ANESTHESIOLOGY

# Mitochondrial Function and Anesthetic Sensitivity in the Mouse Spinal Cord

Christian B. Woods, B.S., Kira A. Spencer, Ph.D., Sangwook Jung, Ph.D., Hailey M. Worstman, B.A., Jan-Marino Ramirez, Ph.D., Philip G. Morgan, M.D., Margaret M. Sedensky, M.D.

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## EDITOR'S PERSPECTIVE

### What We Already Know about This Topic

- Volatile anesthetics–induced inhibition of motor response to painful stimuli is primarily mediated via the neural circuitry of the ventral spinal cord
- Disrupting mitochondrial complex I results in profound hypersensitivity to volatile anesthetics
- The role of mitochondrial complex I in mediating volatile anesthetic action in the spinal cord is incompletely understood

### What This Article Tells Us That Is New

- In transgenic mice lacking a subunit of mitochondrial complex I, isoflurane increased an outwardly rectifying potassium current in the ventral horn of the spinal cord at a concentration much lower than in wild-type controls
- The effects were mediated by noncholinergic neurons and were independent of presynaptic functions
- These observations provide us with a functional link between sensitivity to volatile anesthetics, mitochondrial function, and postsynaptic potassium channel activity

## ABSTRACT

**Background:** *Ndufs4* knockout (KO) mice are defective in mitochondrial complex I function and hypersensitive to inhibition of spinal cord–mediated response to noxious stimuli by volatile anesthetics. It was hypothesized that, compared to wild-type, synaptic or intrinsic neuronal function is hypersensitive to isoflurane in spinal cord slices from knockout mice.

**Methods:** Neurons from slices of the vestibular nucleus, central medial thalamus, and spinal cord from wild-type and the global *Ndufs4* knockout were patch clamped. Unstimulated synaptic and intrinsic neuronal characteristics were measured in response to isoflurane. Norfluoxetine was used to block TREK channel conductance. Cholinergic cells were labeled with tdTomato.

**Results:** All values are reported as means and 95% CIs. Spontaneous synaptic activities were not different between the mutant and control. Isoflurane (0.6%; 0.25 mM; *Ndufs4[KO]* EC95) increased the holding current in knockout (ΔHolding current, 126 pA [95% CI, 99 to 152 pA]; ΔHolding current $P < 0.001$; n = 21) but not wild-type (ΔHolding current, 2 7 pA [95% CI, 9 to 47 pA]; ΔHolding current, $P = 0.030$; n = 25) spinal cord slices. Knockout and wild-type ΔHolding currents were significantly different ($P < 0.001$). Changes comparable to those in the knockout were seen in the wild type only in 1.8% (0.74 mM) isoflurane (ΔHolding current, 72 pA [95% CI, 43 to 97 pA]; ΔHolding current, $P < 0.001$; n = 13), the control EC95. Blockade of action potentials indicated that the increased holding current in the knockout was not dependent on synaptic input (ΔHolding current, 154 pA [95% CI, 99 to 232 pA]; ΔHolding current, $P = 0.506$ compared to knockout without blockade; n = 6). Noncholinergic neurons mediated the increase in holding current sensitivity in *Ndufs4* knockout. The increased currents were blocked by norfluoxetine.

**Conclusions:** Isoflurane increased an outwardly rectifying potassium current in ventral horn neurons of the *Ndufs4(KO)* mouse at a concentration much lower than in controls. Noncholinergic neurons in the spinal cord ventral horn mediated the effect. Presynaptic functions in *Ndufs4(KO)* slices were not hypersensitive to isoflurane. These data link anesthetic sensitivity, mitochondrial function, and postsynaptic channel activity.

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Volatile anesthetics are widely used, yet their molecular mechanisms and critical targets of action remain debated. Although the anesthetized state is defined by several behavioral endpoints, anesthetic potency is commonly defined as the minimum alveolar concentration (MAC) necessary to produce immobility to a noxious stimulus.[1] Studies in multiple animals have established that volatile anesthetics inhibit the motor response to painful stimuli (tail clamp) primarily through action in the ventral spinal cord.[2–6] The neuronal circuitry in the ventral spinal cord includes afferent sensory neurons, efferent motor neurons, and interneurons (glutamatergic, γ-aminobutyric acid–mediated [GABAergic], and glycinergic) that modulate both sensory input and motor output.[6] Early electrophysiologic studies suggested that motor neuron excitability is depressed in the presence of volatile anesthetics,[7,8] most likely by hyperpolarization,[9] and that ventral horn neurons were particularly sensitive to inhibition by an array of

This article is featured in "This Month in Anesthesiology," page A1. This article is accompanied by an editorial on p. 835. Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org). P.G.M. and M.M.S. contributed equally to this article.

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volatile anesthetics.[10] Studies from the Kendig group later indicated that interneurons may be important spinal cord targets, but cholinergic motor neurons were not completely ruled out.[11–13] The precise mechanisms responsible for volatile anesthetic action in the spinal cord remain debated.

One promising candidate for a physiologic target of volatile anesthetics is mitochondrial complex I,[14,15] the rate-limiting step in the electron transport chain. Evidence across the animal kingdom, from invertebrates (worms and flies)[15,16] to mammals (mice and humans),[17,18] indicates that disrupting mitochondrial complex I results in profound hypersensitivity to volatile anesthetics. Because a myriad of processes within the central nervous system are highly energy-dependent,[19,20] inhibiting mitochondrial function has the potential to broadly disrupt function of the nervous system.

The *Ndufs4(KO)* mouse lacks a subunit of mitochondrial complex I[21] and displays the profound hypersensitivity to volatile anesthetics. In response to a nondamaging tail clamp, the MAC for *Ndufs4(KO)* mice is approximately one third that of controls for both isoflurane and halothane.[17] This behavior led us to investigate the action of isoflurane on the spinal cord of this model animal.

Our hypothesis was that volatile anesthetics disrupt presynaptic function (primary) or other neuronal characteristics (secondary) in spinal cord motor neurons in *Ndufs4(KO)* mice in a manner that reflects their profound anesthetic hypersensitivity. Our goal was to exploit the hypersensitive mutant to probe the mechanisms by which a mitochondrial mutation might lead to immobility at lower concentrations of volatile anesthetics than normal animals. We measured both synaptic activity and intrinsic membrane properties of neurons in the mutants for comparison to wild-type neurons.

## Materials and Methods

### Animals

All animal experiments followed the recommendations in the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (Bethesda, Maryland) and were approved by the Institutional Animal Care and Use Committee at Seattle Children's Research Institute (Seattle, Washington). The mice were housed at 22°C with a 12-h light/dark cycle and maintained on a standard rodent diet. Food and water were available *ad libitum*. Lumbar spinal cord slices from male and female mice, 23 to 30 days old, were used for experiments. The total numbers used for each experiment and the numbers of male and female mice are listed at the end of each corresponding figure legend.

Global *Ndufs4(KO)* (*Ndufs4*$^{\Delta/\Delta}$) mice were generated by crossing heterozygotes (*Ndufs4*$^{\Delta/+}$) in a C57Bl/6 genetic background[21,22] with more than 10 backcrosses. Control wild-type animals were C57BL/6 mice originally obtained from The Jackson Laboratory (USA) and maintained at Seattle Children's Research Institute. Sibling wild-type (*Ndufs4*$^{+/+}$) or heterozygous (*Ndufs4*$^{\Delta/+}$) mice were used as controls as previously described.[17,23,24] Mice with cholinergic-specific labeled cells were generated by breeding choline acetyltransferase (*ChAT*)-Cre driver mice, which express Cre-recombinase under the control of the cholinergic-specific promoter (*ChAT*-Cre(B6); 129S6-*ChAT*$^{tm2(Cre)Lowl}$/J; The Jackson Laboratory) and Ai14 mice, which express the red fluorescent protein tdTomato when Cre removes a STOP codon in front of the ROSA26 promotor (*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$; The Jackson Laboratory). Mice with cholinergic-specific labeled cells were then crossed with *Ndufs4* heterozygotes (*Ndufs4*$^{\Delta/+}$) to produce *Ndufs4*$^{\Delta/+}$ containing the cholinergic label. These heterozygotes (*Ndufs4*$^{\Delta/+}$) were then mated to produce homozygous (*Ndufs4*$^{\Delta/\Delta}$) or control mice with tdTomato-labeled cholinergic cells. Offspring genotypes were determined by polymerase chain reaction.

### Slice Preparation

Preparation of slices for the vestibular nucleus and central medial thalamus were as previously described for the hippocampus (CA1).[23,24] Briefly, slices from these three regions were prepared by quickly dissecting the brain, affixing the rostral end to the cutting stage, and supporting the ventral side against an agar block. Coronal slices in the plane of interest, 350 μm thick, were made with a Leica VT1000S vibratome (Leica Biosystems, USA) in ice-cold oxygenated slicing solution (5 mM KCl, 1.25 mM $NaH_2PO_4$, 3.5 mM $MgSO_4$, 0.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 10 mM glucose, 210 mM sucrose) and incubated for 30 min in room-temperature artificial cerebrospinal fluid (118 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM $NaHCO_3$, 1 mM $NaH_2PO_4$, 30 mM glucose) before recording in artificial cerebrospinal fluid.

Methods for preparation of spinal cord slices were adapted from the work of Mitra and Brownstone.[25] Mice between the ages of 23 and 30 days were euthanized after being anesthetized with isoflurane until unresponsive to toe pinch and their breathing rate decreased to 1 breath every 2 s; no other medications were given. All experiments began in the early morning. The animals were tested as they became available by age and genotype. Once anesthetized, the mice were decapitated, and the spinal cord was dissected from the body. To remove the spinal cord, the skin was cut along the midline of the back from neck to tail. The skin was pulled to the sides, exposing the underlying muscle of the back and sides. Each side of the mouse was cut from the neck, through the lateral rib cage, and to the tail above the hind leg. The mouse was eviscerated to expose the vertebral column. Where the two lateral cuts ended near the tail, another transverse cut separated this section of the back, including the vertebral column from the body. This section of back was attached to a chamber filled with ice-cold dissecting solution (208 mM sucrose,

0.75 mM potassium gluconate, 1.25 mM $KH_2PO_4$, 26 mM choline bicarbonate [80% solution], 4 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM glucose, 2 mM kynurenic acid sodium salt [Abcam, United Kingdom], 1 mM [+]-sodium L-ascorbate, 5 mM ethyl pyruvate, 3mM *myo*-inositol) with the ventral side facing up. Using forceps to hold the ventral side of the vertebrae, a vertebrectomy was performed to expose the underlying spinal cord. Once exposed, the spinal cord was removed by gently lifting the cervical end of the cord with forceps and cutting the dorsal and ventral roots. Once isolated from the column, the ends of the cord were trimmed, and a large-bore transfer pipette was used to place the cord onto an agar block with the ventral side resting against the block. Excess solution was blotted away from the cord before a thin strip of cyanoacrylate glue was placed along each side of the agar block. A toothpick was used to gently push small amounts of the glue against the cord to affix it to the agar block. The attached cord was placed into the cutting chamber with ice-cold oxygenated dissecting solution. Using a Leica VT1000S vibratome, 350-μm-thick transverse slices were made. The slices were incubated for 1 min in 30% (w/v) polyethylene glycol solution on ice and washed in ice-cold oxygenated dissection solution for 20 s. The slices were then incubated in a mixture of equal parts dissection solution and recording solution (126 mM NaCl, 3 mM KCl, 1.25 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 1.1 mM $MgCl_2$, 2.2 mM $CaCl_2$, 15 mM glucose, 1 mM [+]-sodium L-ascorbate, 5 mM ethyl pyruvate, 3 mM *myo*-inositol) at 32°C for 30 min. Then the slices were incubated at 32°C recording solution for 30 min, and finally incubated at room temperature in recording solution for at least 30 min before moving to the bath for electrophysiologic recording.

## Whole Cell Patch Clamp

Individual slices were transferred to the recording chamber, which was superfused with oxygenated solution at 3.2 to 3.8 ml/min flow rate and kept at 30°C. Ventral horn spinal cord cells were visualized using differential interference contrast microscopy. In ChAT-Cre;Ai14 mice, the cells were identified as cholinergic or noncholinergic by visualizing the cells with fluorescence microscopy. Whole cell patch clamp experiments were performed using borosilicate glass capillaries pulled on a Sutter Instruments P-97 puller (Sutter Instruments, USA). The cells were held at −60 mV for voltage clamp experiments. Patch pipettes were filled with a potassium gluconate–based solution (140 mM potassium gluconate, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 10 mM EGTA, 4 mM $Na_2ATP$, 0.4 mM NaGTP, 10 mM HEPES, pH 7.3, 302 to 312 mOsm). The conditions were chosen to visualize both excitatory and inhibitory peaks. Patch pipettes had a resistance of 3 to 6 MΩ. Recordings were performed with a MultiClamp700B amplifier (Axon Instruments, USA) and digitized with a Digidata 1400 (Axon Instruments). Patch clamp currents were filtered at 2.1 kHz and sampled at 10 kHz. Upon establishing a whole cell patch, the cells were held for 10 min before starting a recording. Series resistance was monitored during the experiments; recordings with substantially changed series resistance were excluded from the analysis. Because baseline holding currents were quite variable, we normalized the treated holding currents by subtracting untreated from treated values and reporting the difference as ΔHolding current.

## Drug Administration

The slices were first held in the superfusate for 30 min 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 "rundown" 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. For action potential blockade, 0.5 μM tetrodotoxin was used in the recording solution described above (see Whole Cell Patch Clamp section). Norfluoxetine (hydrochloride; catalog No. 15900; Cayman Chemical, USA) was diluted to a final concentration of 20 μM in recording solutions from a stock solution of 10 mM in dimethyl sulfoxide (Sigma-Aldrich, USA), based on previous literature regarding TREK blockade with a concentration causing 50% inhibition of activity of 9 μM.[26]

## Confocal Microscopy

The spinal cord of a ChAT-Cre;Ai14 mouse was dissected and fixed with 4% paraformaldehyde for 1 h and incubated overnight at 4°C in 30 mM sucrose solution. The cord was embedded in optimal cutting temperature medium (Fisher Scientific, USA) and frozen before cutting 50-μm slices on a cryostat. The slices were mounted on slides (Superfrost slides, Fisher Scientific) and incubated overnight at 37°C. They were subsequently incubated for 30 min in a 1× phosphate-buffered saline solution containing 1% bovine serum albumin at 30°C. The slices were then incubated with 4′,6-diamino-2-phenylindole (1 μg/ml in phosphate-buffered saline) for 10 min and then washed three times for 5 min in 1× phosphate-buffered saline. The slides were dried and then covered with mounting media and a coverslip. The slides were imaged on a Zeiss LSM 710 confocal microscope (Carl Zeiss AG, Germany).

## Animal Research: Reporting of In Vivo Experiments Guidelines

The Animal Research: Reporting of In Vivo Experiments (ARRIVE) Guidelines are listed in the Supplemental Materials (Supplemental Digital Content 1, http://links.lww.com/ALN/C612).

### Statistical Analysis

The traces were analyzed with pClamp 10 software (Axon Instruments). Spontaneous excitatory postsynaptic currents and spontaneous inhibitory postsynaptic currents were analyzed for amplitude, frequency, and 90 to 10% decay time. All exposed and wash data were normalized to their average unexposed parameter. Synaptic data are expressed as percentages of the unexposed value, and the holding current data are expressed as differences from unexposed value. The data were tested for normalcy, and once established, the means, SDs, and 95% CIs were determined. The data are expressed in text and graphed as means ± 95% CIs. All data comparing the responses between exposed (isoflurane) and unexposed slices were compared cell by cell before calculating means and 95% CI. Statistical analyses were conducted using *R* version 3.6.1. The figures were generated using the ggplot2 package (GNU general public license) and Microsoft Excel (Microsoft, USA). The two groups were compared with two-tailed, paired *t* tests because the treated and untreated results were from the same cells at different times. Because we did not know which endpoints would differ, we did not do a power analysis before the studies. However, in our previous studies, if a physiologic measurement was of sufficient size to be biologically relevant, we have been able to see significance with n = 5. Thus, we used n = 5 for number of cells studied as our minimum sample sizes for completed measurements; for intermediate anesthetic concentrations, n = 4 was used. In general, each cell came from a separate animal of known genotype. No randomization was used, and the experiments were not blinded. In all studies, all data were reported; there are no missing data other than cells that were unable to be patch clamped. All outliers were included. All data used were raw, unprocessed data. We used the terms "control" or "wild type" to indicate genetically normal C57Bl/6 mice. The term "mutant" refers to *Ndufs4(KO)* mice.

The significance level was selected as 0.01 and then subjected to a Bonferroni correction for multiple comparisons. For synaptic measurements, we used n = 30 for two or three concentrations of anesthetic compared (depending on genotype), two genotypes, three endpoints, and two types of synaptic currents: inhibitory and excitatory. In general, we measured one cell per spinal cord slice and one slice per animal. Thus, our number (n) refers to animals, slices, and cells. Consequently, the final derived threshold for significance for synaptic comparisons was $P < 0.01/29 = 0.0003$. Means, SDs, SEMs, and 95% CI values for all measurements in figures are listed in supplemental table S1 (Supplemental Digital Content 2, http://links.lww.com/ALN/C613). The means and 95% CIs are given in the section "Results" and shown by box plots in the figures. The *P* values for the synaptic comparisons in figures 1 and 4 are listed in supplemental tables S2 (Supplemental Digital Content 3, http://links.lww.com/ALN/C614) and S3 (Supplemental Digital Content 4, http://links.lww.com/ALN/C615), respectively. The *P* values for comparisons of changes in holding currents are noted in the text as (ΔHolding current, *P*).

## Results

### Synaptic Activity Is Not Altered in the Thalamus, Vestibular Nucleus, or Spinal Cord in *Ndufs4(KO)* Compared to Controls

Because we wished to establish synaptic parameters for mutant spinal cord tissue, we compared baseline activity with a concentration of isoflurane appropriate for mutant MAC, 0.6% (0.25 mM) isoflurane (approximate EC95 for *Ndufs4[KO]* animals). For control (wild-type) spinal cord slices only, we also applied 1.8% (0.74 mM) isoflurane (approximate EC95 for inhibition of movement in control animals, lethal for mutants). Although we did not know the specific cell types being studied, we consistently picked large cells in the ventral horn in an attempt to patch motor neurons. Figure 1A is a representative tracing of a continuous whole cell patch clamp recording (top) from a control spinal cord slice, voltage clamped at −60 mV and exposed first to 0% isoflurane (left bottom; unexposed) and then to 0.6% isoflurane (right bottom). We observed no significant differences in the spinal cord for spontaneous synaptic frequencies (fig. 1, B and E), amplitudes (fig. 1, C and F), or decay times (fig. 1, D and G) between genotypes for either spontaneous excitatory postsynaptic currents or spontaneous inhibitory postsynaptic currents in 0, 0.6, or 1.8% isoflurane for controls. For comparison to the spinal cord, we next extended our earlier studies of synaptic function in the hippocampus[23,24] to the vestibular nucleus and central medial thalamus, two regions earlier shown to affect volatile anesthetic sensitivity in the mouse.[27] We did not observe significant differences between genotypes unexposed to isoflurane or exposed to 0.6% isoflurane in the central medial thalamus (supplemental fig. S1, Supplemental Digital Content 5, http://links.lww.com/ALN/C616) or vestibular nucleus (supplemental fig. S2, Supplemental Digital Content 5, http://links.lww.com/ALN/C616). For figure 1 and all subsequent figures, the values for the means, SDs, SEMs, and 95% CIs are listed in supplemental table S1 (Supplemental Digital Content 2, http://links.lww.com/ALN/C613). The *P* values for all comparisons in this and subsequent figures are listed in supplemental tables S2 (Supplemental Digital Content 3, http://links.lww.com/ALN/C614) and S3 (Supplemental Digital Content 4, http://links.lww.com/ALN/C615).

### Isoflurane Increases the Holding Current of Ventral Horn Spinal Cord Neurons

We also measured the intrinsic membrane properties of cells from spinal cord slices from mutant and control spinal cord, from the CA1 region of the hippocampus, from the central medial thalamus, and from the vestibular

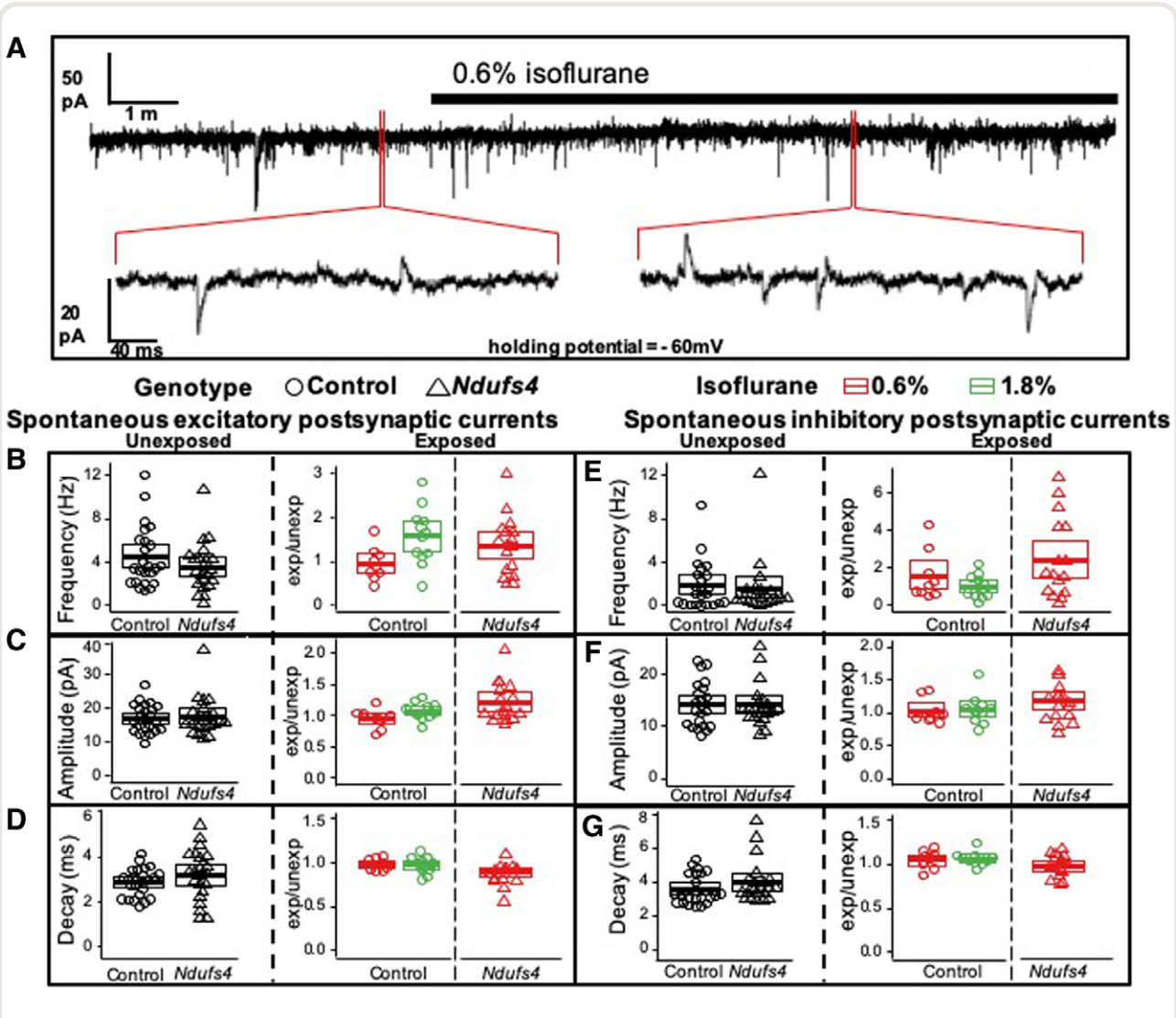


**Fig. 1.** Spontaneous synaptic activity in ventral horn cells of the spinal cord of wild-type control (*circles*) and mutant (*triangles*) adult mice. (*A*) Representative whole cell patch recording of a control cell voltage clamped at –60 mV. Isoflurane, 0.6%, was applied to the slice from minutes 5 to 15 of the recording (*black bar*). Two sections of the recording are shown at enlarged scale to illustrate kinetic properties of the synaptic events (*red lines* to *bottom traces*). (*B–G*) Synaptic event properties of frequency, amplitude, and decay from the spinal cord are quantified and compared between *Ndufs4(KO)* and control with and without isoflurane exposure. Spontaneous excitatory postsynaptic currents are shown in *B–D*; spontaneous inhibitory postsynaptic currents are shown in *E–G*. *Black* and *white symbols* are mean absolute values of the 5 min before isoflurane is applied to the slice. *Colored symbols* are mean normalized values of the last 5 min of isoflurane exposure. *Red* indicates 0.6% isoflurane; *green* indicates 1.8% isoflurane. Normalized mean values were calculated for each cell by dividing its exposed mean by its unexposed mean for the final 5 min before isoflurane exposure. The mean values for each group were then calculated from the individual cell ratios. *Crossbars* show the means, and *boxes* represent the 95% CIs. For *B–D*, n = 24 (wild type; 11 male, 13 female), n = 22 (*Ndufs4[KO]*; 9 male, 13 female). For *E–G*, n = 23 (wild type; 11 male, 12 female), n = 21 (*Ndufs4[KO]*; 8 male, 13 female).

nucleus. (Note that we previously reported transmembrane potentials and input resistance from the hippocampal CA1 region.[24]) In the absence of volatile anesthetic, the holding currents for cells were similar between genotypes for each region (fig. 2, A–D, left). At 0.6% isoflurane, no increases in holding current were observed in controls or mutant cells in the hippocampus (CA1), central medial thalamus, or vestibular nucleus (fig. 2, A–C, right). Because no changes in the mutant at the relevant concentration were observed, we did not extend these studies to a higher concentration in the control slices. In marked contrast, holding currents in patched ventral horn cells rose dramatically upon exposure to 0.6% isoflurane (approximate EC95 for *Ndufs4(KO)* animals[23]) in spinal cord slices from *Ndufs4(KO)* (ΔHolding current, 126 pA [95% CI, 99 to 152 pA]; ΔHolding current, $P < 0.001$; n = 21) but not

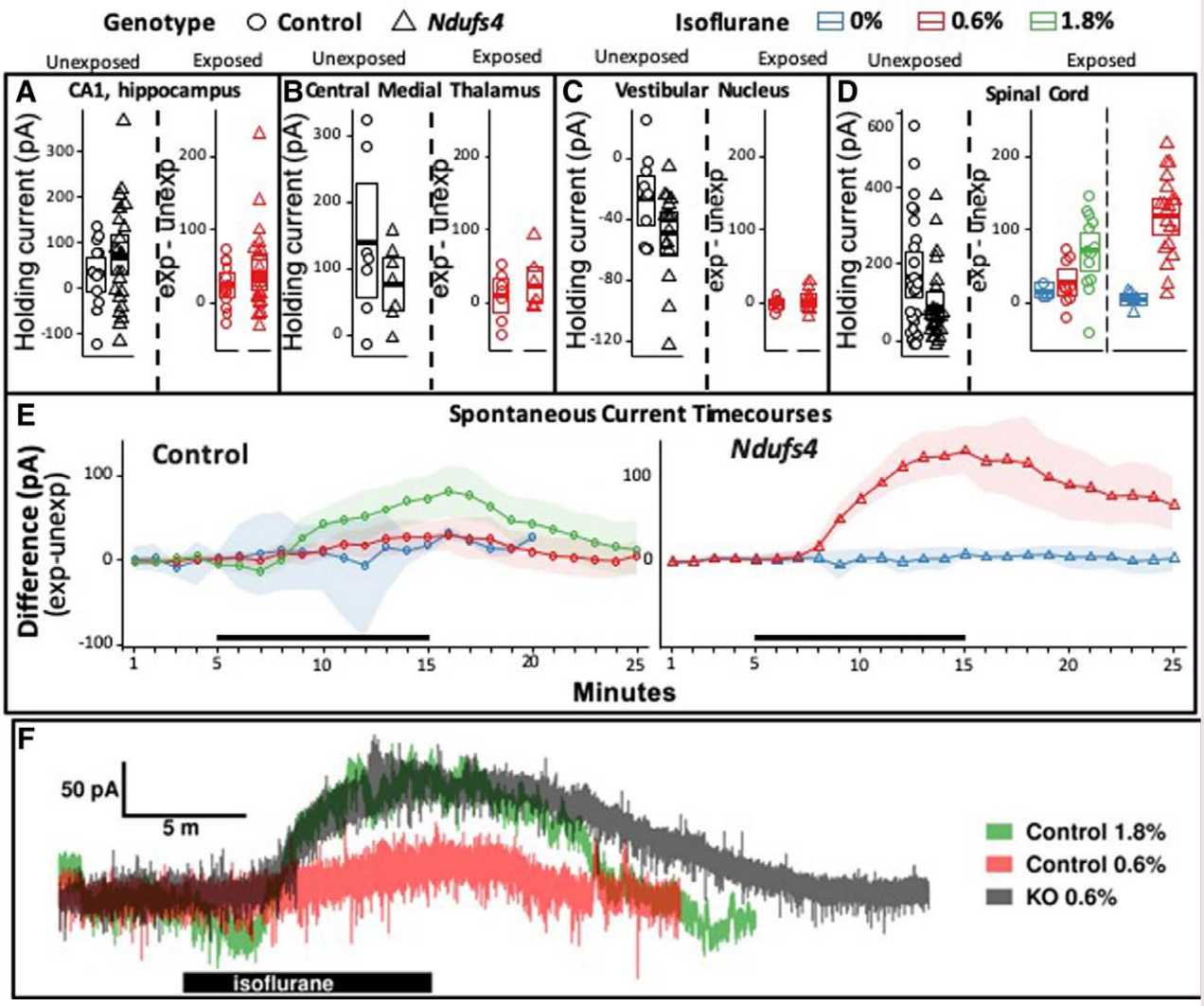


**Fig. 2.** (*A–D*) Holding currents required to voltage clamp wild-type control (*circles*) or mutant (*triangles*) cells at –60 mV. –60 mV *Black* and *white symbols* are mean absolute values of the 5 min before isoflurane is applied to the slice. *Colored symbols* are mean normalized values for each cell during the last 2 min of isoflurane exposure. *Red* indicates 0.6% isoflurane; *green* indicates 1.8% isoflurane. Normalized mean values in this figure and in figures 3, 5, and 6 and supplemental figure S7 (Supplemental Digital Content 5, http://links.lww.com/ALN/C616) were calculated for each cell by subtracting the unexposed mean for the last 5 min before isoflurane exposure from the exposed mean for the last 5 min of exposure. In the CA1 hippocampus (*A*), the central medial thalamus (*B*), and the vestibular nucleus (*C*), the holding current in *Ndusf4(KO)* cells does not change with exposure to 0.6% isoflurane. (*D*) In the spinal cord, the holding current in the mutant rises at a lower concentration (0.6%) of isoflurane than control. (*E*) Differences in holding current over time in controls (*circles*) or mutant (*triangles*) cells with no isoflurane (*blue*) or exposure to either 0.6% (*red*) or 1.8% (*green*) isoflurane. The *black bar* indicates when isoflurane was applied to the slice. *Lines* and *shaded ribbons* are the means and 95% CIs. (*F*) Representative whole cell patch recording of a *Ndusf4(KO)* cell voltage clamped at –60 mV. Isoflurane, 0.6%, applied to slice from minutes 5 to 15 of the recording (*black bar*). Two sections of the recording are shown at enlarged scale to illustrate kinetics of the synaptic events (*red lines* to *bottom traces*). Note the shift in the baseline indicating an increase in holding current necessary to maintain resting membrane potential at –60 mV. For *A*, n = 12 (wild type; 5 male, 7 female), n = 23 (*Ndufs4[KO]*; 18 male, 5 female); for *B*, n = 7 (wild type; 4 male, 3 female), n = 7 (*Ndufs4[KO]*; 3 male, 4 female); for *C*, n = 10 (wild type; 2 male, 8 female), n = 16 (*Ndufs4[KO]*; 5 male, 11 female); and for *D*, unexposed: n = 25 (control; 11 male, 14 female), n = 26 (*Ndufs4[KO]*; 9 male, 17 female); isoflurane-exposed: n = 13 (wild type; 7 male, 6 female), n = 21 (*Ndufs4[KO]*; 8 male, 13 female).

wild type (ΔHolding current, 27 pA [95% CI, 9 to 47 pA]; ΔHolding current, $P = 0.030$; n = 25; fig. 2D, right). The knockout and wild-type ΔHolding currents were significantly different ($P < 0.001$). The time course for these rises are shown in figure 2E for both control (1.8% isoflurane, green; 0.6% isoflurane, red) and mutant (0.6%

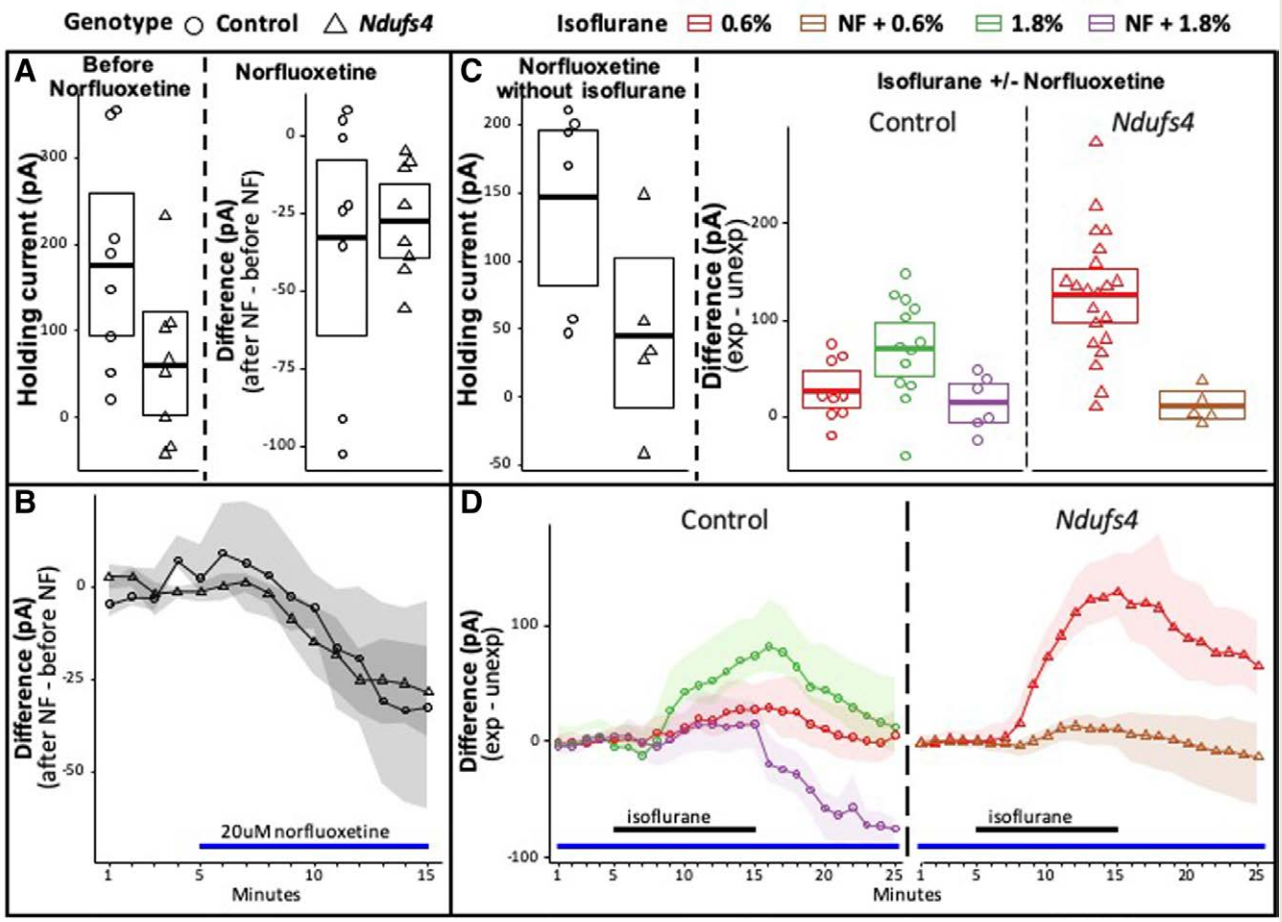


**Fig. 3.** (*A*) Mean holding current in wild-type control (*circles*) and mutant (*triangles*) cells in the 5 min before application of norfluoxetine (*left*) and in the last 2 min of application of norfluoxetine (*right*). The differences plotted on the *right* are the mean changes in holding currents for each cell between the values before and after norfluoxetine application. (*B*) Time course of action of norfluoxetine on holding current. The *bar* shows the time of norfluoxetine application. (*C*) Changes in holding current when isoflurane is applied in the presence of norfluoxetine. The values on the *left* are the mean holding current in control and mutant cells in norfluoxetine in the last 5 min before application of isoflurane. The values on the *right* represent the mean difference in holding current for each cell from its value before isoflurane exposure in the presence of norfluoxetine to its value in the last 2 min of isoflurane exposure in norfluoxetine (*purple* or *brown symbols*). (*D*) Differences in holding current over time of wild-type controls in 1.8% isoflurane (*circles*, *purple plot*) and mutant cells in 0.6% isoflurane (*triangles*, *brown plot*) in the presence of norfluoxetine. *Red* and *green plots* of controls and mutants are of isoflurane exposure alone from figure 4E. For *A* and *B*, n = 8 (wild type; 5 male, 3 female), n = 8 (*Ndufs4[KO]*; 3 male, 5 female); for *C* and *D*, n = 13 (wild type; 7 male, 6 female), n = 21 (*Ndufs4[KO]*; 8 male, 13 female).

isoflurane, black) preparations in isoflurane. In each case, the rise in holding currents returns to baseline when isoflurane is discontinued. A typical recording from the spinal cord of *Ndufs4(KO)* voltage clamped at −60 mV and exposed to 0.6% isoflurane (fig. 2F, black recording) illustrates the large outward current (compared to the absence of an outward current in the control spinal cord upon exposure to 0.6% isoflurane; figs. 1A and 2F, red recording). When the isoflurane concentration was increased to 1.8% (approximate EC95 for tail clamp in control animals,[23] lethal for mutants), the holding current also rose in the control spinal cord (ΔHolding current, 72 pA [95% CI, 43 to 97 pA]; ΔHolding current, $P < 0.001$; n = 13; fig. 2, D–F, green recording).

The differences in the voltage clamp recordings between genotypes at 0.6% isoflurane in spinal cord slices were corroborated by differences in the current clamp recordings (resting membrane potentials). In the absence of anesthetics, the resting membrane potentials of both genotypes were similar (−65 mV for *Ndufs4[KO],* n = 26, and −67 mV for controls, n = 25; supplemental fig. S3A, Supplemental Digital Content 5, http://links.lww.com/ALN/C616). At

0.6% isoflurane, the resting membrane potentials did not significantly change in the controls but decreased by −8 mV in the mutant (supplemental figs. S3, B and C, Supplemental Digital Content 5, http://links.lww.com/ALN/C616). At 1.8% isoflurane, the resting membrane potentials in controls decreased by −5 mV (supplemental figs. S3, B and C, Supplemental Digital Content 5, http://links.lww.com/ALN/C616). No differences in resting membrane potentials existed between genotypes in isoflurane in slices from the vestibular nucleus or central medial thalamus (not shown).

## Dependence of Holding Current on Synaptic Input

We next tested whether the changes in holding current and resting membrane potentials in the patched cell were dependent on synaptic input to that cell. We treated the spinal cord slices from both *Ndufs4(KO)* and control animals with tetrodotoxin (0.5%; see section "Materials and Methods") to inhibit action potentials and synaptic activity. No differences were seen between genotypes in mini-synaptic frequencies, amplitudes, or time decays (data not shown). In *Ndufs4(KO)* slices, tetrodotoxin did not change the dependence of holding current on isoflurane exposure (supplemental fig. S4, Supplemental Digital Content 5, http://links.lww.com/ALN/C616; ΔHolding current, 154 pA [95% CI, 99 to 232 pA] compared to no tetrodotoxin; ΔHolding current, 126 pA [95% CI, 99 to 152 pA]). The ΔHolding currents with and without tetrodotoxin were not significantly different ($P = 0.506$; n = 6). We conclude that in *Ndufs4(KO)* slices, the dependence of current response and resting membrane potential on isoflurane is cell autonomous.

Altogether, these data indicate that isoflurane induces an intrinsic outward current in ventral spinal neurons at lower concentrations of isoflurane in the knockout than in the control. This change is reflected by a more pronounced hyperpolarization in the spinal cord neurons from knockout slices. The genotypic differences in cellular behavior within the slice preparation correspond to the whole animal behavior to tail clamp at effective concentrations of isoflurane.

## Norfluoxetine Blocks the Isoflurane-dependent Increase in Holding Current of Ventral Horn Spinal Neurons

To further elucidate the mechanism for the change in holding currents in the mitochondrial mutant, we determined the class of channels affected. The changes in holding current were reminiscent of previous reports of activation of potassium currents in neurons from rodent brainstem or mollusks in the presence of volatile anesthetics.[28–31] Those reports indicated that enhancement of transmembrane currents was mediated through increased conductance of K2P (TREK, TASK) channels. Therefore, to investigate whether TREK channels mediated the change in holding current in spinal cord slices, we added the TREK-1/TREK-2 inhibitor norfluoxetine[26,32] to the slice preparation. Norfluoxetine had similar effects on control and *Ndufs4(KO)* spinal cord in unexposed slices (fig. 3, A and B). In ventral spinal cord treated with norfluoxetine alone, we observed a decrease in holding current of both mutant and control neurons. Norfluoxetine prevented the increase in holding current observed with 0.6% isoflurane treatment in *Ndufs4(KO)* slices (fig. 3C). Consistent with anesthetic-induced TREK currents at higher isoflurane concentrations, norfluoxetine also inhibited the increased holding current in control ventral spinal cord neurons at 1.8% isoflurane (fig. 3C).[28] The time courses for the effects of norfluoxetine for both genotypes are shown in figure 3D, using data for no norfluoxetine from figure 2E. This decrease in holding current by norfluoxetine was enhanced in control neurons during the washout period after 1.8% isoflurane (fig. 3D, left).

## Synaptic Activity in Cholinergic Cells in the Spinal Cord

Previous work indicated that both hippocampal[33] and spinal cord motor neurons[7,34] were hyperpolarized in response to volatile anesthetics. We therefore studied whether outward currents specifically in motor neurons were hypersensitive to isoflurane in *Ndufs4(KO)* animals. To differentiate the responses to isoflurane between cholinergic and noncholinergic neurons within the ventral horn, we used a ChAT;Ai14 construct that labels specifically cholinergic motor neurons with tdTomato (see section "Materials and Methods"). This construct positively identified cholinergic neurons in the spinal cord. Figure 4A shows the typical distribution of tdTomato in spinal cord slices from *Ndufs4(KO)* animals, which was indistinguishable from that of wild-type slices (data not shown). Figure 4, B–G, shows the frequencies, amplitudes, and decay times for spontaneous excitatory postsynaptic currents and spontaneous inhibitory postsynaptic currents in both cholinergic ($ChAT^+$) and noncholinergic ($ChAT^-$) cells unexposed and in isoflurane in both genotypes. No differences were noted between genotypes for the cell types in synaptic properties.

## Isoflurane Increases the Holding Current of Noncholinergic Ventral Horn Spinal Neurons in *Ndufs4(KO)* Neurons

Cholinergic cells ($ChAT^+$), as identified by tdTomato labeling, differed between genotypes in the baseline holding current required to maintain a voltage clamp of −60 mV (fig. 5A, left; ΔHolding current wild type, 188 pA [95% CI, 139 to 238 pA]; n = 18; ΔHolding current *Ndufs4(KO)*, 60 pA [95% CI, 23 to 97 pA]; $P = 0.001$; n = 7). However, exposure to 0.6% isoflurane did not change the holding current in either genotype from baseline (fig. 5A, right). Cholinergic cells from control animals did show a rise in holding current in 1.8% isoflurane (ΔHolding current, 92 pA [95% CI, 54 to 132 pA]; ΔHolding current, $P < 0.001$; n = 14). The time course for holding current changes in

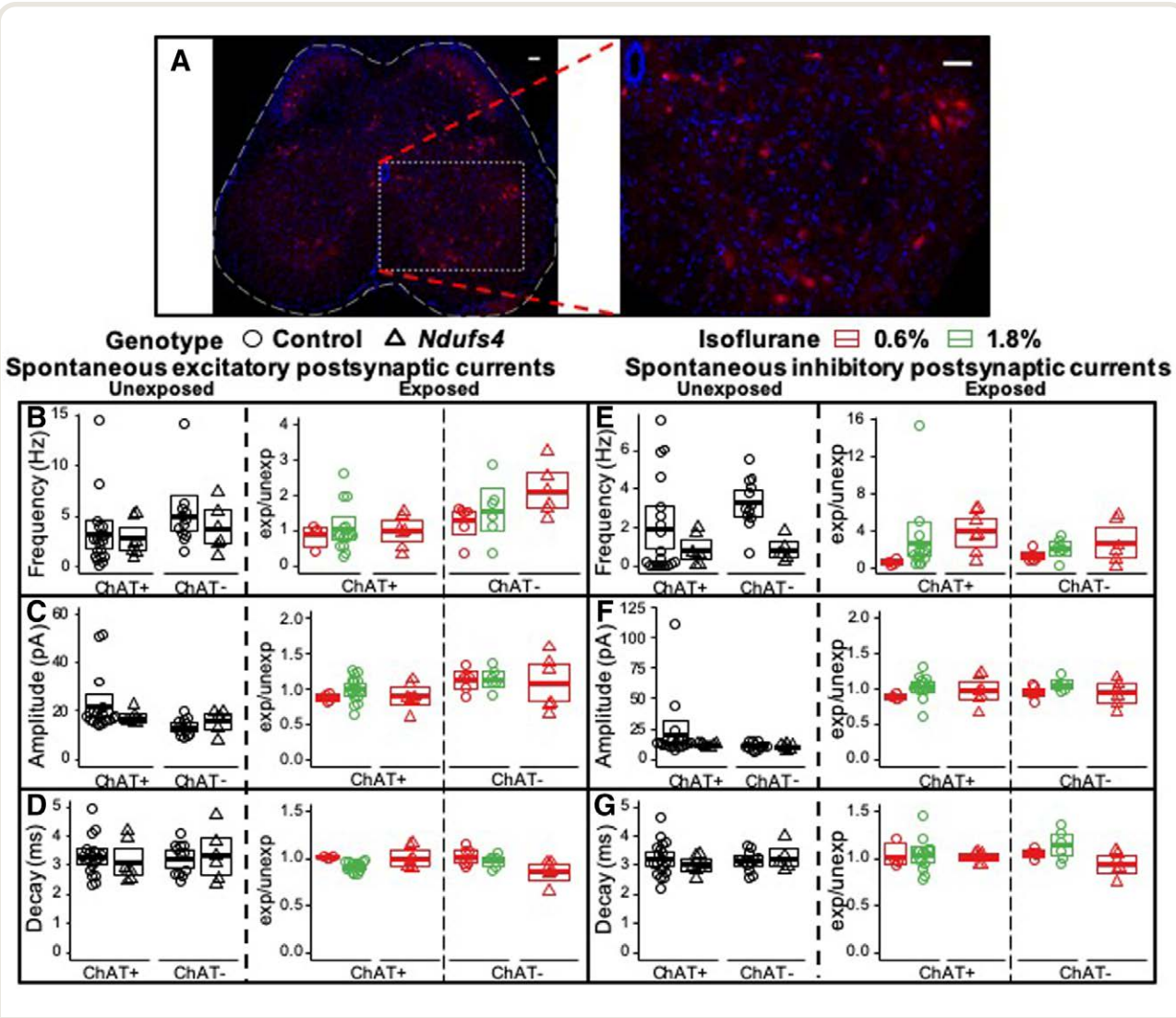


**Fig. 4.** Spontaneous synaptic activity in in the ventral horn of spinal cord cells from wild-type control (*circles*) and mutant (*triangles*) mice. (*A*) Confocal images of a spinal cord slice from ChAT;Ai14 mice. (*Left*) Entire transverse slice. (*Right*) Enlarged portion of the slice (indicated by the *white dotted-line box* in the left image). *White scale bars* in the *upper right corners* are 50 μm. *Red color* indicates cholinergic cells. *Blue color* indicates nuclear stain. (*B–G*) Synaptic event properties of frequency, amplitude, and decay quantified as in figure 1. Cholinergic cells are labeled as ChAT$^+$; noncholinergic cells are labeled ChAT$^-$. Spontaneous excitatory postsynaptic currents are shown in *B–D*; spontaneous inhibitory postsynaptic currents are shown in *E–G*. *Black* and *white symbols* are mean absolute values of the 5 min before isoflurane is applied to the slice. *Colored symbols* are the mean normalized values of the last 5 min of isoflurane exposure. *Red* indicates 0.6% isoflurane; *green* indicates 1.8% isoflurane. Normalized mean values were calculated for each cell by dividing its exposed mean by its unexposed mean for the final 5 min before isoflurane exposure. The mean values for each group were then calculated from the individual cell ratios. The *cross-bars* show the means, and *boxes* represent the 95% CI.s The *symbols* are as in figure 1. For *B–G*, n = 19 (wild type; 10 male, 9 female), n = 7 (*Ndufs4[KO]*; 5 male, 2 female).

cholinergic cells during isoflurane exposure is shown in figure 5B.

Nonlabeled (noncholinergic) neurons did not differ between genotypes in the baseline holding current (fig. 6A, left; ΔHolding current wild type, 10 pA [95% CI, −6 to 25 pA]; n = 12; ΔHolding current *Ndufs4(KO)*, 58 pA [95% CI, 33 to 93 pA]; $P$ = 0.039; n = 7). Nonlabeled (noncholinergic) neurons from wild-type mice did not reach significant changes in their holding current in 0.6% isoflurane (n = 6) or in 1.8% isoflurane (n = 6; fig. 6A, right). However, nonlabeled (noncholinergic) neurons from *Ndufs4(KO)* mice developed significantly increased holding currents in 0.6% isoflurane (ΔHolding current, 98 pA [95% CI, 74 to 123 pA]; ΔHolding current, $P$ = 0.001; n = 6;

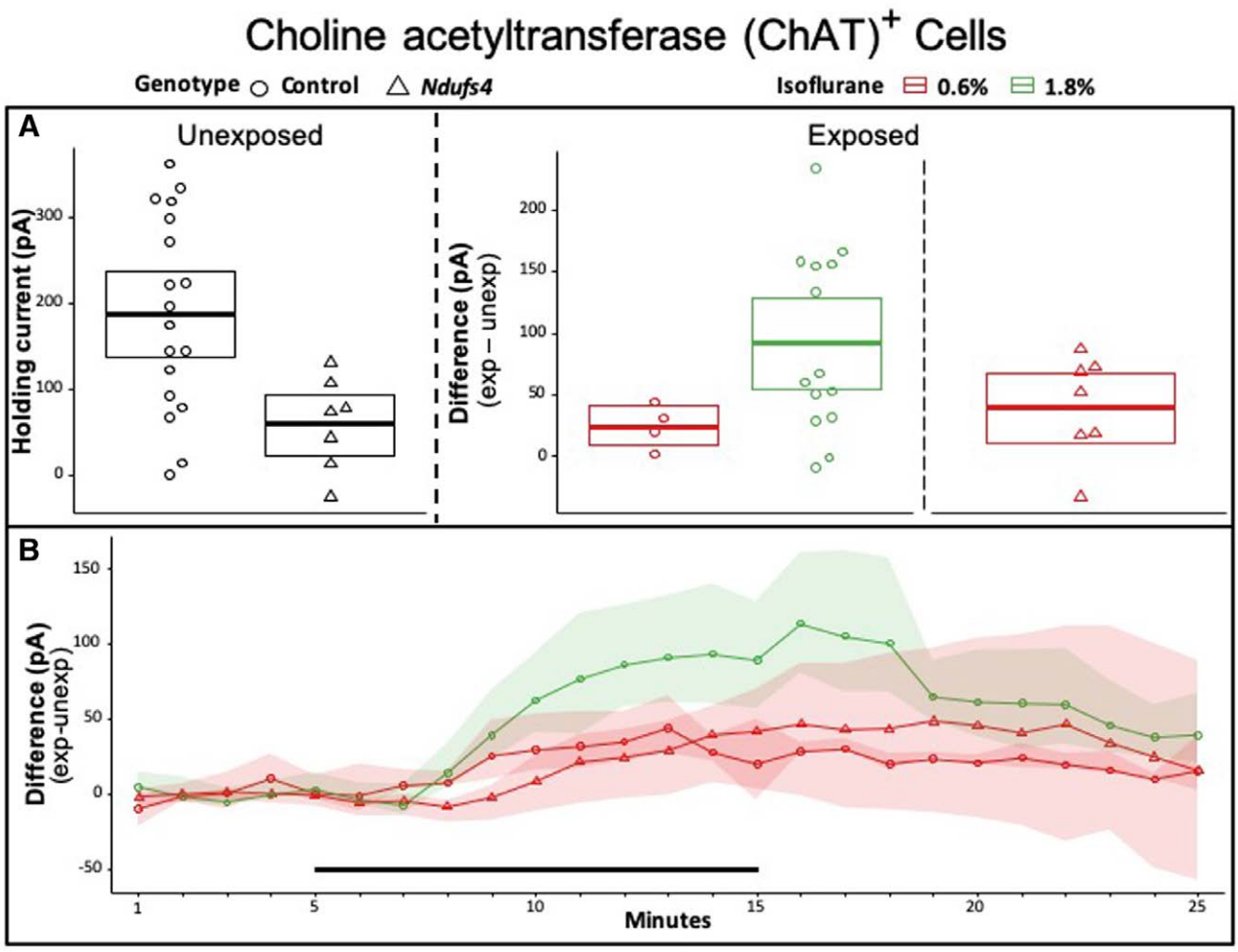


**Fig. 5.** Holding current in cholinergic (ChAT+) cells. (*A*) At baseline, mutant cholinergic cells (*triangles*) require less holding current than control cholinergic cells (*circles*) to voltage clamp at –60 mV ($P = 0.001$); however, their holding current does not rise with 0.6% isoflurane. Control cholinergic cells show a rise in holding current with 1.8% isoflurane ($P = 0.0005$). (*B*) Differences in holding current over time in wild-type control (*circles*) or mutant (*triangles*) cholinergic cells in either 0.6% (*red*) or 1.8% (*green*) isoflurane. The *black bar* indicates when isoflurane was applied to the slice. *Lines* and *shaded ribbons* show the means and 95% CIs. For *A*, n = 18 (wild type; 10 male, 8 female), n = 7 (*Ndufs4[KO]*; 5 male, 2 female).

fig. 6A, right). The time course for changes in holding currents in noncholinergic cells of both genotypes upon exposure to isoflurane is shown in figure 6B. The rise in controls in 1.8% isoflurane is similar in cholinergic and noncholinergic cells (compare figs. 5B and 6B). Changes in resting membrane potentials correlated to the changes in holding current (supplemental figs. S5 and S6, Supplemental Digital Content 5, http://links.lww.com/ALN/C616) with a decrease in resting membrane potential in the nonlabeled (noncholinergic) neurons, but not in the labeled (cholinergic) neurons, in 0.6% isoflurane. Changes in holding current in noncholinergic cells at intermediate concentrations of isoflurane were between those recorded in the absence of isoflurane and those recorded at the EC95 values in both genotypes (supplemental fig. S7, Supplemental Digital Content 5, http://links.lww.com/ALN/C616).

## Discussion

To understand how volatile anesthetics produce immobility, we exploited an animal with an extremely low MAC to understand anesthetic mechanisms. Because the response to tail clamp is thought to be primarily *via* a spinal cord mechanism,[28,29,31,35] we compared spinal cord neuronal responses between wild-type and *Ndufs4(KO)* mice. We also compared spinal cord responses to those from two other regions

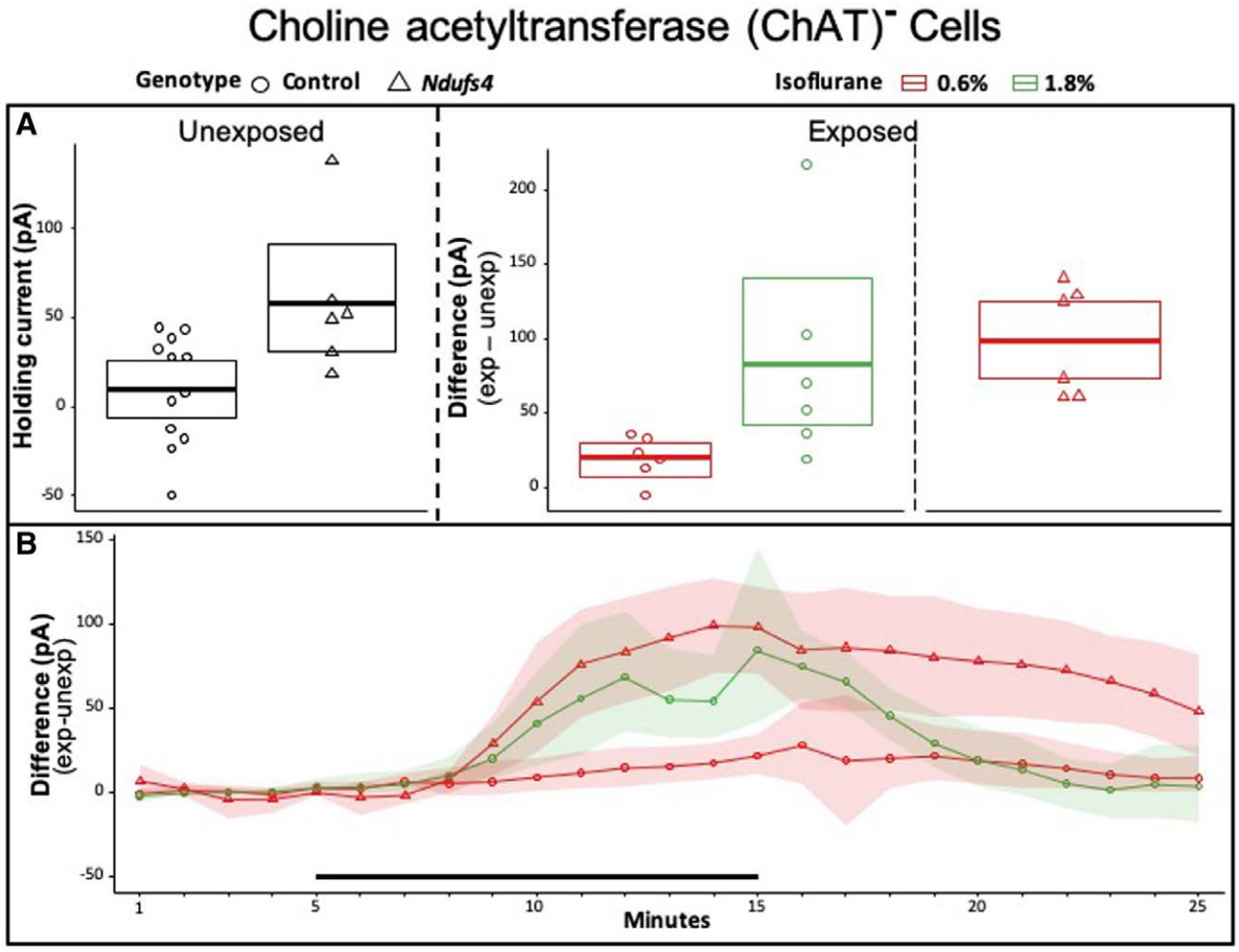


**Fig. 6.** Holding current in noncholinergic (ChAT⁻) cells. (*A*) At baseline, noncholinergic cells for both genotypes require similar holding current to voltage clamp at −60 mV. The holding current of control noncholinergic cells did not reach a significant change with either concentration of isoflurane. Mutant cells increase their holding currents with 0.6% isoflurane (*red triangles*; $P = 0.001$). (*B*) Differences in holding current over time in control (*circles*) or mutant (*triangles*) noncholinergic cells to either 0.6% (*red*) or 1.8% (*green*) isoflurane. The *black bar* indicates when isoflurane was applied to the slice. *Lines* and *shaded ribbons* show the means and 95% CIs. For *A*, unexposed, n = 12 (wild type; 7 male, 5 female), n = 6 (*Ndufs4[KO]*; 3 male, 3 female); exposed, n = 6 (3 male, 3 female, in all cases).

of the brain previously shown to have effects on anesthetic sensitivity in the *Ndufs4(KO)*.[27]

We found no differences between mutant and wild-type slices in any presynaptic functions of spinal cord, vestibular nucleus, or thalamic neurons upon exposure to isoflurane. This ruled out our initial hypothesis regarding the role of presynaptic function in the spinal cord as causative for isoflurane-induced immobility. However, at isoflurane concentrations matching the mutant whole animal EC95, we did discover a marked increase in the holding current of mutant ventral horn neurons compared to wild-type controls; this produced a corresponding transmembrane hyperpolarization of ventral horn neurons. This was not the case in similar recordings in the mutant vestibular nucleus or the thalamus, nor had we seen this in previous studies of the hippocampus. In the *Ndufs4(KO)* spinal cord, an increase in holding current occurs specifically in noncholinergic cells at a much lower concentration of isoflurane than in controls, in both cases matching the EC95 values for immobilization.[17] The increase in holding current also occurs in wild-type spinal cord neurons but at higher isoflurane concentrations and in both cholinergic and noncholinergic cells, implying that either cell type may contribute to immobility in the control genotype. The holding current increases are fully blocked in both genotypes by norfluoxetine, suggesting a role for TREK channels. These data indicate a possible role for TREK channels affecting anesthetic sensitivity *via* a spinal cord mechanism.

In slices from both genotypes, the isoflurane-induced outward currents are prevented by norfluoxetine, an inhibitor of the K2P potassium leak channels, TREK-1 and TREK-2.[26] These results support previous reports that K2P channels may play a role in determining volatile anesthetic sensitivity in normal mice[28,29,35] and that they may play a role in spinal cord neurons. The increases in outward currents are corroborated by the expected hyperpolarization of the transmembrane potential in concentrations of isoflurane that anesthetized the animals. We do not know the precise cell types being measured for either spontaneous excitatory postsynaptic currents (glutamatergic or cholinergic) or spontaneous inhibitory postsynaptic currents (glycinergic or GABAergic). The lack of inhibition of presynaptic frequency is not in conflict with the hyperpolarization of patch clamped neurons, which are postsynaptic cells in this preparation. Nor are the increases in holding currents caused by inhibitory postsynaptic currents because the differences in holding currents are virtually abolished by norfluoxetine treatment, which should not affect inhibitory postsynaptic currents. Finally, blockade of spontaneous synaptic input with tetrodotoxin did not affect the changes in holding currents. We conclude that the observed increased transmembrane current seen in both normal and mutant mice is not dependent on synaptic input and instead is cell autonomous.

In a previous study, we knocked down NDUFS4 in the vestibular nucleus,[27] which produced resistance to the response to tail clamp when using isoflurane. This clearly argues that the spinal cord is not the only site that influences MAC. Notably, that effect in the vestibular nucleus, although clearly statistically significant, was a modest (less than 20%) difference in MAC. We had also discovered a small increase in sensitivity when NUDFS4 was knocked down in the central medial thalamus.[27] The MAC of the global knockout is only a third of that of the wild-type animal (*e.g.*, 0.4% *vs.* 1.3% isoflurane). It is too early to unambiguously compare the effects of mitochondrial dysfunction in different regions of the brain to the effects in the spinal cord. Clearly, additional studies are needed to study the relationship of the brain and spinal cord in controlling MAC. In addition, it would be most interesting to measure MAC in a selective, cell-specific loss of NDUFS4 in the spinal cord to both rule out compensatory effects and determine contributions of other regions of the central nervous system to the anesthetic hypersensitive phenotype.

Our previous data from hippocampal recordings had shown that excitatory presynaptic activity was hypersensitive to isoflurane in the mutant compared to the wild type. The data correlated to the whole animal EC95 values for tail clamp and with the sensitivity of each genotype's mitochondria to isoflurane inhibition. In the three regions we investigate here, no differences were noted in spontaneous excitatory or inhibitory synaptic properties; that is, the spontaneous frequencies, amplitudes, and time decays of spontaneous excitatory postsynaptic currents and spontaneous inhibitory postsynaptic currents were not changed. Stimulated postsynaptic currents were not studied. The potential contribution of the hippocampus as a modulator of spinal cord activity in response to isoflurane is not known.

Earlier studies implicated motor neurons as potential volatile anesthetic targets.[7,8] Thus, we reasoned that the isoflurane-induced hyperpolarization of cells in the ventral horn most likely resided within cholinergic motor neurons. However, we clearly identified that hyperpolarization of noncholinergic cells is responsible for the change in resting membrane potentials recorded in the spinal cord of the mutant. Contrary to our hypothesis, interneurons in the spinal cord were the source of the increase in holding currents in the mutant, consistent with our previous finding that anesthetic sensitivity of an animal that has lost *Ndufs4* only in cholinergic neurons is identical to that of wild-type.[24] These findings inspire follow-up studies to identify the noncholinergic interneurons in the spinal cords of *Ndufs4*, which exhibit increased holding currents and resting membrane potentials in response to low concentrations of isoflurane. Such studies are underway.

These data show the surprising effect of mitochondrial dysfunction on the anesthetic response of any ion channels. Norfluoxetine is promiscuous in its blockade of K2P channels, affecting TREK-1, TREK-2, and, to a lesser extent, other K2P channels.[32] To determine the importance of specific K2P channels in the anesthetic-hypersensitive *Ndufs4(KO)* mouse, loss of specific K2P channels in an *Ndufs4(KO)* background is necessary. The K2P channels are a ubiquitously expressed superfamily of potassium leak channels that includes, among its six main families, TREK and TASK channels, both previously implicated in anesthetic action (for review, see Mathie and Veale[36]). K2P channels (TREK-1, TREK-2, and TRAAK) are most prominent in small cells in the dorsal root ganglion of the spinal cord.[32] Prominent TREK-2 *in situ* staining is seen in the ventral horn of the spinal cord, with a less prominent TREK-1 signal.[37] Staining for each was predominantly in interneuron layers. Previously, we reported that restricted loss of *Ndufs4* in GABAergic cells does not have an effect on anesthetic behavior.[24] However, because glutamatergic-specific loss of *Ndufs4* does give the full increase in volatile anesthetic sensitivity,[24] we hypothesize that glutamatergic interneurons give rise to the change in holding currents. Glutamatergic cells in the spinal cord can be probed for their role in the anesthetic behavior of *Ndufs4(KO)*. It may be that in the mutant, isoflurane induces a quiescence, *via* hyperpolarization, of interneurons upstream of motor neurons that ordinarily maintain cholinergic transmission to muscle. Our data are consistent with reports of anesthetic resistance in TREK-1 knockout animals. Animals lacking TREK-1 are reported to be partially resistant to a variety of volatile anesthetics, including a reported 16% resistance to isoflurane.[35] If a TREK channel is missing, one would predict that the ability of a volatile anesthetic to modulate motor activity *via*

excitatory interneurons would be largely lost because of the loss of TREK-mediated hyperpolarization.

It is not clear how a mitochondrial defect is transduced into a change in transmembrane currents through the K2P channels. We did not see a difference in TREK1 transcription *via* quantitative reverse transcriptase–polymerase chain reaction between the two genotypes (data not shown). We are now determining whether the phosphorylation status, which modulates activity of K2P channels,[31] is decreased in the *Ndufs4(KO)* mouse. Because phosphorylation of TREK channels is dependent on the adenosine monophosphate kinase,[38] it is possible that the energetic output from mitochondria could directly affect the function of the channels. However, it continues to be curious that profound increased volatile anesthetic sensitivity is seen primarily in complex I deficiency and is not a general result from other mitochondrial defects, nor are sensitivities to nonvolatile anesthetics similarly affected.[14–18,39]

In conclusion, in spinal cord slices, increases in holding current in ventral neurons occur at a much lower concentration of isoflurane in *Ndufs4(KO)* mice than in wild-type controls, matching the whole animal hypersensitivity of *Ndufs4(KO)* to tail clamp.[17] Surprisingly, this hypersensitivity is mediated by interneurons in the ventral horn. In slices from both wild-type and *Ndufs4(KO)*, the increases in holding currents are prevented by norfluoxetine, an inhibitor of the potassium leak channels TREK-1 and TREK-2.[26] Mitochondrial function is linked to activity of potassium leak channels.

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## Competing Interests

The authors declare no competing interests.

## Correspondence

Address correspondence to Dr. Morgan: University of Washington, Seattle, Washington 98101. pgm4@uw.edu. 

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