**Title:** Mitochondrial Function and Anesthetic Sensitivity in the Spinal Cord

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**Supplemental Methods.**

**Animals**

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*. Lumbar spinal cord slices from male and female mice, 23-30 days old, were used for experiments.

*Ndufs4(KO)* (*Ndufs4∆/∆*) mice were generated by crossing heterozygotes (*Ndufs4∆/+*) in a C57Bl/6 genetic background 22, 23 with more than 10 backcrosses. Control wildtype animals were c57BL/6 mice originally obtained from Jackson Laboratory and maintained at SCRI.

**Slice Preparation**

Preparation of slices for the vestibular nucleus (VN) and central medial thalamus (CMT) were as previously described for the hippocampus (CA1) [32, 33]. 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. 350 µm thick coronal slices in the plane of interest were made with a Leica VT1000S vibratome in ice-cold oxygenated slicing solution (in mM: 5 KCl, 1.25 NaH2PO4, 3.5 MgSO4, 0.5 CaCl2, 26 NaHCO3, 10 glucose, 210 sucrose), and incubated for 30 minutes in room temperature artificial cerebrospinal fluid (ACSF in mM: 118 NaCl, 3 KCl, 1.5 CaCl2, 1 MgCl2, 25 NaHCO3, 1 NaH2PO4, 30 glucose) before recording in artificial cerebrospinal fluid (ACSF) .

Methods for preparation of spinal cord slices were adapted from Mitra and Brownstone [34]. Mice between the ages of 23 and 30 days were anesthetized with isoflurane until unresponsive to toe pinch and their breathing rate decreased to 1 breath every two seconds. 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 (in mM: 208 sucrose, 0.75 K-gluconate, 1.25 KH2PO4, 26 choline bicarbonate (80% solution), 4 MgSO4, 1 CaCl2, 20 glucose, 2 kynurenic acid sodium salt (Abcam, Cambridge, UK), 1 (+)-sodium L-ascorbate, 5 ethyl pyruvate, and 3 *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 placing a thin strip cyanoacrylate glue 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. 350 µm thick transverse slices were made using a Leica VT1000S vibratome. 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 seconds. Slices were then incubated in a mixture of equal parts dissection solution and recording solution (in mM: 126 NaCl, 3 KCl, 1.25 NaH2PO4, 25 NaHCO3, 1.1 MgCl2, 2.2 CaCl2, 15 glucose, 1 (+)-sodium L-ascorbate, 5 ethyl pyruvate, and 3 *myo*-inositol) at 32°C for 30 minutes. Then the slices were incubated at 32°C recording solution for 30 minutes, and finally at room temperature in recording solution for at least 30 minutes before moving to the bath for electrophysiological recording.

**Whole-cell patch-clamp**

Individual slices were transferred to the recording chamber, which was superfused with oxygenated solution at 3.2-3.8 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, 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 Sutter Instruments P-97 puller. Cells were held at -60 mV for voltage-clamp experiments. Patch pipettes were filled with a potassium gluconate-based solution (in mM: potassium gluconate 140, CaCl2 1, MgSO4 2, EGTA 10, Na2ATP 4, NaGTP 0.4, HEPES 10, pH 7.3, 302-312 mOsm). 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) and digitized with a Digidata 1400 (Axon Instruments, CA, USA). Patch-clamp currents were filtered at 2.1 kHz and sampled at 10 kHz. Upon establishing a whole-cell patch, cells were held for 10 minutes before starting a recording. Series resistance was monitored during the experiments; recordings with substantially changed series resistance were excluded from the analysis.

**Drug Administration**

Isoflurane was applied in the superfusate at equilibrated concentrations delivered by passing carbogen (a mixture of 95% O2 and 5% CO2) through a calibrated isoflurane vaporizer. The superfusate was sampled during isoflurane exposure, and the isoflurane concentration was determined using gas chromatography. For action potential blockade, 0.5 µM tetrodotoxin (TTX) used in the recording solution described above.  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).

**Confocal microscopy**

The spinal cord of a ChAT-Cre;Ai14 mouse was dissected and fixed with 4% paraformaldehyde for an hour and incubated overnight at 4°C in 30mM sucrose solution. The cord was embedded in Optimal Cutting Temperature medium (Fisher Scientific, USA) and frozen before cutting 50 µm slices on a cryostat. Slices were mounted on slides (Superfrost) and incubated overnight at 37°C. They were subsequently incubated for 30 minutes in a 1X sodium phosphate buffer (PBS) solution containing 1% bovine serum albumin at 30oC. Slices were then incubated with DAPI (1ug/ml in PBS) for 10 minutes and then washed three times for five minutes in 1X PBS. The slides were dried and then covered with mounting media and a coverslip. Slides were imaged on a Zeiss LSM 710 Confocal Microscope. (983 words)