stimulant Compounds Inhibit TASK-3 Potassium Channel Function Likely by Binding at a Common Site in the Channel Pore. *Mol Pharmacol*, 88, 926-34.
- Cohen, A., Y. Ben-Abu, S. Hen & N. Zilberberg (2008) A novel mechanism for human K2P2.1 channel gating. Facilitation of C-type gating by protonation of extracellular histidine residues. *J Biol Chem*, 283, 19448-55.
- Conway, K. E. & J. F. Cotten (2012) Covalent modification of a volatile anesthetic regulatory site activates TASK-3 (KCNK9) tandem-pore potassium channels. *Mol Pharmacol*, 81, 393-400.
- Cotten, J. F. (2013) TASK-1 (KCNK3) and TASK-3 (KCNK9) tandem pore potassium channel antagonists stimulate breathing in isoflurane-anesthetized rats. *Anesth Analg*, 116, 810-6.
- Davies, L. A., C. Hu, N. A. Guagliardo, N. Sen, X. Chen, E. M. Talley, R. M. Carey, D. A. Bayliss & P. Q. Barrett (2008) TASK channel deletion in mice causes primary hyperaldosteronism. *Proc Natl Acad Sci U S A*, 105, 2203-8.

Decher, N., B. Ortiz-Bonnin, C. Friedrich, M. Schewe, A. K. Kiper, S. Rinne, G. Seemann, R. Peyronnet, S. Zumhagen, D. Bustos, J. Kockskamper, P. Kohl, S. Just, W. Gonzalez, T. Baukowitz, B. Stallmeyer & E. Schulze-Bahr (2017) Sodium permeable and "hypersensitive" TREK-1 channels cause ventricular tachycardia. *EMBO Mol Med*, 9, 403-414.

Dong, Y. Y., A. C. Pike, A. Mackenzie, C. McClenaghan, P. Aryal, L. Dong, A. Quigley, M. Grieben, S. Goubin, S. Mukhopadhyay, G. F. Ruda, M. V. Clausen, L. Cao, P. E. Brennan, N. A. Burgess-Brown, M. S. Sansom, S. J. Tucker & E. P. Carpenter (2015) K2P channel gating mechanisms revealed by structures of TREK-2 and a complex with Prozac. *Science*, 347, 1256-9.

Eckenhoff, R. G., J. Xi, M. Shimaoka, A. Bhattacharji, M. Covarrubias & W. P. Dailey (2010) Azisoflurane, a Photolabel Analog of the Commonly Used Inhaled General Anesthetic Isoflurane. *ACS Chem Neurosci*, 1, 139-45.

Enyedi, P. & G. Czirjak (2010) Molecular background of leak K⁺ currents: two-pore domain potassium channels. *Physiol Rev*, 90, 559-605.

Fourati, Z., R. J. Howard, S. A. Heusser, H. Hu, R. R. Ruza, L. Sauguet, E. Lindahl & M. Delarue (2018) Structural Basis for a Bimodal Allosteric Mechanism of General Anesthetic Modulation in Pentameric Ligand-Gated Ion Channels. *Cell Rep*, 23, 993-1004.

Franks, N. P. & W. R. Lieb (1988) Volatile general anaesthetics activate a novel neuronal K⁺ current. *Nature*, 333, 662-4.

Friedrich, C., S. Rinne, S. Zumhagen, A. K. Kiper, N. Silbernagel, M. F. Netter, B. Stallmeyer, E. Schulze-Bahr & N. Decher (2014) Gain-of-function mutation in TASK-4 channels and severe cardiac conduction disorder. *EMBO Mol Med*, 6, 937-51.

Gruss, M., T. J. Bushell, D. P. Bright, W. R. Lieb, A. Mathie & N. P. Franks (2004) Two-pore-domain K⁺ channels are a novel target for the anesthetic gases xenon, nitrous oxide, and cyclopropane. *Mol Pharmacol*, 65, 443-52.

Gurney, A. & B. Manoury (2009) Two-pore potassium channels in the cardiovascular system. *Eur Biophys J*, 38, 305-18.

Heginbotham, L., L. Kolmakova-Partensky & C. Miller (1998) Functional Reconstitution of a Prokaryotic K⁺ Channel. *J Gen Physiol*, 111, 741-9.

Hemmings, H. C., Jr., P. M. Riegelhaupt, M. B. Kelz, K. Solt, R. G. Eckenhoff, B. A. Orser & P. A. Goldstein (2019) Towards a Comprehensive Understanding of Anesthetic Mechanisms of Action: A Decade of Discovery. *Trends Pharmacol Sci*, 40, 464-481.

Henin, J., G. Brannigan, W. P. Dailey, R. Eckenhoff & M. L. Klein (2010) An atomistic model for simulations of the general anesthetic isoflurane. *J Phys Chem B*, 114, 604-12.

Heurteaux, C., N. Guy, C. Laigle, N. Blondeau, F. Duprat, M. Mazzuca, L. Lang-Lazdunski, C. Widmann, M. Zanzouri, G. Romey & M. Lazdunski (2004) TREK-1, a K⁺ channel involved in neuroprotection and general anesthesia. *Embo j*, 23, 2684-95.

Heusser, S. A., M. Lycksell, X. Wang, S. E. McComas, R. J. Howard & E. Lindahl (2018) Allosteric potentiation of a ligand-gated ion channel is mediated by access to a deep membrane-facing cavity. *Proc Natl Acad Sci U S A*, 115, 10672-10677.

Honore, E. (2007) The neuronal background K2P channels: focus on TREK1. *Nat Rev Neurosci*, 8, 251-61.

1059 Honore, E., F. Maingret, M. Lazdunski & A. J. Patel (2002) An intracellular proton sensor
1060 commands lipid- and mechano-gating of the K(+) channel TREK-1. *Embo j*, 21, 2968-76.

1061 Jo, S., T. Kim, V. G. Iyer & W. Im (2008) CHARMM-GUI: a web-based graphical user interface for
1062 CHARMM. *J Comput Chem*, 29, 1859-65.

1063 Jo, S., J. B. Lim, J. B. Klauda & W. Im (2009) CHARMM-GUI Membrane Builder for mixed bilayers
1064 and its application to yeast membranes. *Biophys J*, 97, 50-8.

1065 Kang, D., C. Choe & D. Kim (2005) Thermosensitivity of the two-pore domain K⁺ channels TREK-
1066 2 and TRAAK. *J Physiol*, 564, 103-16.

1067 Klauda, J. B., R. M. Venable, J. A. Freites, J. W. O'Connor, D. J. Tobias, C. Mondragon-Ramirez, I.
1068 Vorobyov, A. D. MacKerell, Jr. & R. W. Pastor (2010) Update of the CHARMM all-atom
1069 additive force field for lipids: validation on six lipid types. *J Phys Chem B*, 114, 7830-43.

1070 Krivov, G. G., M. V. Shapovalov & R. L. Dunbrack, Jr. (2009) Improved prediction of protein side-
1071 chain conformations with SCWRL4. *Proteins*, 77, 778-95.

1072 Lafreniere, R. G., M. Z. Cader, J. F. Poulin, I. Andres-Enguix, M. Simoneau, N. Gupta, K. Boisvert,
1073 F. Lafreniere, S. McLaughlan, M. P. Dube, M. M. Marcinkiewicz, S. Ramagopalan, O.
1074 Ansorge, B. Brais, J. Sequeiros, J. M. Pereira-Monteiro, L. R. Griffiths, S. J. Tucker, G.
1075 Ebers & G. A. Rouleau (2010) A dominant-negative mutation in the TRESK potassium
1076 channel is linked to familial migraine with aura. *Nat Med*, 16, 1157-60.

1077 Lazarenko, R. M., M. G. Fortuna, Y. Shi, D. K. Mulkey, A. C. Takakura, T. S. Moreira, P. G.
1078 Guyenet & D. A. Bayliss (2010) Anesthetic activation of central respiratory
1079 chemoreceptor neurons involves inhibition of a THIK-1-like background K(+) current. *J*
1080 *Neurosci*, 30, 9324-34.

1081 Lolicato, M., C. Arrigoni, T. Mori, Y. Sekioka, C. Bryant, K. A. Clark & D. L. Minor, Jr. (2017)
1082 K2P2.1 (TREK-1)-activator complexes reveal a cryptic selectivity filter binding site.
1083 *Nature*, 547, 364-368.

1084 Lolicato, M., P. M. Riegelhaupt, C. Arrigoni, K. A. Clark & D. L. Minor (2014) Transmembrane
1085 helix straightening and buckling underlies activation of mechanosensitive and
1086 thermosensitive K(2P) channels. *Neuron*, 84, 1198-212.

1087 Luethy, A., J. D. Boghosian, R. Srikantha & J. F. Cotten (2017) Halogenated Ether, Alcohol, and
1088 Alkane Anesthetics Activate TASK-3 Tandem Pore Potassium Channels Likely through a
1089 Common Mechanism. *Mol Pharmacol*, 91, 620-629.

1090 Ma, L., D. Roman-Campos, E. D. Austin, M. Eyries, K. S. Sampson, F. Soubrier, M. Germain, D. A.
1091 Tregouet, A. Borczuk, E. B. Rosenzweig, B. Girerd, D. Montani, M. Humbert, J. E. Loyd, R.
1092 S. Kass & W. K. Chung (2013) A novel channelopathy in pulmonary arterial
1093 hypertension. *N Engl J Med*, 369, 351-361.

1094 Maingret, F., M. Fosset, F. Lesage, M. Lazdunski & E. Honore (1999) TRAAK is a mammalian
1095 neuronal mechano-gated K⁺ channel. *J Biol Chem*, 274, 1381-7.

1096 Maingret, F., I. Lauritzen, A. J. Patel, C. Heurteaux, R. Reyes, F. Lesage, M. Lazdunski & E.
1097 Honore (2000) TREK-1 is a heat-activated background K(+) channel. *Embo j*, 19, 2483-
1098 91.

1099 McClenaghan, C., M. Schewe, P. Aryal, E. P. Carpenter, T. Baukrowitz & S. J. Tucker (2016)
1100 Polymodal activation of the TREK-2 K2P channel produces structurally distinct open
1101 states. *J Gen Physiol*, 147, 497-505.

1102 McGaughey, G. B., M. Gagne & A. K. Rappe (1998) pi-Stacking interactions. Alive and well in
1103 proteins. *J Biol Chem*, 273, 15458-63.

1104 Nury, H., C. V. Renterghem, Y. Weng, A. Tran, M. Baaden, V. Dufresne, J.-P. Changeux, J. M.
1105 Sonner, M. Delarue & P.-J. Corringer (2011) X-ray structures of general anaesthetics
1106 bound to a pentameric ligand-gated ion channel. *Nature*, 469, 428-431.

1107 Pang, D. S., C. J. Robledo, D. R. Carr, T. C. Gent, A. L. Vyssotski, A. Caley, A. Y. Zecharia, W.
1108 Wisden, S. G. Brickley & N. P. Franks (2009) An unexpected role for TASK-3 potassium
1109 channels in network oscillations with implications for sleep mechanisms and anesthetic
1110 action. *Proc Natl Acad Sci U S A*, 106, 17546-51.

1111 Patel, A. J., E. Honore, F. Lesage, M. Fink, G. Romey & M. Lazdunski (1999) Inhalational
1112 anesthetics activate two-pore-domain background K⁺ channels. *Nat Neurosci*, 2, 422-6.

1113 Pavel, M. A., E. N. Petersen, H. Wang, R. A. Lerner & S. B. Hansen (2019) Studies on the
1114 mechanism of membrane mediated general anesthesia.

1115 Phillips, J. C., R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, R. D. Skeel, L.
1116 Kale & K. Schulten (2005) Scalable molecular dynamics with NAMD. *J Comput Chem*, 26,
1117 1781-802.

1118 Piechotta, P. L., M. Rapedius, P. J. Stansfeld, M. K. Bollepalli, G. Erhlich, I. Andres-Enguix, H.
1119 Fritzenschaft, N. Decher, M. S. P. Sansom, S. J. Tucker & T. Baukrowitz. 2011. The pore
1120 structure and gating mechanism of K2P channels. In *EMBO J*, 3607-19.

1121 Pope, L., C. Arrigoni, H. Lou, C. Bryant, A. Gallardo-Godoy, A. R. Renslo & D. L. Minor, Jr. (2018)
1122 Protein and Chemical Determinants of BL-1249 Action and Selectivity for K2P Channels.
1123 *ACS Chem Neurosci*, 9, 3153-3165.

1124 Rajan, S., E. Wischmeyer, C. Karschin, R. Preisig-Muller, K. H. Grzeschik, J. Daut, A. Karschin & C.
1125 Derst (2001) THIK-1 and THIK-2, a novel subfamily of tandem pore domain K⁺ channels.
1126 *J Biol Chem*, 276, 7302-11.

1127 Rinne, S., A. K. Kiper, K. S. Vowinkel, D. Ramirez, M. Schewe, M. Bedoya, D. Aser, I. Gensler, M.
1128 F. Netter, P. J. Stansfeld, T. Baukrowitz, W. Gonzalez & N. Decher (2019) The molecular
1129 basis for an allosteric inhibition of K(+) -flux gating in K2P channels. *Elife*, 8.

1130 Rinné, S., A. Kiper, K. Vowinkel, D. Ramírez, M. Schewe, M. Bedoya, D. Aser, I. Gensler, M.
1131 Netter, P. Stansfeld, T. Baukrowitz, W. Gonzalez & N. Decher (2019) The Molecular
1132 Basis for an Allosteric Inhibition of K⁺-flux Gating in K²P Channels. *eLife*, 8.

1133 Royal, P., A. Andres-Bilbe, P. Avalos Prado, C. Verkest, B. Wdziekonski, S. Schaub, A. Baron, F.
1134 Lesage, X. Gasull, J. Levitz & G. Sandoz (2019) Migraine-Associated TRESK Mutations
1135 Increase Neuronal Excitability through Alternative Translation Initiation and Inhibition
1136 of TREK. *Neuron*, 101, 232-245.e6.

1137 Sandoz, G., D. Douguet, F. Chatelain, M. Lazdunski & F. Lesage (2009) Extracellular acidification
1138 exerts opposite actions on TREK1 and TREK2 potassium channels via a single conserved
1139 histidine residue. *Proc Natl Acad Sci U S A*, 106, 14628-33.

1140 Sauguet, L., R. J. Howard, L. Malherbe, U. S. Lee, P.-J. Corringer, R. A. Harris & M. Delarue
1141 (2013) Structural basis for potentiation by alcohols and anaesthetics in a ligand-gated
1142 ion channel. *Nature Communications*, 4, 1-10.

1143 Scheller, M., J. Bufler, H. Schneck, E. Kochs & C. Franke (1997) Isoflurane and sevoflurane
1144 interact with the nicotinic acetylcholine receptor channels in micromolar
1145 concentrations. *Anesthesiology*, 86, 118-27.

1146 Schewe, M., E. Nematian-Ardestani, H. Sun, M. Musinszki, S. Cordeiro, G. Bucci, B. L. de Groot,
1147 S. J. Tucker, M. Rapedius & T. Baukowitz (2016) A Non-canonical Voltage-Sensing
1148 Mechanism Controls Gating in K2P K(+) Channels. *Cell*, 164, 937-49.
1149 Sonner, J. M. & R. S. Cantor (2013) Molecular mechanisms of drug action: an emerging view.
1150 *Annu Rev Biophys*, 42, 143-67.
1151 Soussia, I. B., F. S. Choveau, S. Blin, E. J. Kim, S. Feliciangeli, F. C. Chatelain, D. Kang, D. Bichet &
1152 F. Lesage (2018) Antagonistic Effect of a Cytoplasmic Domain on the Basal Activity of
1153 Polymodal Potassium Channels. *Front Mol Neurosci*, 11, 301.
1154 Steinberg, E. A., K. A. Wafford, S. G. Brickley, N. P. Franks & W. Wisden (2015) The role of K(2)p
1155 channels in anaesthesia and sleep. *Pflugers Arch*, 467, 907-16.
1156 Tong, L., M. Cai, Y. Huang, H. Zhang, B. Su, Z. Li & H. Dong (2014) Activation of K(2)P channel-
1157 TREK1 mediates the neuroprotection induced by sevoflurane preconditioning. *Br J*
1158 *Anaesth*, 113, 157-67.
1159 Trott, O. & A. J. Olson (2010) AutoDock Vina: improving the speed and accuracy of docking with
1160 a new scoring function, efficient optimization and multithreading. *J Comput Chem*, 31,
1161 455-61.
1162 Webb, B. & A. Sali (2017) Protein Structure Modeling with MODELLER. *Methods Mol Biol*, 1654,
1163 39-54.
1164 Woll, K. A., X. Zhou, N. V. Bhanu, B. A. Garcia, M. Covarrubias, K. W. Miller & R. G. Eckenhoff
1165 (2018) Identification of binding sites contributing to volatile anesthetic effects on GABA
1166 type A receptors. *Faseb j*, 32, 4172-4189.
1167 Zhuo, R. G., P. Peng, X. Y. Liu, H. T. Yan, J. P. Xu, J. Q. Zheng, X. L. Wei & X. Y. Ma (2016)
1168 Allosteric coupling between proximal C-terminus and selectivity filter is facilitated by
1169 the movement of transmembrane segment 4 in TREK-2 channel. *Sci Rep*, 6, 21248.
1170 Zilberberg, N., N. Ilan & S. A. Goldstein (2001) KCNKO: opening and closing the 2-P-domain
1171 potassium leak channel entails "C-type" gating of the outer pore. *Neuron*, 32, 635-48.
1172