Supporting Information

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SI Materials and Methods

Primary Neuronal Culture Preparation. Primary cortical cell cultures were prepared from gestational day 15 mouse embryos (1). Cortical neurons were cultured in Neurobasal medium (Life Technologies) supplemented with B27 and 2% horse serum. On day in vitro (DIV) 4, the cultures were treated with 5-fluoro 2-deoxyuridine (30 μM) to inhibit glial growth and proliferation. Experiments were performed at DIV 12. Under these conditions, mature neurons represent 90% of the cells in the culture. *N*-methyl-*N*-nitroso-*N*-nitroguanidine (MNNG) (50 μM) was applied to neurons for 15 min, and cells were washed and collected at several time points for seahorse and biochemical analysis. In a subset of experiments, cell viability was determined on the following day by unbiased objective computer-assisted cell counting after staining of all nuclei with 7 μM Hoechst 33342 (Invitrogen) and dead cell nuclei with 2 μM propidium iodide (Invitrogen). The numbers of total and dead cells were counted with the Axiovision 4.6 software (Carl Zeiss). At least three separate experiments using at least six separate wells were performed with a minimum of 15,000–20,000 neurons or cells counted per data point. For neuronal toxicity assessments, glial nuclei fluoresced at a different intensity than neuronal nuclei and were gated out. The percentage of cell death was determined as the ratio of live to dead cells compared with the percentage of cell death in control wells to account for cell death attributed to mechanical stimulation of the cultures. All experiments were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (2) and were approved by the The Johns Hopkins University School of Medicine institutional animal care and use committee.

Measurements of Oxygen Consumption Rate. The mitochondrial oxygen consumption rate (OCR) in mouse cortical neurons was measured in an XF24 Extracellular Flux Analyzer (Seahorse Bioscience), as described previously (3–5). On DIV 12, the culture medium in cortical neurons plated at a density of $\sim 0.5 \times 10^6$ per well in XF24 cell-culture microplates was replaced with XF24 DMEM containing 10 mM glucose with or without 1 mM L-glutamine (Life Technologies) and 1 mM sodium pyruvate (Life Technologies). OCR was measured at 37 °C with a 1-min mix, 1-min wait, and 2-min measurement protocol. OCR was analyzed in an XF24 analyzer after 45-min incubation in a CO_2-free incubator. The first OCR measurement was recorded following 11-min equilibration, 1-min mix, and 1-min wait (total time after MNNG-treatment ~60 min). Oligomycin, carbonyl cyanide m-chlorophenylhydrazone, and rotenone were injected into each well sequentially to access basal respiration, coupling of respiratory chain, and mitochondrial respiratory capacity. OCRs were normalized relative to the protein concentration in each well. Data are presented as the percentage of change as compared with control.

Measurement of Glycolysis as Extracellular Acidification Rate. Culture medium in neuronal cultures plated at a density of $\sim 0.5 \times 10^6$ per well in XF24 cell-culture microplates was replaced with glucose-free XF24 Seahorse medium. Glycolytic flux (basal glycolysis, glycolytic capacity, and glycolytic reserve) as assessed by extracellular acidification rate (ECAR) was analyzed by the sequential addition of glucose, oligomycin, and 2-deoxyglucose in an XF24 flux analyzer (4). ECAR was measured at 37 °C with a 1-min mix, 1-min wait, and 2-min measurement protocol. Seahorse analysis was started after 45-min incubation in a CO_2-free incubator. The first ECAR measurement was recorded after 11-min equilibration, 1-min mix, and 1-min wait (total time after MNNG-treatment ~60 min). ECAR values were normalized relative to the protein concentration in each well. Data are presented as the percent of change compared with control.

Lactate Measurements. Lactate production was measured directly in the medium. One hundred microliters of the culture medium was aspirated into the injection module of the pHOXplusL analyzer (Nova Biomedical) to measure lactate flux in the neuronal cultures treated with MNNG or DMSO. Data were normalized to the cell number in each culture well and are expressed as millimoles of lactate per one million cells.

Immunoblot Analysis. Neuronal cultures were exposed to MNNG for 15 min and then were collected after 0, 15, 30, 60, and 120 min in lysis buffer (PBS containing 1 mM EDTA, 1 mM EGTA, 0.5% SDS, 1% Nonidet P-40, 0.25 mM sodium orthovanadate, 0.25 mM PMSF, 2.5 μg/mL leupeptin, 2.5 μg/mL aprotinin). Cell lysates were subjected to centrifugation at $10,000 \times g$ for 10 min at 4 °C. The resulting supernatant was subjected to SDS/PAGE, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was washed in Tris-buffered saline solution containing 0.1% Tween 20 (TBST) and was blocked for 1 h at room temperature in TBST containing 5% nonfat dry milk. The membrane then was incubated overnight at 4 °C with anti-poly(ADP-ribose) (anti-PAR; clone #19, custom designed at Bio-Rad AbD Serotec GmbH), anti–HK-1 (Cell Signaling), and HRP-conjugated anti–β-actin (Sigma). After primary antibody incubation, the membranes were incubated with HRP-conjugated goat anti-human IgG (Fab')2 (Abcam) and HRP-conjugated donkey anti-rabbit IgG (GE Healthcare) secondary antibodies for 1 h at room temperature. The immunoblots were visualized with enhanced chemiluminescence (Pierce) in an ImageQuant LAS 4000 Mini imaging analyzer (GE Healthcare).

NAD^+ and ATP Measurements. NAD^+ was measured using an NAD/NADH quantification kit (Sigma). Cells were treated with 50 μM MNNG for 15 min in presence or absence of 3,4-dihydro-5[4-(1-piperidinyl)butoxy]-1(2H)-isoquinoline. In the FK866 experiment, neuronal cultures were treated with 10 μM FK866 or DMSO, and samples were collected at 0, 1, 3, and 5 h. Approximately 1×10^6 cells were collected in 400 μL of extraction buffer at 0, 15, 30, 120, and 360 min after MNNG-treatment or 0, 1, 3, and 5 h after FK866 treatment. The cofactors were extracted following two cycles of freezing on dry ice for 20 min followed by 10 min at room temperature. The samples were centrifuged at $13,000 \times g$ for 10 min to remove insoluble materials. NAD^+ was quantified according to the manufacturer's guidelines provided with the kit (Sigma). ATP levels were assessed by luminescence assay using an ATP Determination Kit (Life Technologies). Cells were washed with ice-cold PBS and lysed in ATP-releasing buffer [100 mM Tris (pH 7.8), 4 mM EDTA, 1 mM DTT, and 1% TritonX-100]. The assay was performed according to the manufacturer's guidelines. Ten microliters of the lysate was used in ATP assay, and the values were normalized by the amount of protein in each sample. The data are presented as the percentage of control.

Hexokinase Activity Assay. Hexokinase activity was measured according to the Worthington protocol (Worthington Biochemical Corporation) with slight modifications. After MNNG treatment, the cells were washed with ice-cold PBS and lysed in

hexokinase cell lysis buffer [50 mM Tris (pH 7.8), 13.3 mM $MgCl_2$, 0.05% Triton X-100, and a mixture of protease inhibitors]. The assay buffer consisted of 50 mM Tris, 13.3 mM $MgCl_2$, 1 mM NAD, 1 mM ATP, 0.05% Triton X-100, and 1 U/mL G-6-P dehydrogenase (pH 8.0). For the hexokinase enzymatic reaction, 25 μL (50 μg protein) of sample was added to 155 μL of the assay buffer in a 96-well plate. After 1-min equilibration at room temperature, the reaction was initiated by the addition of 20 μL of 2 M glucose. The total volume of the assay was 200 μL. Reading were taken at 1-min intervals for 10 min at 340 nm at 30 °C in a kinetics mode in a 96-well plate reader. The enzymatic activity was calculated in accordance with the guidelines provided in the Worthington protocol.

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