APPENDIX

Resistance of glia-like central and peripheral neural stem cells to genetically-induced mitochondrial dysfunction - differential effects on neurogenesis

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MATERIALS AND METHODS

Tissue preparation and histochemistry

Mice were heavily anesthetized and then killed by decapitation. Dissected tissues were fixed overnight in paraformaldehyde (4% in PBS) and embedded in paraffin (brain) or OCT (Tissue-Tek; peripheral tissues) before sectioning. Coronal mouse brain sections (20 μm thick) were used for NeuN or GFAP immunostaining. Peripheral tissue sections (10 μm thick) were used for immunostaining with the different antibodies. Staining procedures were performed as previously described [1, 2]. Nuclei were detected by DAPI staining. Primary antibodies and the dilution factors applied were as follows: mouse anti-NeuN (Millipore; 1:200), rabbit anti-GFAP (Dako; 1:500), rabbit anti-TH (Novus Biological; 1:1000), mouse anti-HuC/D (Invitrogen; 1:1000). Secondary antibodies and dilutions applied were: For 3,3'-diaminobenzidine-based detection, an Envision+ kit (Dako) was used according to the manufacturer's recommended protocol. For fluorescence detection the secondary antibodies used were Alexa568-goat-anti-mouse-IgG (Molecular Probes, 1:500) and Alexa568-goat-anti-rabbit-IgG (Molecular Probes, 1:500).

X-gal staining

Sections of Superior cervical ganglia (SCG) from hGFAP-Cre/floxed LacZ mice were stained to detect the expression of the β-galactosidase by incubating them 12-24 hours at 37ºC in X-gal buffer. The buffer was made of PBS (pH=7.4) supplemented with 2 mM MgCl2 (Sigma), 5 mM potassium ferrocyanide (Sigma), and 5 mM potassium ferricyanide (Sigma). X-gal (Molecular Probes) was added to the buffer right before use, at a final concentration of 1 mg/ml. Once X-gal staining was performed, sections were processed for antibody staining as indicated previously.

Tissue dissociation and neurosphere assays

Mouse CBs were dissociated by enzymatic treatment in PBS solution containing 0.6 mg/ml collagenase type II (Sigma) and 0.3 mg/ml trypsin (Sigma) for 20 min at 37 ºC in a glass flask at 600 rpm in a Thermomixer Comfort (Eppendorf). After enzymatic treatment, two volumes of staining solution were added to quench enzymes. The staining solution contained (per 50 ml): 44 ml L15 medium (GIBCO), 0.5 ml penicillin/streptomycin (GIBCO), 0.5 ml 1 M HEPES buffer (GIBCO), 0.1 g BSA (Sigma), and 5 ml distilled and deionized water to volume. Dissociated CB cells were centrifuged at 300 x g for 5 min at 4 ºC. The cell pellet was then resuspended and plated in an ultra-low binding well for neurosphere assays, or on cover slips for immunocytochemistry.

For the growth of neurospheres, dissociated mouse CB cells were typically cultured in ultra-low binding 6-well plates (Corning Inc.) at a clonal density of 10000 cells per well so that individual neurospheres were spatially separated from each other. The culture medium contained D-MEM:F-12 (GIBCO BRL) with 15% FBS (GIBCO), 1% N2 supplement (GIBCO), 2% B27 supplement (GIBCO), 1% penicillin/streptomycin (GIBCO), 20 ng/ml recombinant human bFGF (R&D Systems), 20 ng/ml recombinant human IGF-1 (R&D Systems), and 20 ng/ml recombinant human EGF (R&D Systems). All cultures were maintained in O_2- and CO_2-controlled incubators (Thermo Electron Corp) at 21% O_2, and 37 ºC.

To prepare cultures of brain NSCs, the SVZ area of freshly dissected mouse brains was removed and suspended after mechanical dissociation in PBS with 25 mg/mL deoxyribonuclease type 1 (DNAse1, Sigma) and 0.3 mg/mL of trypsin (Sigma) for 20 min at 37 ºC. After quenching and centrifuging, the cells were

resuspended, filtered through nylon mesh (pore size:45 µm, SefarMaissa), counted with the aid of a hemocytometer, and plated.

Free-floating cultures of brain neurospheres were obtained by plating SVZ cells in ultra-low binding 6-well plates (Corning Incorporated, Corning, NY), at clonal density to avoid neurosphere fusion. The culture medium was based on DMEM:F12 and was supplemented with 1% penicillin/streptomycin (Biowhittaker), 1% N_2 supplement (Gibco), 2% B27 supplement (Gibco), 20 ng/ml recombinant human bFGF (R&D Systems), 20 ng/ml EGF (R&D Systems) and 10 ng/ml IGF (R&D Systems). All cultures were maintained at 37 ºC in 5% CO_2/balance air. After 10 days in culture, the diameters of neurospheres were quantified using ImageJ software (NIH). For differentiation assays, SVZ neurospheres were plated under adherent conditions in 6-well plates previously treated with 0.15 mg/ml human fibronectin (Biomedical Technologies). Mitogens (FGF, EGF and IGF) were removed from the culture medium [3].

Cell sorting

Dissected cortex and striatum from mouse brains were mechanically dissociated and resuspended in staining solution (per 50 ml: 44 ml L15 medium (GIBCO), 0.5 ml penicillin/streptomycin (Cambrex), 0.5 ml 1 M HEPES buffer (GIBCO), 0.1 g BSA (Sigma), and 5 ml distilled and deionized water to volume). Cell sorting of GFAP+ cells was performed in a MoFlow three-laser flow cytometer (DakoCytomation). During sorting, 7-AAD+ (Molecular Probes) dead cells were eliminated. For RNA extraction, cells were harvested and stored frozen in liquid nitrogen until use.

Immunocytochemistry

SVZ cultured cells were fixed in 4% PFA (prepared in PBS) for 15 min at room temperature. Primary antibodies were incubated overnight at 4 ºC, and secondary antibodies were incubated for 1-2 hr at room temperature. The antibodies used were rabbit anti-Tuj1 (Abcam; 1:1000), Alexa488-donkey-anti-rabbit-IgG (Molecular Probes, 1:500), rabbit anti-GFAP (DAKO, 1:500), Alexa568-goat-anti-rabbit-IgG (Molecular Probes, 1:500), mouse anti-O4 (Chemicon, 1:2000), and Alexa568-Goat-anti-Mouse-IgM (Invitrogen, 1:1000).

***SdhD* DNA and mRNA analyses**

DNA and RNA analyses were performed as described previously [1]. Genomic DNA

was extracted from nuclear fractions obtained from the different brain or peripheral tissues by overnight incubation at 37 ºC in 0.1 M Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 0.2 M NaCl, and 100 µg/ml proteinase K. The relative amount of the *SdhD*^{flox} allele was estimated by qPCR using the following primers: 5'-CTATGTAGGAGTCTGCAGCCAAGCT-3', 5'-ACTCAAGGTCAGCCTCACCTACCTAT-3', and normalized to the PCR product of the *GusB* gene. Total RNA was prepared from frozen tissues by using an RNA asymicrokit (Qiagen) according to the manufacturer's directions. Reverse transcription of mRNA was performed with a Superscript II reverse transcriptase kit (Invitrogen), and the *SdhD* cDNA was amplified by quantitative PCR with the primers 5' CCAGCACATTCACCTGTCA-3' and 5'-ATCAGCCCCAAGAGCAGAA-3' in the presence of SYBRgreen (Life Technologies). The *Arbp* housekeeping gene was used for normalization.

Mitochondria isolation and complex II activity

Mitochondrial complex II activity was determined according to Piruat et al. [4] with slight modifications. Briefly, 30-50 µg of protein were assayed at 30 ºC. Samples were diluted 1:4 in the essay reaction buffer (25 mM KH_2PO_4 pH 7.2, 5 mM $MgCl_2$, 3 mM potassium cyanide, and 2.5 mg/ml bovine serum albumin) and liquid nitrogen frozen-thawed three times before the essay. Succinate-ubiquinone oxidoreductase (SQR) activity of mitochondrial complex II was measured for a period of two minutes as the decrease in absorbance at 600 nm due to the reduction of 50 µM 2,6-dichlorophenol-indophenol (DCPIP) coupled to reduction of 130 µM ubiquinone-1. The reaction was carried out in presence of 3.6 µM antimycin, 5 µM rotenone and 10 mM succinate.

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