HHS Public Access

Author manuscript

Anesthesiology. Author manuscript; available in PMC 2024 July 01.

Published in final edited form as:

Anesthesiology. 2023 July 01; 139(1): 63–76. doi:10.1097/ALN.0000000000004577.

TREK-1 and TREK-2 knockout mice are not resistant to halothane or isoflurane

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Abstract

Background.—A variety of molecular targets for volatile anesthetics have been suggested including the anesthetic-sensitive K^+ leak channel, TREK-1. Knockout of TREK-1 is reported to render mice resistant to volatile anesthetics, making TREK-1 channels compelling targets for anesthetic action. Spinal cord slices from mice, either wildtype or an anesthetic-hypersensitive mutant, *Ndufs4*, display an isoflurane-induced outward K^+ leak that correlates with their minimum alveolar concentrations (MAC) and is blocked by norfluoxetine. We hypothesized that TREK-1 channels conveyed this current and contribute to the anesthetic hypersensitivity of *Ndufs4.* Our results led to evaluation of a second TREK channel, TREK-2, in control of anesthetic sensitivity.

Methods.—We measured anesthetic sensitivities of mice carrying knockout alleles of *Trek-1* and *Trek-2*, the double knockout *Trek-1;Trek-2,* and *Ndufs4;Trek-1.* Neurons from spinal cord slices from each mutant were patch clamped to characterize isoflurane-sensitive currents. Norfluoxetine was used to identify TREK-dependent currents.

Results.—We compared mean values for MAC (+/− SD) between wildtype and two *Trek-1* knockout alleles in mice (p-values, *Trek-1* compared to wildtype). Wildtype: (MAC(Hal), 1.30%(0.10); MAC(Iso), 1.40%(0.11): *Trek-1*^{tm1Lex} (MAC(Hal), 1.27%(0.11); p=0.387; MAC(Iso), 1.38%(0.09); p=0.268): *Trek-1*^{tm1Lzd} (MAC(Hal); 1.27%(0.11); p=0.482: MAC(Iso); 1.41%(0.12); p=0.188). Neither allele was resistant for loss of righting reflex. The

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Details of authors' contributions. KAS, CBW planned and performed experiments and edited the manuscript. SCJ, JMR planned experiments, evaluated data, and edited the manuscript. HMW planned and performed the immunohistochemistry and microscopy and edited the manuscript. PGM and MMS planned and performed experiments, obtained funding, evaluated data, and wrote and edited the manuscript.

Conflicts of interests. The authors claim no competing financial interests.

EC50s of *Ndufs4;Trek-1*^{tm1Lex} did not differ from *Ndufs4. Ndufs4*: (EC50(Hal), 0.65%(0.05); EC50 (Iso), 0.63%(0.05): *Ndufs4;Trek-1*^{tm1Lex} (EC50(Hal), 0.58%(0.07); p=0.004; EC50(Iso); 0.61%(.06); p=0.442). Loss of TREK-2 did not alter anesthetic sensitivity in a wildtype or *Trek-1* genetic background. Loss of TREK-1 or TREK-2, or both, did not alter the isoflurane-induced currents in wildtype cells but did cause them to be norfluoxetine-insensitive.

Conclusions.—Loss of TREK channels did not alter anesthetic sensitivity in mice, nor did it eliminate isoflurane-induced transmembrane currents. However, the isoflurane-induced currents are norfluoxetine-resistant in *Trek* mutants indicating that other channels may function in this role when TREK channels are deleted.

Keywords

mitochondria; volatile anesthetic; genetics; anesthetic sensitivity; TREK channels

Introduction

The minimum alveolar concentration (MAC) required to prevent response to a painful stimulus is a standard reference point to determine volatile anesthetic potency.[1,2] Studies in animal models have established that volatile anesthetics act in the spinal cord to induce this immobility.[3–7] Nevertheless, the molecular components responsible for anesthetic-induced immobility remain unclear. In general, studies have focused on anesthetic enhancement of inhibitory signaling, or depression of excitatory activity.[8,9] However, genetic manipulation of several putative anesthetic targets *in vivo* has failed to produce the changes in anesthetic response predicted by *in vitro* results,[10–13] complicating the search for a molecular target. One frequently cited exception reported that knocking-out the anesthetic sensitive K^+ leak channel, TREK-1, rendered mice resistant to an array of volatile anesthetics. [14]

The Trek channels (TREK-1 and TREK-2) are members of the two-pore domain potassium (K^+) channel (K_{2P}) superfamily, which contribute to outward "leak" K^+ currents. They are important for maintaining the resting membrane potential in neurons[15] and are pharmacologically identified by inhibition with the drug, norfluoxetine.[16,17] Activation of Trek channels increases K^+ efflux and therefore hyperpolarizes neurons, decreasing their excitability.[18,19] This makes the TREK-1 channel an extremely compelling target for volatile anesthetic action.[20,21]

Our interest in TREK channels stems from findings in the mitochondrial knockout mutant,[22] *Ndufs4*, which is profoundly hypersensitive to volatile anesthetics.[23] *Ndufs4* is a deletion of the NDUFS4 subunit of mitochondrial complex I, causing an ~60% decrease in MAC compared to wildtype mice. In spinal cord slices from *Ndufs4* mice, we identified an isoflurane-dependent increase in the holding currents of ventral non-cholinergic neurons at low isoflurane concentrations, correlating with their lower MAC.[24] These cells were hyperpolarized, consistent with an increased outward K^+ current. A similar isoflurane-dependent increase in outward current was found in cells from the wildtype animals at isoflurane concentrations reflecting their higher MAC.[24] Application of norfluoxetine, which inhibits TREK-1 and TREK-2 channels,[16,17] prevented these increases of holding currents in both WT and *Ndufs4* genotypes, suggesting isoflurane induces the outward

current through TREK-1, TREK-2, or both.[25] These results are consistent with the known activation of the TREK channels by volatile anesthetics [26,27] including the identified site of action for isoflurane on TREK-1.[28] Given the report of volatile anesthetic resistance in *Trek-1* mice, we hypothesized that this outward cation current was carried by TREK-1 and was a molecular mechanism contributing to volatile anesthetic suppression of movement and the profound anesthetic hypersensitivity of *Ndufs4*.

We investigated the anesthetic response of mice in which NDUFS4 and TREK-1 were deleted. We predicted that multiple *Trek-1* alleles would be resistant to volatile anesthetics, in keeping with previous reports, and that the magnitude of the outward currents produced by volatile anesthetics would be reduced in *Trek-1* animals. We also predicted that *Ndufs4;Trek-1* double mutants would be resistant to anesthetics relative to *Ndufs4* and would not display increased holding current as *Ndufs4* in spinal cord slices at low concentrations of isoflurane. These predictions were incorrect. Our results led us to study mutants in the second TREK channel, TREK-2.

Methods.

Additional Methods are found in the Supplemental Digital Content 1.

Animals

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Seattle Children's Research Institute. Mice were housed at 22°C with a 12-hour light-dark cycle and maintained on a standard rodent diet. Food and water were available *ad libitum*. Male and female mice were used for each experiment and the total number of mice used are listed in the Tables. Female and male mice were used in approximately equal numbers in all experiments.

Mouse strains—When referring to the protein product of a gene, all letters are capitalized and not italicized (*e.g.,* TREK-1). When referring to the gene or the mutant strain, the first letter is capitalized, and the term is italicized (*e.g., Trek-1*). In general, all mutants used are in a C57Bl/6 background. Since there were no sensitivity differences between the homozygote wildtype and heterozygotes for either *Trek-1, Trek-2,* or *Trek-1;Trek-2*, heterozygotes are included in the wildtype designation in our tables.

The generation[22] and functional [23,24,29] characteristics of the *Ndufs4* strain have been previously described. *Trek-1*^{tm1Lex} was purchased from the Mutant Mouse Resource and Research Center at UC Davis (mmrrc.ucdavis.edu). *Trek-1*^{tm1Lzd} is the allele studied by Heurteaux *et al.,14* and was carried in a previously characterized triple mutant, *Trek-1*^{tm1Lzd}*,Trek-2,Traak.*[30,31] It was the kind gift of Drs. Florian Lesage (French Institute of Health and Medical Research) and Dr. Andreas Schwingshackl (UCLA, USA). In the previous version of this manuscript which used this strain, several reviewers requested that we isolate *Trek-1*^{tm1Lzd} from a potentially confounding genetic background. Therefore, *Trek-1*^{tm1Lzd} was isolated from the triple mutant by outcrossing and its loss confirmed by PCR. This isolated allele is the source for all data reported here as *Trek-1*^{tm1Lzd}.

Using CRISPR/CAS9 in a C57Bl/6 background, we contracted with Taconic-Cyagen (Leverkusen, Germany) to construct two alleles of *Trek-2*, each of which unequivocally produced knockout alleles and removed all of exon 2 (*Fcc1 and Fcc2*). CRISPR guide sequences, which flanked exon 2, are available on request. The two new alleles were used for all studies reporting results for individual *Trek-2* data. Genotypes of *Trek-1, Trek-2* and *Ndufs4* were confirmed by PCR in each mouse studied. The loss of the TREK-1 and TREK-2 proteins was confirmed by immunohistochemistry (See Results). Primer sequences are also available on request.

Anesthetic sensitivity

Anesthetic endpoints were loss of righting reflex (LORR) or nonmovement during a non-crushing tail clamp (TC) using the methods described by Sonner.[32,33] Mice between p23–30 (if containing *Ndufs4*), or p23-30 and p57-70 (not containing *Ndufs4*) were anesthetized with either halothane or isoflurane, while their temperature was maintained by a water filled heating pad. The anesthetic exposures did not affect survival and all animals intended for study were successfully included. Temperature of the mice was monitored with a skin monitor and maintained between 37 and 38°C. Mice were exposed to both anesthetics, separated by 48 hours between exposures; the order of anesthetic treatment was randomized. The concentrations of halothane and isoflurane were monitored using a calibrated inline AA-8000 Anesthetic Agent Analyzer (BC Biomedical, Vancouver, BC, CA).

Responses to both the loss of righting reflex (LORR) and tail clamp (TC) assays were measured at each dose of anesthetic after 10 minutes of equilibration, beginning with a concentration of 0.6% for each anesthetic (or 0.2% for *Ndufs4* containing mice) and increasing in steps of 0.2%. Once the endpoint was reached, concentrations were decreased to determine when the animals regained their response. MAC was calculated as described by Sonner.[32]

The exceptions were the *Ndufs4*-containing animals which were anesthetized for both halothane and isoflurane for two behavioral endpoints. In *Ndufs4,* in pilot studies we observed that this battery of two long repeated exposures of one individual animal introduced confounding morbidity and mortality, something not observed in our previous paradigm of a single anesthetic exposure for one endpoint in one individual.[23,34] In addition, the rare births of the double mutants (*Ndufs4;Trek-1)* necessitated modifying the protocol to maximize data obtained from any one individual. Therefore, in this study of *Ndufs4* and *Ndufs4;Trek-1* animals, only the induction concentrations were determined, not the emergence values. Induction is defined as the midpoint between the last concentration at which the behavior was present and the first at which it is lost. Since this method is not the standard for determining MAC, we termed this endpoint the EC50 for induction. Using this method for *Ndufs4,* all animals survived, and those animals intended for study were all successfully included.

For a subset of experiments the anesthetic sensitivity was determined using the method described in the report of resistance of *Trek-1* to volatile anesthetics.[14] Briefly, this protocol rapidly increased anesthetic concentrations using clinical overpressure techniques to quickly induce anaesthesia in p57-70 mice. Then the anesthetic was slowly decreased to determine

the emergence concentration (the midpoint between the last concentration at which the behavior was absent and the first at which it is regained) at which the animal responded to a tail clamp. This concentration was reported as the MAC in the original publication. We report it as the emergence concentration in these studies.

ARRIVE Guidelines

Arrive Guidelines are listed in the Supplemental Digital Content 1.

Holding Currents

In all cases cells of the lateral ventral horn were patched. Ventral horn spinal cord cells were visualized using differential interference contrast microscopy. Cells could be identified as cholinergic or noncholinergic in some genotypes (*Ndufs4* and *Ndufs4;Trek-1*) that incorporated a fluorescent tag (Ai14) in cholinergic neurons as described previously.[24] This signal could be seen with fluorescence microscopy and was used in *Ndufs4*-containing slices to ensure use of non-cholinergic neurons. This selection was used since prior studies showed that the increased holding currents at 0.6% isoflurane in *Ndufs4* slices were seen only in non-cholinergic cells. Increased currents at 1.8% isoflurane in other genotypes were seen in all neuronal types of the spinal cord.

Drug Administration to spinal cord slices

Slices of lumbar spinal cord were isolated and studied as previously described. [24] They were first held in the superfusate for 30 minutes without isoflurane for baseline, unexposed measurements. Isoflurane was then applied in the superfusate at equilibrated concentrations delivered by passing carbogen (a mixture of 95% O_2 and 5% CO_2) through a calibrated isoflurane vaporizer. The superfusate was sampled during isoflurane exposure, and the isoflurane concentration was determined using gas chromatography. To rule out “run down” of the preparation, recordings were also made with no isoflurane exposure for the same period of time that matched the sum of experimental exposure and wash.

Norfluoxetine (hydrochloride) (Cayman Chemical #15900) was diluted to a final concentration of 20 μM in recording solutions from a stock solution of 10 mM in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA), based on prior published IC_{50}=9mcM for TREK blockade.[16] Norfluoxetine was added to the bath following a previously published protocol hat compared the effects of isoflurane with and without norfluoxetine.[22] In short, this protocol recorded at 0% isoflurane, then isoflurane exposure followed by its washout, then application of norfluoxetine for 15 minutes, followed by resumption of isoflurane exposure, now in the presence of norfluoxetine. The exception to this protocol in this study isin the *Trek-1;Trek-2* double mutant. These cells were very difficult to maintain in a patched status for a long period of time. Thus, after baseline measurements *Trek-1;Trek-2* slices were exposed to isoflurane and, after 15 minutes, norfluoxetine was added and currents measured in the presence of both agents for 10 minutes. *Trek-2* slices were repeated under this protocol to ensure that the two techniques gave comparable results.

Statistical analysis

Since heterozygotes for the *Trek* channels (*e.g. Trek-1*$^{+/-}$ and *Trek-2*$^{+/-}$) behave like wildtype in isoflurane and halothane, in this report we use the term wildtype to denote both C57Bl/6 (*Trek*$^{+/+}$ or *Trek*$^{+/-}$) mice. These heterozygotes are included in the wildtype designation in our tables. The term mutant refers to *Ndufs4(knockout), Trek-1(knockout)* or *Trek-2(knockout)* containing mice. All induction, emergence and MAC values for behavioral testing are expressed as the mean anesthetic concentration with standard deviation in parentheses (mean (SD followed by N (in the Tables) for number of animals studied and corresponding p-value. Effect sizes (ES) calculated by dividing the mean of the Trek mutant to the mean of the control using same endpoint and anesthetic and are listed in the tables. In the graphs, error bars refer to 95% confidence intervals. Since including all these values in the prose made the manuscript difficult to read, we included four tables with the values and referenced those tables when appropriate.

We used % anesthetic for comparison to prior reports; however, we also included temperature corrected aqueous concentrations of isoflurane for comparison in holding current results. In labels, “I or Iso” always refers to isoflurane, “H or Hal” to halothane. For normally distributed data we used a paired, one tailed t-test when comparing two groups, and a one-way ANOVA with Dunnet’s multiple comparisons test for greater than two groups. p-values were determined in Excel or in Prism and calculated for each paired group individually. For non-normally distributed data we used the Kolmogorov-Smironov test when comparing two groups, and the Kruskal-Wallis test with Dunn’s multiple comparison test for greater than two groups.

p-values refer to mutant values compared to CB57Bl/6 values determined in paired experiments (control and mutant done side by side) unless explicitly stated otherwise. In general, *Ndufs4;Trek-1* double mutants were limited in number such that having matched side-by-side measurements was not possible. In those cases, comparisons were made between the double mutant and *Ndufs4* groups but not as matched pairs. In addition, the EC50s for wildtype and *Ndufs4* strains listed in Table 1 are the cumulative means for those two strains from all experiments. We grouped all values from each of the paired tests which led to a larger N than for any individual comparison. Significance level was selected as a p-value less than 0.05. Post-hoc values were subjected to a Bonferroni correction for multiple comparisons to adjust significance for multiple comparisons. For comparisons to C57Bl/6 there were six groups leading to a corrected p-value for significance of 0.01. For comparisons to *Ndufs4* there were four groups leading to a corrected p-value for significance of 0.017.

In general, we measured one cell per spinal cord slice and one slice per animal. Thus, our N refers to animals, slices, and cells. For holding current studies, means and 95% confidence intervals are given in the Results and shown by box plots in the figures. p-values for comparisons of changes in holding currents are noted in the text and in Table 4. Further statistical considerations are listed in the Supplementary Materials. For measurements of EC50s, we defined a change of >10% as an effect size of biological significance. We used a difference of 10% from the EC50s for WT mice, a standard deviation of 0.1, an alpha of

0.05 and a desired power of 0.8 to determine adequate sample size (generally 7). In most cases, our sample sizes exceeded these values.

Results

Effect of TREK-1 on wildtype sensitivity

As controls for the effects of TREK-1 loss on *Ndufs4,* we first measured both induction and emergence in the presence of volatile anesthetics in *Trek-1* p23-p30 mice in a wildtype background for two knockout alleles, *tm1Lex*35 and *tm1Lzd*.[14] In response to a non-damaging tail clamp, we did not observe significant changes in either induction or emergence concentrations of isoflurane or halothane when comparing either allele of *Trek-1* mice to wildtype controls (Figures 1 A,B; Tables 1,2). Minimum alveolar concentrations (MACs) calculated from averaging the induction and emergence concentrations closely matched those previously published for wildtype mice:[32] Wildtype: (MAC(Hal), 1.30%(0.10); MAC(Iso), 1.40%(0.11): *Trek-1*^{tm1Lex} (MAC(Hal), 1.27%(0.11); p=0.387; MAC(Iso), 1.38%(0.09); p=0.268): *Trek-1*^{tm1Lzd} (MAC(Hal); 1.27%(0.11); p=0.482: MAC(Iso); 1.41%(0.12); p=0.188). p-values compare mutant values to paired wildtype values. Similarly, we also did not detect significant differences between the TREK-1 mutant strains and controls for loss of righting reflex in either anesthetic (Tables 1, 2).

The original reported resistance of *Trek-1*^{tm1Lzd} to volatile anesthetics was based on determining an average anesthetic concentration at which emergence occurred in p57-70 mice following a rapid induction.[14] We determined whether mice anesthetized under that protocol, which measured emergence as anesthetic concentrations were decreased, might display a resistance in *Trek-1*^{tm1Lex} *or Trek-1*^{tm1Lzd} compared to wildtype. Since the differences reported between wildtype and *Trek-1*^{tm1Lzd} were greatest in halothane, we specifically tested response to tail clamp in halothane, trying to exactly match the published protocol. We were unable to find any difference in sensitivity between the three genotypes using this emergence protocol (Figure 1C, Table 3). Wildtype (MAC(Hal); 1.23% (0.18)): *Trek-1*^{tm1Lex} (MAC(Hal); 1.28% (0.09); p=0.582): *Trek-1*^{tm1Lzd} (MAC(Hal); 1.25% (0.09); p=0.943. Of note, the MAC for the *Trek-1*^{tm1Lex} and *Trek-1*^{tm1Lzd} strains were similar to that earlier reported for *Trek-1*^{tm1Lzd}.[14] However, in that report of halothane resistance for *Trek-1,* the wildtype MAC was low (0.87%)[14] compared to values in the literature.[32,33] In our measurements the wildtype MAC was not shifted from other reports nor from that of either *Trek-1* allele.

The reported resistance of *Trek-1*^{tm1Lzd} mice also resulted from studies done in older mice than those used for our determinations of MACs.[14] We therefore also applied the standard MAC determination[32] to p57-70 mice for wild type and *Trek-1*^{tm1Lex} mice. When we repeated the studies in wildtype and *Trek-1*^{tm1Lex} mouse strains at the older age, we again found no difference between the mutant and wildtype in either isoflurane (Figure 1D, Table 3) or halothane (Figure 1E, Table 3). Wildtype (MAC(Hal); 1.23% (0.07), MAC(Iso); 1.22% (0.13): *Trek-1*^{tm1Lex} (MAC(Hal); 1.31% (0.16); p=0.401, MAC(Iso); 1.18% (0.12); p=0.492). p-values compare mutant values to paired wildtype values.

Effect of TREK-1 on *Ndufs4* sensitivity

It remained unclear to what extent the increased holding current seen in spinal neurons from *Ndufs4* mice[24] at low doses of isoflurane contributed to the profound hypersensitivity of the animal to isoflurane and halothane. We generated *Ndufs4;Trek-1*^{tm1Lex} and *Ndufs4;Trek-1*^{tm1Lzd} mice to test whether loss of TREK-1 expression would attenuate the profound anesthetic hypersensitivity of *Ndufs4* mice using two behavioral endpoints. As noted in Methods, for technical reasons we used only the induction concentrations to determine anesthetic sensitivities of these double mutants; we termed these results EC50 instead of MAC for that reason. There were no differences between *Ndufs4* mice and *Ndufs4;Trek-1*^{tm1Lex} or *Ndufs4;Trek-1*^{tm1Lzd} mice in the concentrations of isoflurane or halothane necessary to induce inhibition of the tail clamp response (Figure 2A, Tables 1 and 2). *Ndufs4* (EC50(Hal); 0.65% (0.05): EC50(Iso); 0.63% (0.05)): *Ndufs4;Trek-1*^{tm1Lex} (EC50(Hal); 0.58% (0.07); p=0.004; EC50 (Iso); 0.61% (.06); p=0.442): *Ndufs4;Trek-1*^{tm1Lzd} (EC50(Hal); 0.63% (0.09); p=0.529; EC50 (Iso); 0.64% (0.07); p=0.394). p-values report double mutant values compared to *Ndufs4* values. We did measure a small, but significant, difference in the concentration of halothane required to inhibit the tail clamp response. However, *Ndufs4;Trek-1*^{tm1Lex} mice exhibited an ***increase*** in sensitivity to halothane compared to *Ndufs4* mice (0.58% versus 0.65%), rather than a resistance as hypothesized (Figure 2A, Tables 1 and 2). We did not observe a difference between *Ndufs4* mice and *Ndufs4;Trek-1*^{tm1Lex} or *Ndufs4;Trek-1*^{tm1Lzd} mice in the loss of righting reflex response when exposed to isoflurane or halothane (Figure 2B, Tables 1 and 2).

Effect of TREK-2 on wildtype sensitivity

Trek-1 animals are not resistant to anesthetics. However, TREK-2 is of interest for its potential role in anesthetic behavior in wild type animals as a cause for the isoflurane-induced, norfluoxetine-inhibitable, increase in holding current. We measured both induction and emergence in the presence of volatile anesthetics in *Trek-2* mice for two knockout alleles, *Fcc1* and *Fcc2*. In response to tail clamp, we did not observe significant changes in the EC50s of isoflurane or halothane when comparing either allele of *Trek-2* mice to wildtype controls (Figures 1A,B, Tables 1,2). MACs closely matched those previously published for wildtype mice and our values reported above:[32] (*Trek-2*$^{(Fcc1)}$ (MAC(Hal); 1.29%(0.14); p=0.073; MAC(Iso); 1.41%(0.09); p=0.412) (*Trek-2*$^{(Fcc2)}$ (MAC(Hal); 1.36%(0.12); p=0.330: MAC(Iso); 1.36%(0.09); p=0.086). p-values refer to mutant values compared to paired wildtype values. In the two cases where there was a trend toward significance, the *Trek* mutants tended to increased sensitivity, not resistance. Similarly, we did not detect any significant differences between the *Trek-2* mutant strains and controls for loss of righting reflex (Tables 1,2).

In addition, and in response to the editor's request, although our study was not specifically designed to detect sex-specific differences in anesthetic sensitivity, *post-hoc* evaluation of our data does not suggest differences between male and female animals in terms of EC50s to either halothane or isoflurane. While we detected statistically significant differences in mean concentrations for *Trek-1* male and female responses to isoflurane for loss of righting reflex (MAC(Iso); male 0.73(0.04), female 0.82(0.10); p=0.004), these small differences

pose little functional significance and did not warrant further investigation. (Supplemental Digital Content 2, Table S1).

Effect of TREK-1 and TREK-2 together on wildtype sensitivity

We finally considered whether the loss of both TREK-1 and TREK-2 channels might affect anesthetic sensitivity of the wildtype animal. We constructed the double mutant *Trek-1*^{tm1Lex}*;Trek-2*^{Fcc1} and measured the MACs for halothane and isoflurane. In response to tail clamp, we did not observe significant changes in the EC50s of isoflurane or halothane when comparing *Trek-1;Trek-2* mice to controls (Figure 1A,B Tables 1,2). MACs calculated from averaging the induction and emergence concentrations closely matched those previously published for wildtype mice and our values reported above:[32] (*Trek-1*^{tm1Lex}*;Trek-2*^{Fcc1} (MAC(Hal); 1.26%(0.14); p=0.661: MAC(Iso); 1.46%(0.06); p=0.373)). p-values refer to double mutant values compared to paired control values. Similarly, we did not detect any significant differences between the double mutant strains and controls for loss of righting reflex (Tables 1 and 2).

Holding currents.—Our interest in the TREK-1 channel was initially the result of a norfluoxetine-inhibitable increase in leak current in non-cholinergic neurons of the ventral spinal cord of *Ndufs4*[24] upon exposure to low concentrations of isoflurane, coupled with a report that a *Trek-1* mouse was resistant to anesthetics.[14] Since norfluoxetine blocks both TREK-1 and TREK-2 channels,[16,17] we considered whether TREK-1 or TREK-2 might be up-regulated in *Ndufs4* mice. After verifying that our alleles of Trek-1 and Trek-2 did not produce a protein product (Figures 3A, B) we found that there was no increase in TREK-1 or TREK-2 staining in *Ndufs4* spinal cords (Figures 3C,D) compared to those from wildtype animals.

While norfluoxetine affects other targets,[36–38] the isoflurane-induced currents were blocked by norfluoxetine. This indicates that the increases in holding current upon anesthetic exposure, indicative of hyperpolarization of the cell, are most likely carried by TREK channels. Blocking TREK channels therefore eliminates the rise in holding current upon isoflurane exposure. We determined whether the isoflurane-induced holding current was still present in *Ndufs4;Trek-1*^{tm1Lex} and *Trek-1*^{tm1Lex} mice, and whether an isoflurane-induced increase in these animals would be sensitive to norfluoxetine. Baseline spinal cord holding currents did not differ significantly between wildtype and mutant strains (Table 4).

In agreement with our previous results, holding currents increased in *Ndufs4* slices at 0.6% isoflurane but not in wildtype slices (Supplemental Digital Content 3, Figure S1).[24] Holding currents (HC) also increased in *Ndufs4;Trek-1*^{tm1Lex} exposed to 0.6% isoflurane (~0.25mM) (Isoflurane 0.6%; ΔHC 110.9 (61.45 to 169.18), p=0.015 compared to no isoflurane) (Figure 4A). Norfluoxetine approached significance in blocking the rise in holding current in *Ndufs4;Trek-1*^{tm1Lex} (ΔHC 42.3 (23.1 to 61.7), p=0.077 norfluoxetine plus isoflurane compared to isoflurane (Table 4). Representative curves are shown for all comparisons in Figure 4A–D.

In slices of *Trek-1*^{tm1Lex}, *Trek-2*^{Fcc1} and *Trek-1*^{tm1Lex};*Trek-2*^{Fcc1} slices, isoflurane (0.74mM) increased holding currents, just as it did in wildtype slices (Figures 4B–D).

However, norfluoxetine did not block the increase seen in *any of the mutants compared to the increases* at 1.8% isoflurane in the absence of norfluoxetine (Figures 4B–D, Table 4). These data implicate recruitment of norfluoxetine-insensitive channels to increase holding currents caused by isoflurane in spinal cord neurons.

Discussion.

In mice, loss of TREK-1 or TREK-2 channels individually, or as a double mutant, has no significant impact on the tail clamp response to isoflurane or halothane. Neither induction nor emergence values separately were significantly different in *Trek(KO)* mice compared to wildtype controls, and there were no significant differences in MAC values. The same was true for loss of righting reflex; loss of TREK channels did not affect that endpoint. Furthermore, we failed to replicate the resistance seen during emergence from volatile anesthetics observed in *Trek-1* mice following a nonstandard induction protocol.[14] However, the resistance to halothane reported previously for *Trek-1*^{tm1Lzd} appears to be based on a low MAC value in control animals compared to that customarily found in the literature.[32] Since those data have been frequently cited as evidence for the K2P channels as anesthetic targets, it is important to note that we were unable to repeat these findings of anesthetic resistance.

Interest in the TREK channels and behavior in anesthetic stems from initial work in the marine mollusk, *Aplysia californica*. It was discovered there that volatile anesthetics activate an outwardly rectifying K^+ channel, the S channel.[39] Mammalian TREK-1 and TREK-2 are proposed orthologues of the *Aplysia* S channel; they share many biophysical and pharmacological properties.[40–42] Their activation hyperpolarizes neurons causing a predicted decrease in activity. In addition, TREK-1 and TREK-2 are activated by several volatile anesthetics including isoflurane [26,27] and both channels are widely expressed in the mammalian spinal cord.[43] Recent data have also shown the important sites in TREK-1 for transducing the effect of isoflurane on the channel to increase conductance.[28]

It is well-established that volatile anesthetics can hyperpolarize neurons,[44,45] and our data corroborate those findings in the spinal cord of adult mice.[24] These data thus fit well with the previous report of resistance to volatile anesthetics of *Trek-1*^{tm1Lzd} mice.[14] Cells in the spinal cord expressing TREKs would be predicted to become hyperpolarized, and therefore less active, with exposure to VAs. We therefore expected that this hyperpolarization, induced by VA concentrations that correlated to whole animal phenotypes would contribute to the anesthetic response in both wildtype and the mitochondrial mutant *Ndufs4*. A role for TREK-1 channels in the hypersensitivity of *Ndufs4* would have indicated a potential unifying model for anesthetic mechanisms involving those channels.

However, loss of either or both TREK channels did not change behavior of wild type or *Ndufs4* animals in isoflurane or halothane. In addition, an isoflurane-inducible rise in holding current remained present with TREK loss in both wildtype and *Ndufs4* animals. However, removal of TREK channels in wildtype animals caused the isoflurane-inducible increase in holding currents to become norfluoxetine-insensitive. Other (norfluoxetine-insensitive) potassium channels, such as the TASK channels, have also been shown to be induced by volatile anesthetics and may also affect volatile anesthetic sensitivity.[46,47] We

have not ruled out compensatory changes in TASK channels, or other possible effectors, when TREK channels are removed; in fact, our data indicate that they likely exist. They may be recruited to conduct the current seen when either TREK is removed. However, the blockade of the holding current in the wildtype by norfluoxetine would appear to rule out the TASK family of K2P channels for the potassium current seen in “normal,’” wildtype slices.[48]

Loss of TREK-1 also failed to inhibit the increase in holding current of *Ndufs4* neurons to 0.6% isoflurane. However, the interpretation of the effect of norfluoxetine on the rise is less clear In *Ndufs4;Trek-1*^{tm1Lex} (Figure 4B). It may be that there is a partial block, but clarification awaits further studies. At this time, we hypothesize that if our data represent a partial block, then the hyperpolarizing current is a mix of both norfluoxetine-sensitive and -insensitive channels. Construction of a *Trek-1;Trek-2;Ndufs4* triple knockout is underway clarify our data. It is noteworthy that since TASK channels affect current in cholinergic neurons, we did not see any rise in holding currents when we measured signal from cholinergic cells of the spinal cord in *Ndufs4*.[24] This makes a role for TASK channels unlikely in mediating an isoflurane induced hyperpolarizing current in *Ndufs4.*

Most importantly, the whole animal behavior of two different knockout alleles of both *Trek-1* and *Trek-2*, as well as in the *Trek-1;Trek-2* double mutant did not reveal any resistance to either of two different volatile anesthetics for two different endpoints. The original report of resistance to volatile anesthetics in a *Trek-1*^{tm1Lzd} animal was performed nearly two decades ago.[14] It is possible that the background genetics of that animal were different than the current C57Bl/6, or that genetic drift has occurred over time, causing a novel compensatory change and a return to wildtype sensitivity. Regardless, loss of these particular K2P channels does not change anesthetic resistance in our hands. The interpretation of the increase in holding current seen with the application of isoflurane to spinal cord slices also needs re-evaluation The electrophysiologic phenomenon of isoflurane-induced increased potassium currents is maintained even in the absence of TREK channels and may contribute to the action of volatile anesthetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements.

The authors are indebted to Florian Lesage, PhD (Professor, Universit C te d’Azurm, FR) and Andreas Schwingshackl, PhD (Professor, UCLA, USA) for sharing the *Trek-1*^{tm1Lzd}*,Trek-2(KO),Traak(KO)* mice. The authors thank Beatrice Predoi, MD, PhD and Miranda Howe for their excellent technical assistance, and Julia Stokes and Angelina Zimenko for technical support. They also thank Sangwook Jung, PhD for his insightful discussions and reading of the manuscript.

Funding.

MMS, JMR, SJ, KAS, and CW were supported in part by NIH grant R01GM105696 and by continued support from the Northwest Mitochondrial Research Guild. KAS was supported in part by T32 GM086270. PGM, CW were supported in part by NIH grant R35GM139566.

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Summary Statement.

Mice carrying knockout alleles of *Trek-1* and/or *Trek*-2 are not resistant to volatile anesthetics. TREK-1 does not affect the hypersensitivity of *Ndufs4* mice. The roles of TREK channels in volatile anesthetic sensitivity need re-evaluation.

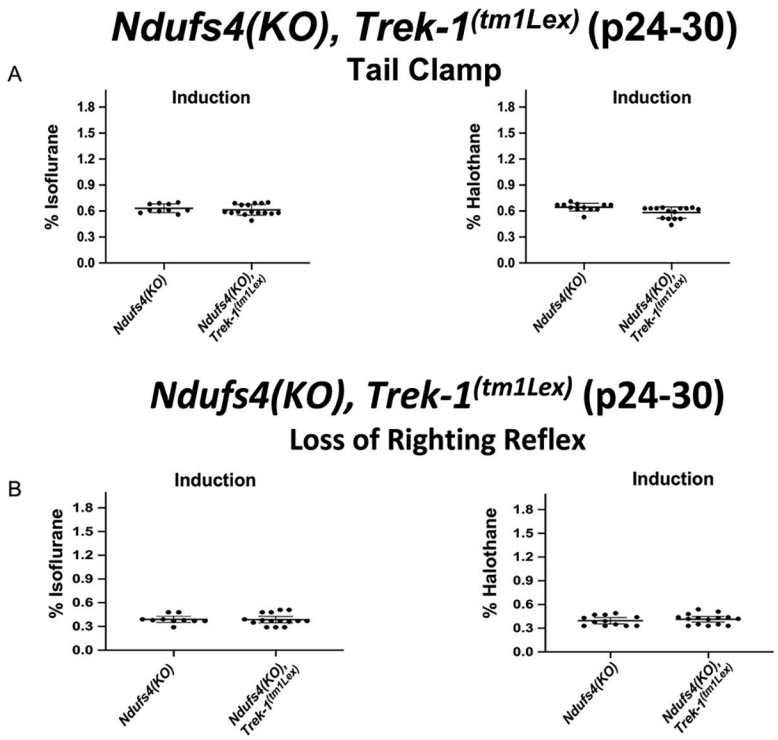

Figure 1. Knocking out *Trek-1* or *Trek-2* does not significantly change induction or emergence values for either halothane or isoflurane in mouse responses to a tail clamp.

A. Graphs show standard induction (left panel), emergence (middle panel) and MAC (right panel) values for mouse strains in isoflurane. Each panel shows values for wildtype, two knockout alleles of *Trek-1,* one allele of *Trek-2*, and the double knockout *Trek-1;Trek-2.* In this and subsequent graphs, solid line indicates mean values, error bars=+/−95% confidence intervals (CI). MAC and p-values are listed in Tables 1 and 2 and p values compare MAC values for mutant to wildtype means. **B.** Standard induction (left panel), emergence (middle panel) and MAC (right panel) values for mouse strains in halothane. As in Figure 1A, each panel shows values for wildtype, two knockout alleles of *Trek-1,* one allele of *Trek-2*, and the double knockout *Trek-1;Trek-2.* MAC and p-values are listed in Tables 1 and 2. **C.** Emergence EC50s for wildtype and *Trek-1*^{tm1Lex} in halothane. The technique approximated that described by Heurteaux *et al.* [14] using a high concentration induction followed by a slow decrease in concentrations to determine the emergence EC50s. Wildtype (MAC(Hal); 1.23%(0.18); *Trek-1*^{tm1Lex} (MAC(Hal); 1.28%(0.09); p=0.582. EC50 and p-values are listed in Table 3. **D.** Standard induction (left panel), emergence (middle panel) and MAC (right panel) values for p57-70 mouse strains wildtype and *Trek-1*^{tm1Lex} in isoflurane. Wildtype MAC(Iso); 1.22%(0.13); *Trek-1*^{tm1Lex} MAC(Iso); 1.18%(.12); p=0.492. MAC and p-values are listed in Table 3. **E.** Standard induction (left panel), emergence (middle panel) and MAC (right panel) values for p57-70 mouse strains wildtype and *Trek-1*^{tm1Lex} in halothane. Wild type MAC(Hal); 1.23%(0.07); *Trek-1*^{tm1Lex} MAC(Hal); 1.31%(0.16); p=0.401. In Figure 1, no comparisons reached significance. (See Tables 1 and 2) **Abbreviations**: Iso, Isoflurane; Hal, Halothane.

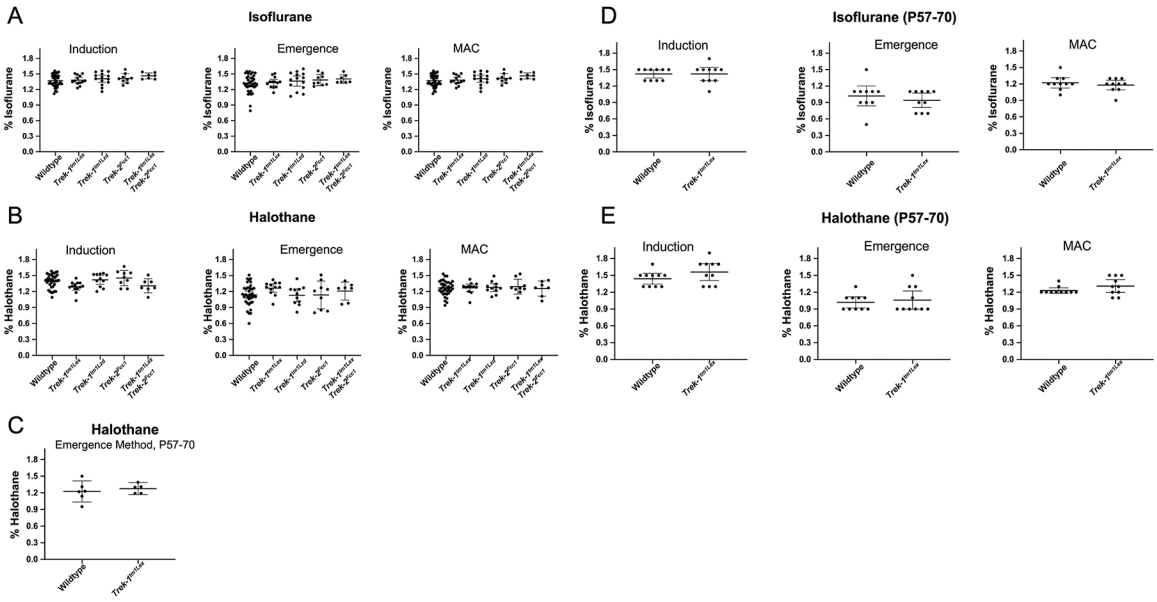

Figure 2. The loss of *Trek-1* does not change the increased sensitivity of *Ndufs4* mice to isoflurane or halothane for two behavioral endpoints.

The endpoints are as labeled, the term induction denotes the midpoint between the last concentration at which the behavior was present and the first at which it is lost. For technical reasons, only induction values were measured, see Methods, and are termed EC50 rather than MAC. **A.** Induction values for loss of tail clamp response in mouse strains *Ndufs4* and *Ndufs4, Trek-1*^{tm1Lex} in isoflurane (left panel) and halothane (right panel). *Ndufs4* EC50(Hal); 0.65%(0.05); EC50(Iso); 0.63%(0.05): *Ndufs4;Trek-1*^{tm1Lex} (EC50(Hal); 0.58%(0.07), p=0.045; EC50(Iso); 0.61%(.06), p=0.442). **B.** Induction values for loss of righting reflex response in mouse strains *Ndufs4* and *Ndufs4;Trek-1*^{tm1Lex} in isoflurane (left panel) and halothane (right panel). *Ndufs4* (EC50 (Hal); 0.40%(0.06): EC50 (Iso); 0.39%(0.06): *Ndufs4;Trek-1*^{tm1Lex} (EC50 (Hal); 0.41%(0.07), p=0.453; EC50 (Iso); 0.39%(0.08), p=0.924. p-values report double mutant values compared to *Ndufs4* values. In Figure 2, no comparisons reached significance. See Tables 1 and 2 which contain data for both alleles of *Trek-1.* **Abbreviations**: Iso, Isoflurane; Hal, Halothane.

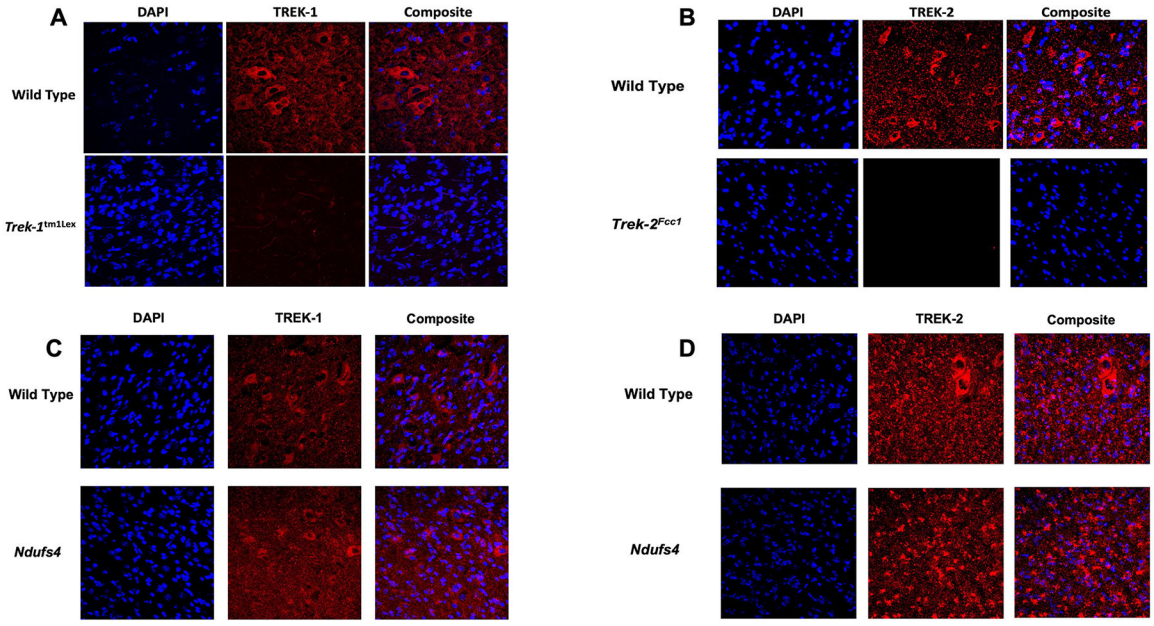

Figure 3. Effects of loss of *Ndufs4* or TREK-1 on TREK-2 expression.

In all figures, the left panels are DAPI staining to locate nuclei, the middle panels are stained with the antibody to the protein labeled at top (TREK-1 or TREK-2) and the right panels are the merged figures. The genotypes are labeled on the left. **A.** Representative staining in red of TREK-1 (middle panels) in wildtype (upper row) or *Trek-1*^{tm1Lex} (lower row) spinal cords. Absence of staining in *Trek-1*^{tm1Lex} confirms that it is a knockout for TREK-1. **B.** Representative staining in red of TREK-2 (middle panels) in wildtype (upper row) or *Trek-2*^{Fcc1} (lower row) spinal cords. Absence of staining in *Trek-2*^{Fcc1} confirms that it is a knockout for TREK-2. **C.** Representative staining in red of TREK-1 (middle panels) in wildtype or *Ndufs4* spinal cord. There is no evidence of upregulation of the TREK-1 channel in an *Ndufs4* spinal cord. **D.** Representative staining in red of TREK-2 in wildtype or *Ndufs4* spinal cord. There is no evidence of upregulation of the TREK-2 channel in an *Ndufs4* spinal cord. **Abbreviations**: DAPI, 4′,6-diamidino-2-phenylindole used to stain DNA

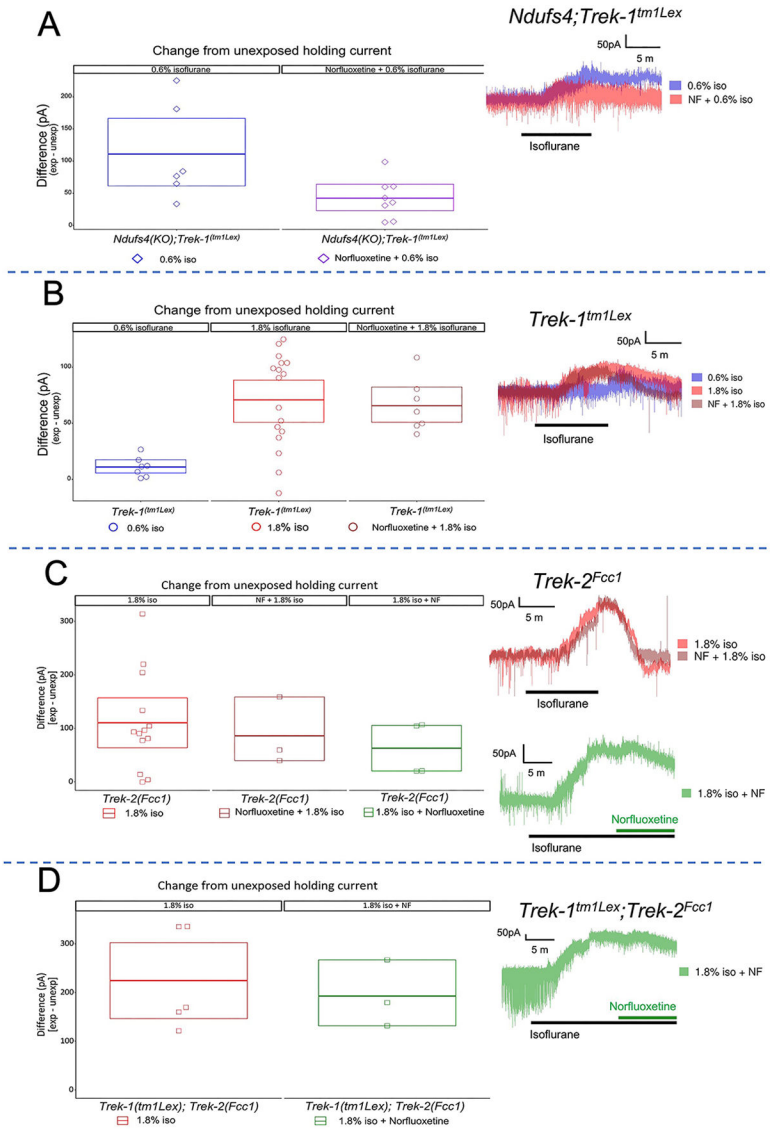

Figure 4. Effect of *Trek (KOs)* on holding currents in mouse spinal cord neurons.

No significant differences in resting holding currents were noted between the genotypes at baseline (See Table 4). All box plots show mean (center line) +/− 95% confidence intervals. **A.** Holding currents (HC) were increased in *Ndufs4(KO),Trek-1^{tm1Lex}* exposed to 0.6% Isoflurane (ΔHC 110.9 (61.45 to 169.18), p=0.015 compared to unexposed, left boxplot); the rise was blocked by norfluoxetine (ΔHC 42.3 (23.1 to 61.7), p=0.006 compared to isoflurane without norfluoxetine, right boxplot). A representative tracing is shown to the right. The initial recording period is shown in blue, and the method is described in reference #24. The pink tracing shows the continuation of the experiment. After isoflurane washout, norfluoxetine was added to the bath, and 15 minutes later, isoflurane exposure was resumed. Norfluoxetine was present throughout the time course of the tracing shown in pink. **B.** Holding currents were increased in *Trek-1^{tm1Lex}* exposed to 1.8% Isoflurane (ΔHC 70.8 (50.7 to 88.6), p<0.001 compared to unexposed, middle boxplot) but the rise was not blocked by norfluoxetine (ΔHC 65.6 (51.6 to 82.7), p<0.001 compared to unexposed, right

boxplot.) Representative tracings are shown to the right. The tracing shown in blue shows a lack of response of *Trek-1*^{tm1Lex} to 0.6% isoflurane. The pink tracing shows a rise in response to 1.8% isoflurane. Its continuation, after washout, norfluoxetine equilibration, and resumption of 1.8% isoflurane (brown tracing) revealed that norfluoxetine did not inhibit the rise in holding current. **C.** Holding currents were increased in *Trek-2*^{Fcc1} exposed to 1.8% Isoflurane (ΔHC 110.2 (67.3 – 161.8), p=0.004 compared to unexposed, left boxplot) but the rise was not blocked by norfluoxetine (Norfluoxetine plus isoflurane, ΔHC 86.0 (46.1 – 158.8), p=0.616 compared to isoflurane without norfluoxetine, middle boxplot). The protocol was as in **4B.** Representative tracings are shown on the right as red and brown tracings. The right boxplot represents data collected in a shortened protocol, needed for a comparison to recordings necessitated by *Trek-1;Trek-2* double mutant – see below, **4D**. In this case isoflurane was present throughout the experiment, and norfluoxetine added after fifteen minutes of isoflurane exposure. This much shorter protocol, with a representative tracing in green on the right, also demonstrated lack of inhibition by norfluoxetine of the rise in holding current in 1.8% isoflurane (Isoflurane plus norfluoxetine, ΔHC 62.9 (20.3 – 105.5), p=0.210). **D.** Holding currents were increased in *Trek-1*^{tm1Lex}*;Trek-2*^{Fcc1} exposed to 1.8% Isoflurane (ΔHC 224.3 (148.2 – 302.3), p<0.001 compared to unexposed, left boxplot) but the rise was not blocked by norfluoxetine (ΔHC 192.8 (131.6 – 267.2), p=0.623 compared to isoflurane without norfluoxetine, right boxplot). As noted in the Methods, *Trek-1;Trek-2* cells were very difficult to maintain in a patched status for a long period of time. Thus, for these slices, the norfluoxetine was added to the bath following 15 minutes of isoflurane exposure. A representative tracing is shown in the right panel. **Abbreviations:** HC, holding current to maintain transmembrane potential at −60 millivolts; NF, norfluoxetine; Iso, Isoflurane.

Table 1.

The EC50s (+/−S.D., N) for Tail Clamp and loss of righting reflex of multiple genotypes in isoflurane and halothane.

Strain	LORR Iso	LORR Hal	Tail Clamp Iso	Tail Clamp Hal
Wildtype Controls	0.81(0.10,37)	0.78(0.07,35)	1.40(0.11,37)	1.30(0.10,34)
Trek-1 ^{tm1Lex}	0.78 (0.07,11)	0.77(0.06,11)	1.38(0.09,13)	1.27(0.11,12)
Trek-1 ^{tm1Lzd}	0.80(0.11,14)	0.76(0.07,11)	1.41(0.12,14)	1.27(0.11,11)
Trek-2 ^{Fcc1}	0.83(0.07,9)	0.81(0.05,9)	1.41(0.09,9)	1.29(0.14,9)
Trek-2 ^{Fcc2}	0.82(0.08,9)	0.80(0.07,9)	1.36(0.09,9)	1.36(0.12,9)
Trek-1 ^{tm1Lex} *;Trek-2* ^{Fcc1}	0.73 (0.07,7)	0.68(0.05,7)	1.46 (0.06,7)	1.26(0.14,7)
Ndufs4	0.37(0.05,19)	0.39(0.04,15)	0.63(0.05,20)	0.65(0.05,18)
Ndufs4; Trek-1 ^{tm1Lex}	0.39(0.07,15)	0.41(0.06,12)	0.61(0.06,16)	0.58(0.07,15)
Ndufs4;Trek-1 ^{tm1Lzd}	0.40(0.12,6)	0.41(0.07,5)	0.64(0.07,6)	0.63(0.09,5)

No p-values reached significance (see Table 2) and are reported for the comparison to wildtype or to *Ndufs4*, as appropriate. *Trek* knockouts did not cause significant differences from either wildtype or *Ndufs4* genetic backgrounds. All MACs on this chart were determined by the up/down method described by Sonner *et al.*[23,24] while the EC50s (those containing the *Ndufs4* mutation) were determined by induction only. Superscripts denote alleles. Wildtype controls are C57Bl/6 or heterozygotes for *Trek-1* or *Trek-2* in a C57Bl/6 background.

Abbreviations. S.D., Standard Deviation; N, Number of animals studied; LORR, Loss of Righting Reflex; Iso, Isoflurane; Hal, Halothane.

Table 2.

The p-values and effect sizes [mean(p, Effect Size (ES)) for comparison of *Trek* strains to wildtype controls (MACs) and of *Ndufs4;Trek* strains to *Ndufs4* (EC50s) reported in Table 1.

Strain	LORR Iso (p, ES)	LORR Hal (p, ES)	Tail Clamp Iso (p, ES)	Tail Clamp Hal (p, ES)
Wildtype	0.81	0.78	1.40	1.30
Trek-1^{tm1Lex}	0.78 (p=0.167, 0.96)	0.77 (p=0.251, 0.99)	1.38 (p=0.268, 0.99)	1.27 (p=0.387, 0.98)
Trek-1^{tm1Lzd}	0.80 (p=0.385, 0.99)	0.76 (p=0.701,0.97)	1.41 (p=0.188, 1.01)	1.27 (p=0.482, 0.98)
Trek-2^{Fcc1}	0.83 (p=0.619, 1.02)	0.81 (p=0.314,1.04)	1.41 (p=0.412, 1.01)	1.29 (p=0.073, 0.99)
Trek-2^{Fcc2}	0.82 (p=0.364, 1.01)	0.80 (p=0.278,1.03)	1.36 (p=0.086, 0.97)	1.36 (p=0.330, 1.05)
Trek-1^{tm1Lex};*Trek-2*^{Fcc1}	0.73 (p=0.143, 0.90)	0.68 (p=0.709,0.87)	1.46 (p=0.373, 1.04)	1.26 (p=0.661, 0.97)
Ndufs4	0.37	0.39	0.63	0.65
Ndufs4;Trek-1^{tm1Lex}	0.39 (p=0.886, 1.05)	0.41 (p=0.453, 1.05)	0.61 (p=0.442, 0.97)	0.58 (p=0.004, 0.89)
Ndufs4;Trek-1^{tm1Lzd}	0.40 (p=0.528, 1.08)	0.41 (p=0.606, 1.05)	0.64 (p=0.394, 1.02)	0.63 (p=0.529, 0.97)

No significant differences were caused by a *Trek* knockout. Effect sizes (ES) calculated by dividing the mean of the Trek mutant to the mean of the control using same endpoint and anesthetic. All MACs on this chart were determined by the up/down method described by Sonner *et al.*[23,24] while the EC50s (those containing the *Ndufs4* mutation) were determined by induction only. Superscripts denote alleles. Controls are C57Bl/6 or heterozygotes for *Trek-1* or *Trek-2* in a C57Bl/6 background.

Abbreviations. ES, Effect Size; Iso, Isoflurane; Hal, Halothane.

Table 3.

Minimum alveolar concentrations (MACs) or EC50s for isoflurane and halothane in *Trek-1* using different methods and ages.

Strain	Isoflurane (S.D.,N, p value)	Halothane (S.D.,N, p value)	Notes
Wildtype	1.37(0.11,39)	1.27(0.14,37)	P23–65, Up/down method
Wildtype	ND	1.23%(0.18,N=6)	P57–70, emergence method
Trek-1 ^{tm1Lex}	ND	1.28%(0.09,N=5; p=0.757)	P57–70, emergence method
Trek-1 ^{tm1Lzd}	ND	1.25%(0.09,N=5; p=0.943)	P57–70, emergence method
Wildtype	1.22%(0.13;N=10)	1.23%(0.07,N=10)	P57–70, Up/down method
Trek-1 ^{tm1Lex}	1.18%(0.12;N=10,p=0.492)	1.31%(0.16,p=0.401)	P57–70, Up/down method

Protocols for determining MACs are given in the Methods. Wildtype controls are C57Bl/6 or heterozygotes for *Trek-1* or *Trek-2* in a C57Bl/6 background. p=values report comparisons between mutant and wildtype anesthetized in the same manner. No comparison between *Trek-1* and wildtype reached significance.

Abbreviations. S.D., Standard Deviation; N, Number of animals studied; ND, Not Done; P##, Post Delivery age.

Table 4.

Effects of Isoflurane and of norfluoxetine on change in holding currents (ΔHC) in spinal cord neurons of Ndufs4 and Trek alleles. 95% confidence intervals in parentheses.

Strain	Unexposed baseline holding current (pA)	0.6% Isoflurane (CI) (ΔHC)	1.8% isoflurane (CI) (ΔHC)	Isoflurane plus norfluoxetine (ΔHC)
Wildtype	5.4 (−15.3 – 25.2)	NC	71.6 (45.1 – 96.7), p<0.001 [a]	14.7 (−6.5 – 35.4), p=0.007 [b]
Trek-1 [(tm1Lex)]	17.5 (6.8 – 28.6)	NC	70.8 (50.7 – 88.6), p<0.001 [a]	65.6 (51.6 – 82.7), p=0.705 [b]
Trek-2 [(Fcc1)]	65.1 (18.6 – 133.8)	ND	110.2 (67.3 – 161.8), p=0.004 [a]	86.0 (46.1 – 158.8), p=0.616 [b] [*]62.9 (20.3 – 105.5), p=0.210 [b]
Trek-1;Trek-2	12.1 (−26.2 – 42.4)	ND	224.3 (148.2 – 302.3), p<0.001 [a]	[*]192.8 (131.6 – 267.2), p=0.623 [b]
Ndufs4	58.2 (34.6 – 91.3)	126.1(97.9 – 151.6), p<0.001 [a]	ND	10.6(-1.4 – 26.2), p<0.001 [b]
Ndufs4;Trek-1	26.8 (9.7 – 52.1)	110.9 (61.45 – 169.18), p=0.015 [a]	ND	42.3 (23.1 – 61.7), p=0.077 [b]

Wildtype are C57Bl/6 or heterozygotes for *Trek-1* or *Trek-2* in a C57Bl/6 background.

a. p-value compares holding current in the presence of isoflurane to that in absence of isoflurane.

b. p-value compares holding current in the presence of isoflurane plus norfluoxetine to that in presence of isoflurane without norfluoxetine.

[*] (For second value of *Trek-2* and for *Trek-1;Trek-2* refers to the (1.8% isoflurane + Norfluoxetine) protocol as described in the legend for Figure 4C and D. This corresponds to the green tracings in Figures 4C,D.)

Abbreviations. pA, picoamps; HC, Holding Current; CI, 95% Confidence Intervals, ND, Not Done; NC, No Change from Baseline.