Supporting Information

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SI Materials and Methods

Generation of *Ndufs4*-null (*Ndufs4*$^{-/-}$) Mice. Generation and characterization of the *Ndufs4*$^{-/-}$ mice has been described (26). Briefly, a mouse line carrying a conditional allele of *Ndufs4* gene was created by flanking exon 2 with loxP sites such that Cre-mediated recombination removes exon 2, resulting in a transcript with a premature stop codon. Mice with the conditional *Ndufs4* allele (*Ndufs4*$^{lox/lox}$) were crossed with mice in which the *Cre* recombinase gene is targeted to the *Mox2* locus that is ubiquitously expressed early in development. The *Ndufs4* heterozygotes (*Ndufs4*$^{+/-}$) were then bred to generate littermates of *Ndufs4*$^{+/+}$, *Ndufs4*$^{+/-}$, and *Ndufs4*$^{-/-}$ embryos. The mice were on a mixed 129/Sv × C57/BL6 background. PCR genotyping of the embryos was performed by a second investigator and the results were matched to each embryo at the end of the experiment. Therefore, all experiments were performed double-blinded regarding the status of *Ndufs4* genotype.

Primary Mesencephalic Neuron Cultures. To prepare primary cultured dopaminergic neurons from single embryos, we modified the cell culture protocol of Gille *et al.* (58) to increase the yield of surviving TH^{+} dopaminergic neurons obtained from a single embryo. Briefly, the embryos were placed in PBS (pH 7.2) (Invitrogen) on ice, and the mesencephalon of each embryo was dissected and handled separately. The dissected tissue was washed with Dulbecco's modified Eagle medium (DMEM; Sigma), incubated at 37°C for 10 min, then placed in culture media consisting of DMEM supplemented with 4 mM glutamine, 10 mM Hepes buffer, 30 mM glucose, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated FBS (Invitrogen). The tissue was then triturated six times with a narrow pipette tip (P-3207; ISC BioExpress). The dissociated cells (3 to 5 × 10^4 /100 μl) were plated as a "microisland" on 9 mm diameter, Aclar embedding film (Electron Microscopy Sciences). The Aclar films were precoated with 100 μg/ml polyD-lysine and 4 μg/ml laminin (BD Biosciences) and placed in 48-well plates. The cultures were maintained at 37°C in a humidified 7% CO_2 atmosphere. At 24 h after plating, 300 μl of fresh culture media was added to each well. Half of the medium was changed every 48 h thereafter.

Isolation of Mitochondria. Isolation of mitochondria was performed as described (59). In brief, primary E14 mesencephalic cells at 5–6 DIV were scraped in isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM EGTA, 5 mM Hepes (pH 7.3), and 2 mg/ml fat-free BSA). Cells of the same genotype were pooled and homogenized using a Dounce homogenizer with 10 up-and-down strokes. The homogenate was centrifuged at 1,000 *g* for 10 min. For the crude mitochondria preparation, the supernatant was centrifuged again at 13,000 *g* for 20 min. For the purified mitochondria (used in all figures), the 1,000 *g* supernatant was transferred onto a layered Ficoll gradient (5 ml of 7.5% Ficoll medium on top of 5 ml of 10% Ficoll medium containing 0.3 M sucrose, 50 μM EGTA, and 10 mM Hepes) and centrifuged at 79,000 *g* for 30 min. The resulting mitochondrial pellet was resuspended in isolation buffer (without BSA), and the protein concentrations were assayed using the bicinchoninic assay (Pierce) with BSA standards.

Polarography. Monitoring of mitochondrial oxygen consumption was performed as described (59). Briefly, healthy, intact E14 cultured neurons grown on coverslips (five each) or freshly purified mitochondria were placed in respiration buffer (pH 7.3) consisting of 225 mM mannitol, 75 mM sucrose, 10 mM KCl, 5 mM Hepes, 5 mM K_2HPO_4, and freshly added 1 mg/ml defatted BSA at 37°C (cells) or 30°C (mitochondria). Oxygen consumption was measured in a 3 ml vessel of an oxygen monitoring apparatus (5300A system; YSI). Mitochondrial complex I activity was defined as the rotenone (1.25 μM) or piercidin (3 μM)-sensitive oxygen consumption rate in the presence of complex I-specific substrates (10 mM pyruvate /2 mM malate/1 mM ADP). Complex II activity was calculated as the antimycin A (2.5 μM)-sensitive oxygen consumption rate in the presence of complex II-specific substrates succinate (10 mM). State 2 or state 3 respiration was initiated by the addition of 10 mM pyruvate/2 mM malate or 1 mM ADP, respectively. The amount of oxygen consumed was calculated by assuming the initial oxygen concentration in the buffer to be 0.223 μmol O_2/ml.

Oxygen Biosensor System. Oxygen consumption was also measured using the oxygen-sensitive, fluorescent dye-embedded 96-well microplate of the BD Oxygen Biosensor System (BD Biosciences) as described per manufacturer's instructions (60–62). Briefly, cells or mitochondria were incubated in respiration buffer in the 96-well assay microplate (BD Biosciences). The fluorescence was measured using a fluorescent microplate reader (Molecular Probes; Ex/Em = 485/595 nm). The data were normalized and calculated according to manufacturer's protocol. Complex I activity and the state 2 and state 3 respiration were determined as described in *Polarography*.

NADH Dehydrogenase Activity Assay. We modified published protocols for NADH dehydrogenase activity assay in intact cells (59, 63) to measure complex I activity in Fig. 4 and Fig. S1. E14 mesencephalic cells (3 to 5 × 10^4/100 μl) were cultured in 98-well plates precoated with polyD-lysine and laminin. Cells were incubated for 1 h with soluble tetrazolium (Promega) as an NADH dehydrogenase substrate, with or without a high-dose complex I inhibitor (10 μM rotenone or 2 μM piericidin). After incubation, the optical density (OD) of the culture media was measured at 490 nm using a multiwell plate reader (Molecular Devices), and mitochondrial complex I (CI) activity among total NADH dehydrogenase activity (NDA) was calculated as follows:

$$\frac{\text{NDA w/o CI inhibitor–NDA w/CI inhibitor}}{\text{NDA w/o CI inhibitor}} \times 100(\%)$$

Drug Treatments. Rotenone (Sigma) was dissolved in dimethyl sulfoxide (DMSO) as 10 mM stock or in ethanol as 1 mM stock; the same results were obtained with either stock solution. Paraquat (Sigma) was dissolved in water as a 400 mM stock; MPP^{+} iodide (Sigma) was dissolved in N2 medium (Invitrogen) as a 10 mM stock. All drugs were diluted in N2 media immediately before the drug treatments. When cell cultures were treated with rotenone, the final concentration of DMSO or ethanol did not exceed 0.0001% or 0.001%, respectively. All drug treatments were performed in defined serum-free N2 medium. Half of the media was replaced with N2 medium supplemented with BSA (10 μg/ml; Sigma) on the day before drug treatment, and then again at the time of drug treatment. Cultures treated with vehicle alone were used as controls.

Immunocytochemistry. Neuron cultures were fixed with 4% paraformaldehyde/4% sucrose and blocked with 5% BSA/5% normal

goat serum and 0.1% Triton X-100 in PBS. Primary antibodies included mouse monoclonal antibody against tyrosine hydroxylase (TH; 1:500; Sigma), rabbit polyclonal antibody against TH (1:50,000; Pel-Freez), rabbit polyclonal antibody against GABA (1:5,000; Sigma), mouse monoclonal antibody against NeuN (1:750; Chemicon), rabbit polyclonal antibody against GFAP (1:400; DAKOCytomation), rabbit polyclonal antibody against Iba1 (1:500; Wako Pure Chemical), rabbit polyclonal antibody against Olig-2 (1:5,000; generously provided by Dr. John Alberta), and rabbit polyclonal antibody against activated caspase-3 (1:3,000; R&D Systems). Secondary antibodies were Alexa Fluor 488 (or 568) goat anti-rabbit IgG and Alexa Fluor 568 (or 488) goat anti-mouse IgG (1:200; Molecular Probes).

Quantification of TH^+, GABAergic, or Total Neurons. Cells immunostained positive for TH antibody and having neurites twice the length of the soma were scored as TH^+ cells. All TH^+ cells on a 9 mm diameter Aclar embedding film were scored. The number of TH^+ neurons in one Aclar embedding film was ≈100–200 in control cultures (either untreated or vehicle control treated). Cells immunostained positive for GABA or NeuN antibody were counted from five representative fields from each Aclar embedding film and scored as the GABAergic or total neuron population.

Quantification of Total Apoptosis. Apoptosis was determined for the entire cell population by nuclear condensation and/or fragmentation after Hoechst staining (64). Healthy cells have evenly and uniformly stained nuclei.

Terminal Deoxynucleotidyl Transferase-Mediated Biotinylated UTP Nick End Labeling (TUNEL). TUNEL staining was performed using TDT (Promega) as described (64). The number of TUNEL-positive cells in the TH^+ neuron population was quantified. All TH^+ neurons on each Aclar film were scored.

ROS Labeling and Quantification. Cells were incubated at 37°C for 15 min with 5 μM 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-DCFDA; Molecular Probes), washed, and left in the incubator for another 15 min. Alternatively, cells were incubated with MitoSOX (Molecular Probes) for 10 min and washed. The images of eight fields from each well were captured by a microscope equipped by digital camera (Leica or Zeiss). Cells were then fixed and processed for anti-TH immunostaining. After staining, images from the same eight fields were captured and compared. The staining intensity of CM-DCFDA dye (ROS) in the TH^+ neuron population was quantified using NIH Image program (http://rsb.info.nih.gov/ij/). All TH^+ neurons in captured images were scored, and the average ROS level per TH^+ neuron was calculated.

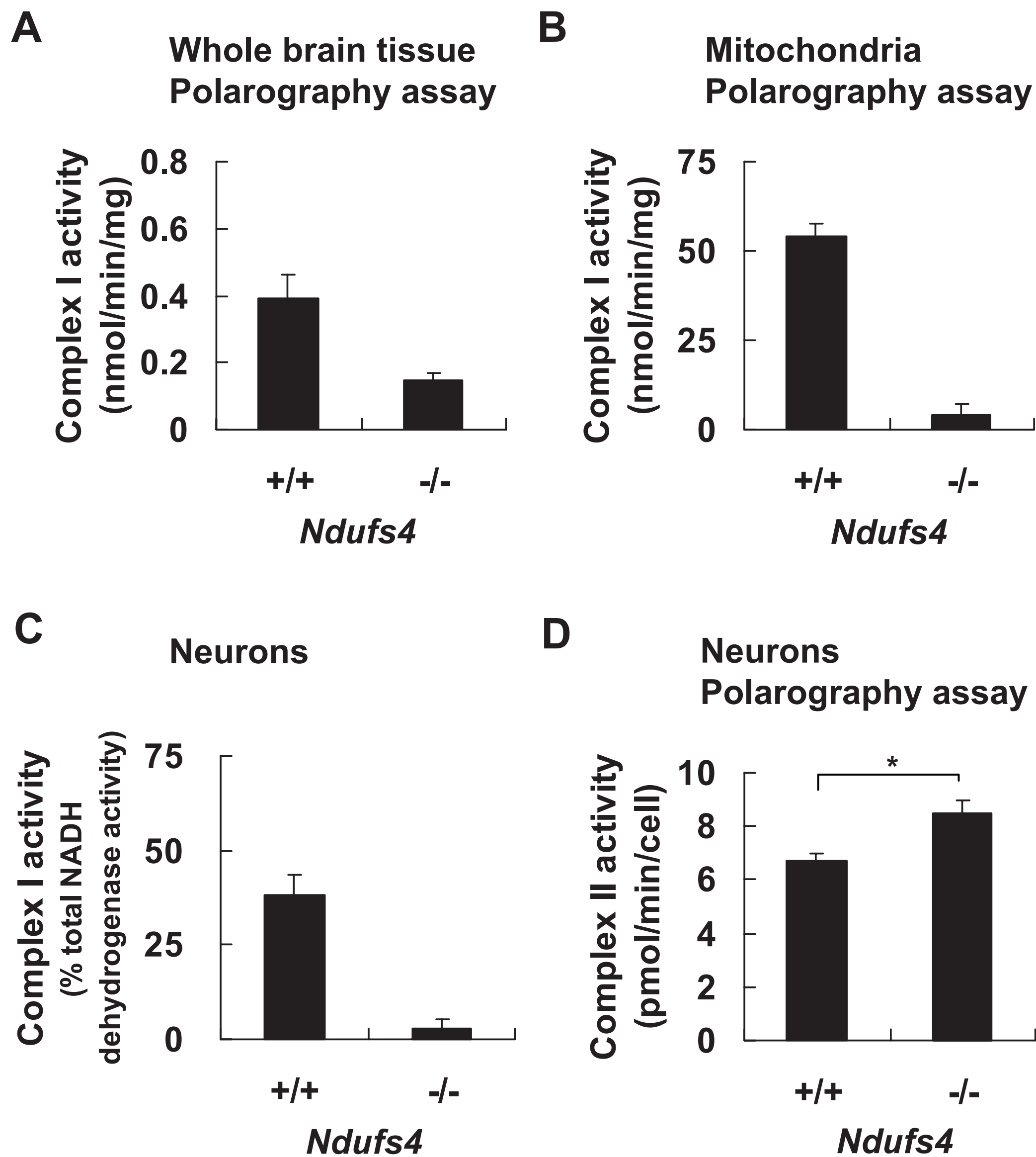

Fig. S1. Deletion of the *Ndufs4* ablates mitochondrial complex I activity from purified mitochondria and neuronal cultures. (*A*) Brain tissue from newborn mice (P1) was finely chopped with scissors, and tissue fragments were incubated with complex I substrates (plus or minus rotenone) for measurement of O_2 consumption by polarography as described in *Methods*; rotenone-sensitive O_2 consumption was reduced compared with WT controls, but still significantly above background. Crude mitochondria (see *Methods*) isolated from neonatal mouse brain of *Ndufs4*-null mice also retained some rotenone-sensitive O_2 consumption (data not shown). (*B*) Rotenone-sensitive O_2 consumption was lost in purified mitochondria isolated from P1 brain of *Ndufs4*-null mice. (*C*) E14 mesencephalic neurons (DIV6) cultured from *Ndufs4*-null mice have no complex I activity, measured by the NADH dehydrogenase activity assay using nitroblue tetrazolium as a substrate. (*D*) Succinate-driven, antimycin A-sensitive complex II activity in E14 mesencephalic neuron cultures (DIV6) from *Ndufs4*-null mice is not compromised.

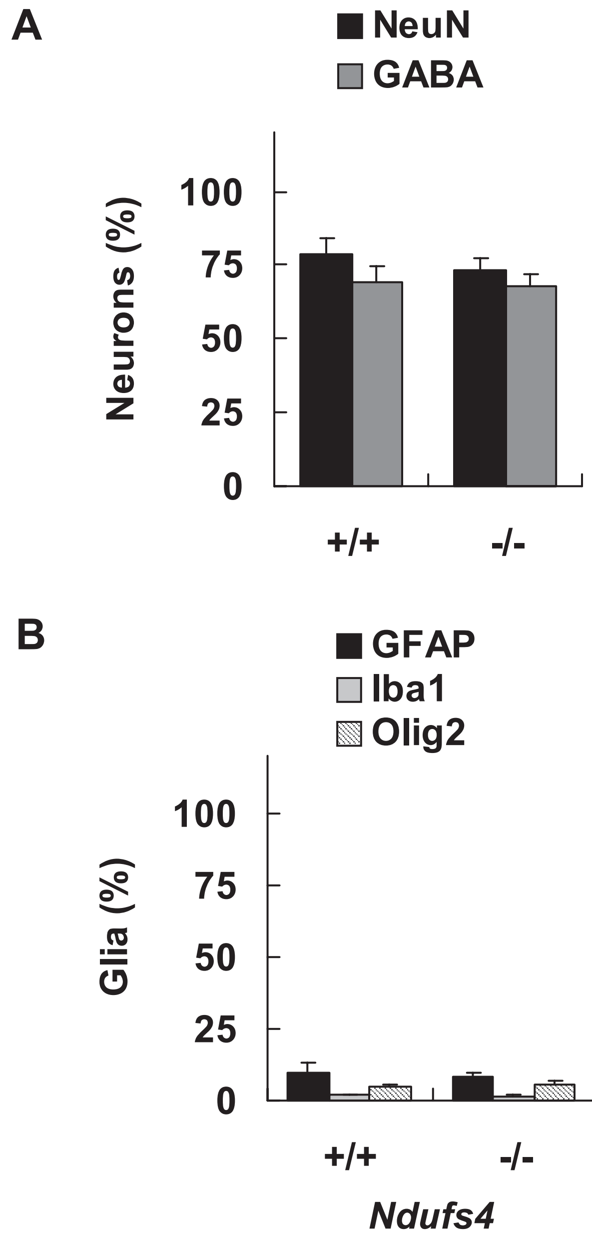

Fig. S2. Deletion of *Ndufs4* does not change the composition of neurons or glial. E14 mesencephalic neurons were cultured for 6 days. (*A*) The percentage of GABAergic ($GABA^+$) or total ($NeuN^+$) neurons. (*B*) The percentage of astroglia ($GFAP^+$), microglia ($Iba1^+$), and oligodendrocytes ($Olig2^+$).

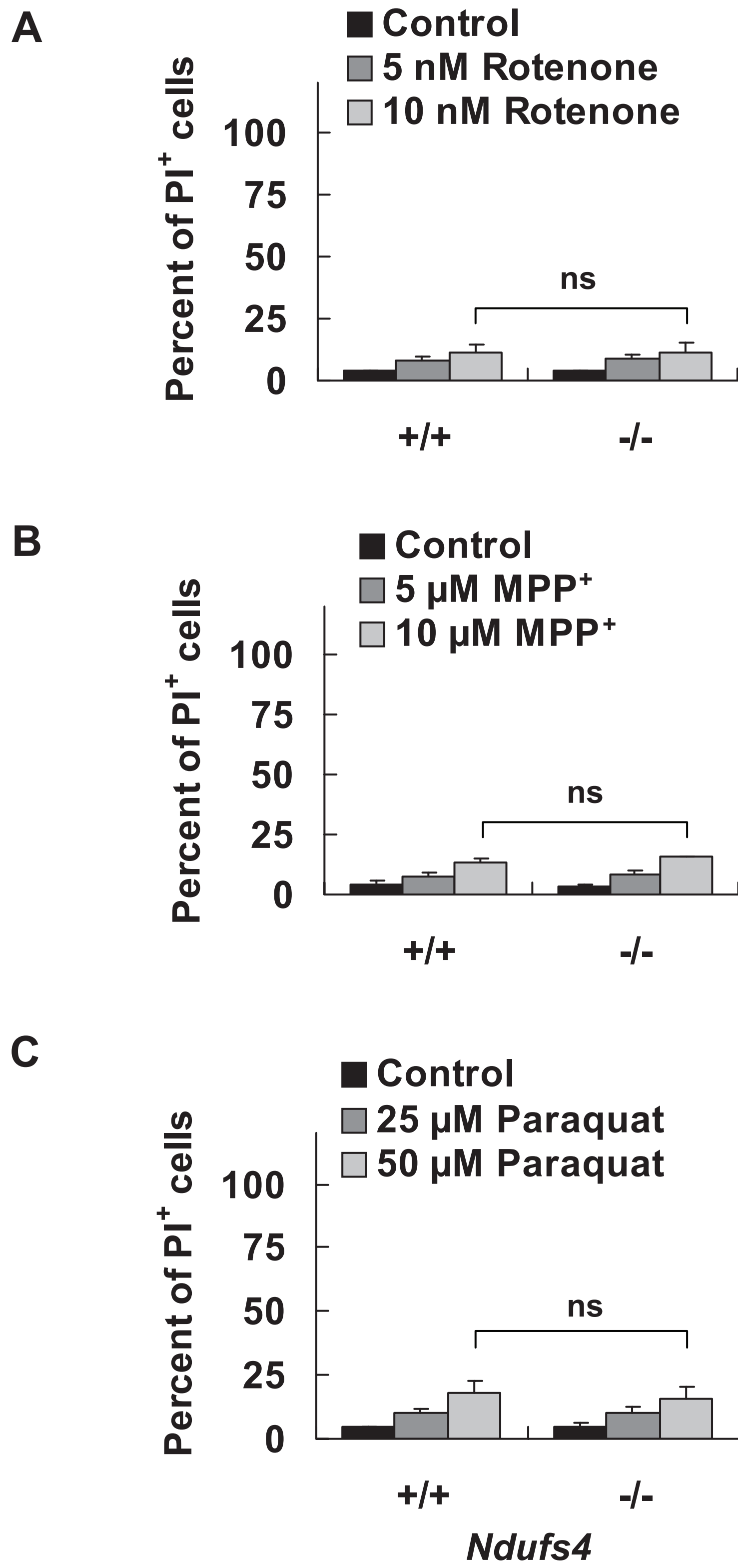

Fig. S3. *Ndufs4* deletion does not change the degree of total cell death quantified by propidium iodide (PI) staining. (*A*) Treatment with rotenone (24 h). (*B*) Treatment with MPP^+ (48 h). (*C*) Treatment with paraquat (24 h). Cells were stained with 0.5 μg/ml propidium iodide (PI) for 15 min and then washed with DMEM. PI-stained cells were counted in eight random fields from each treatment ($n = 3$).

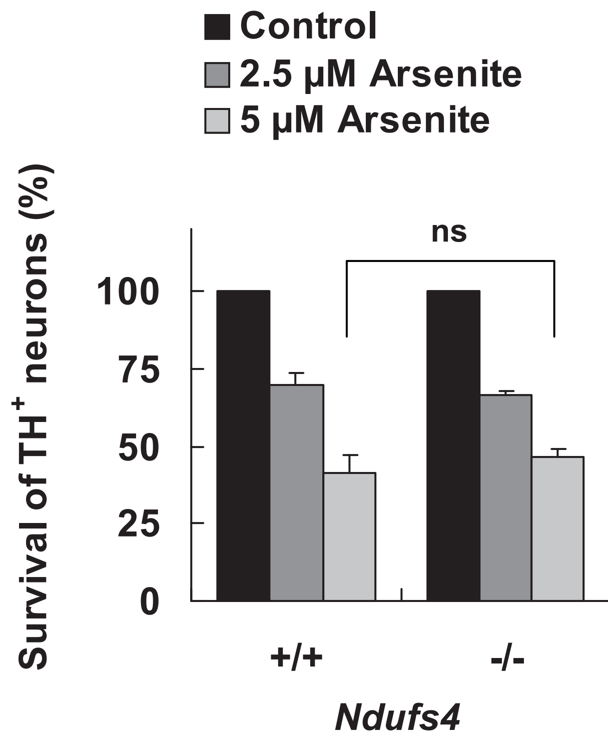

Fig. S4. TH$^+$ dopaminergic neurons from *Ndufs4*$^{-/-}$ mice exhibit the same sensitivity to arsenite toxicity as those from the wild-type littermates. Cells were treated with sodium arsenite (arsenite) or vehicle control on DIV 5 for 24 h.

Table S1. Quantification of cell morphology of TH^+ dopaminergic neurons cultured from *Ndufs4*$^{-/-}$ and the wild-type littermates

	Ndufs4$^{+/+}$	*Ndufs4*$^{-/-}$	*P* value*
Length of primary neurite, μm	131.6 ± 12.6	123.2 ± 8.8	0.66
Soma diameter, μm	12.7 ± 1.9	12.6 ± 1.8	0.44
No. of primary neurite	2.1 ± 0.4	2.3 ± 0.5	0.55
No. of branch points on primary neurite	1.6 ± 1.5	1.9 ± 1.6	0.74

Images of TH^+ neurons were captured and morphometric parameters, including length of the longest primary neurite, soma diameter, number of neurite, and number of branch points on the longest primary neurite, were quantitated by using NIH Image and Slidebook (Olympus). Values are mean ± SEM.

**P* values were obtained from Student's *t* test.