

Mitochondrial Protection Attenuates Inflammation-Induced Impairment of Neurogenesis *In Vitro* and *In Vivo*

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The impairment of hippocampal neurogenesis has been linked to the pathogenesis of neurological disorders from chronic neurodegenerative disease to the progressive cognitive impairment of children who receive brain irradiation. Numerous studies provide evidence that inflammation downregulates neurogenesis, with multiple factors contributing to this impairment. Although mitochondria are one of the primary targets of inflammatory injury, the role of mitochondrial function in the modulation of neurogenesis remains relatively unstudied. In this study, we used neurosphere-derived cells to show that immature doublecortin (Dcx)-positive neurons are uniquely sensitive to mitochondrial inhibition, demonstrating rapid loss of mitochondrial potential and cell viability compared with glial cells and more mature neurons. Mitochondrial inhibition for 24 h produced no significant changes in astrocyte or oligodendrocyte viability, but reduced viability of mature neurons by 30%, and reduced survival of Dcx⁺ cells by 60%. We demonstrate that protection of mitochondrial function with mitochondrial metabolites or the mitochondrial chaperone mtHsp75/mortalin partially reverses the inflammation-associated impairment of neurogenesis *in vitro* and in irradiated mice *in vivo*. Our findings highlight mitochondrial mechanisms involved in neurogenesis and indicate mitochondria as a potential target for protective strategies to prevent the impairment of neurogenesis by inflammation.

Introduction

Neurogenesis continues throughout life, with thousands of new neurons generated daily in the dentate gyrus subgranular zone (Gage, 2000; Cameron and McKay, 2001). Impairment of hippocampal neurogenesis is implicated in aging-associated cognitive decline, Alzheimer's disease, and ionizing radiation damage (Haughey et al., 2002; Monje and Palmer, 2003; Rola et al., 2004; Galvan and Jin, 2007), all settings in which inflammation is thought to play a role (Nelson et al., 2002; Sparkman and Johnson, 2008). The number of activated microglia directly correlates with the impairment of neurogenesis in irradiated animals (Mizumatsu et al., 2003) and treatment with anti-inflammatory drugs reversed impairment of neurogenesis caused by inflammation or irradiation (Ekdahl et al., 2003; Monje et al., 2003). Cerebral ischemia stimulates neurogenesis in adult brain (Liu et al., 1998; Türeyen et al., 2004; Tonchev et al., 2005), and the nonsteroidal antiinflammatory drug indomethacin increases survival of these newly generated cells (Takasawa et al., 2002; Sun et al., 2003; Hoehn et al., 2005).

Only activated, not resting, microglia inhibit neurogenesis (Monje et al., 2003). Activated microglia produce proinflammatory factors, including IL-6 and tumor necrosis factor- α (TNF- α) (Pocock and Liddle, 2001; Hanisch, 2002), reactive oxygen spe-

cies (ROS), and nitric oxide (Rock et al., 2004). TNF- α induces mitochondrial damage through suppression of mitochondrial complexes I and IV and pyruvate dehydrogenase (PDH) activities (Stadler et al., 1992; Zell et al., 1997; Samavati et al., 2008). Exposure to increased ROS impairs oxidative phosphorylation through oxidation of mitochondrial lipids and respiratory enzymes (Wagner et al., 1990; Halliwell, 2006), and IL-6 stimulates increased ROS production in brain (Behrens et al., 2008). Nitric oxide is a potent inhibitor of mitochondrial cytochrome *c* oxidase (complex IV) (Brown, 1995; Giuffrè et al., 1996).

Inflammation induces mitochondrial dysfunction *in vitro* and *in vivo* (Xie et al., 2004; Hunter et al., 2007), and exposure to IL-6, TNF- α , and nitric oxide impair *in vitro* neurogenesis (Monje et al., 2003; Covacu et al., 2006; Peng et al., 2008), so we postulated that impairment of mitochondrial function by inflammatory mediators is a mechanism by which inflammation impairs neurogenesis.

Two recent studies highlight the involvement of mitochondrial function in neurogenesis. First, mice deficient in α -ketoglutarate dehydrogenase complex activity were shown to have reduced hippocampal neurogenesis with fewer doublecortin-positive (Dcx⁺) cells (Calingasan et al., 2008). Second, treatment with the mitochondrial antioxidant α -lipoic acid partially reversed the radiation-induced reduction of immature Dcx⁺ neurons in hippocampus *in vivo* (Fike et al., 2007). These studies led us to postulate that Dcx⁺ cells might represent the stage in neurogenesis most susceptible to inhibition by mitochondrial impairment, such as that triggered by inflammation. We therefore tested which cells produced in the course of neural progenitor differentiation were most vulnerable to inhibition of mitochondrial function and identified the Dcx⁺ early neuron as most susceptible.

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Several strategies to protect mitochondria, including overexpressing a mitochondrial chaperone, heat shock protein 75 (mtHsp75), or providing mitochondrial fuels and cofactors, were found here to rescue the impairment of neurogenesis caused by activated microglial medium-induced inflammation *in vitro* and radiation *in vivo*.

Materials and Methods

Cell culture. Neural precursor cells were isolated from newborn mice. The brains were removed, freed of meninges, and diced with a sterile razor blade in dissociation buffer [DMEM/F12 medium containing 2.5 U/ml papain (Worthington), 1 U/ml Dispase II (Roche Diagnostics), and 250 U/ml DNase I (Worthington)]. After a 1 h incubation, the cells were washed once with DMEM/F12 supplemented with 10% fetal bovine serum (FBS) (HyClone) and then twice with DMEM/F12. The cells were resuspended in growth medium, Neurobasal A (Invitrogen), with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen), B-27 without vitamin A (Invitrogen), 20 ng/ml fibroblast growth factor-2 (PeproTech), and 20 ng/ml epidermal growth factor (PeproTech), and plated at a density of 1 brain per six-well plate. Neural precursor cells proliferated and started to form neurospheres in 2–3 d. One-half of the growth medium was changed every 2 d. To prevent attachment of the neurospheres to the bottom, the medium was pipetted to resuspend the plated neurospheres. The neurospheres were passaged when the diameter of neurospheres reached \sim 100 μ m. For passaging, the floating neurospheres were collected by centrifugation at $400 \times g$ for 5 min. The supernatant was removed and the cells were gently triturated with a 200 μ l pipette tip 20–25 times, resuspended in growth medium, and plated in six-well plates after 1:2 dilution. Neural precursor cells were induced to differentiate by plating dissociated neurospheres (second or third passage) into laminin-coated 24-well plates. The plates were coated with 10 μ g/well laminin (Invitrogen) in dH₂O for 2–3 h, and rinsed two times with dH₂O. The floating neurospheres were again collected by centrifugation, and the cell pellet was resuspended in a small volume of differentiation medium, Neurobasal A (Invitrogen), B27 without vitamin A (Invitrogen), 1% FBS, 100 nM all-*trans* retinoic acid (Sigma-Aldrich), 1 ng/ml fibroblast growth factor-2 (PeproTech), 10 ng/ml brain-derived neurotrophic factor (PeproTech), and 10 ng/ml neurotrophic factor-3 (PeproTech). The neurospheres were gently triturated with a 200 μ l pipette tip. The cells were subsequently resuspended into 5 ml of differentiation medium and passed through a 40 μ m cell strainer (BD Biosciences). The cells were plated onto laminin-coated 24-well plates at a density of \sim 100,000 cells/well. One-half of the differentiation medium was changed every 2 d. For the mitochondrial inhibition experiments, the cells were allowed to differentiate for 8–9 d and treated with 2 μ M antimycin A for 24 h. For the microglial conditioned media (CM) experiments (see below), the cells were allowed to differentiate for 5–6 d before the 24 h CM exposure.

BV-2 cells and CM. BV-2 murine microglia were plated on uncoated plastic tissue culture plates and grown in DMEM supplemented with 10% FBS, and 100 U/ml penicillin and 100 μ g/ml streptomycin. BV-2 cells were treated with fresh differentiation medium with added lipopolysaccharide (LPS) (1 μ g/ml; Sigma-Aldrich) to prepare CM from activated microglia, or without LPS for control medium, for 6–8 h. The medium was collected, sterile filtered, and applied to neural progenitor cells as described above. For *in vitro* mitochondrial protection experiments, lactate (Sigma-Aldrich), pyruvate (Sigma-Aldrich), thiamine-HCl (Sigma-Aldrich), glutathione (GSH) (Sigma-Aldrich), glutathione ethyl ester (GSH-EE) (Sigma-Aldrich), cyclosporin A (CsA) (Sigma-Aldrich), α -lipoic acid (Geronova Research), and NIM811 (Novartis) were added to CM for the duration of treatment.

In vitro immunocytochemistry. Fluorescence immunocytochemistry was performed on cell cultures in 24-well plates. The cultures were washed with PBS and then fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature. The cells were then washed twice with PBS, and nonspecific binding was blocked with 3% bovine serum albumin, 0.3% Triton X-100 in PBS for 1 h. The cells were subsequently incubated with primary antibodies diluted in blocking buffer overnight at 4°C. The

cell-specific antibodies used were Dcx for immature neurons (1:500; sc-8066; Santa Cruz Biotechnology); glial fibrillary acid protein (GFAP), an intermediate filament protein expressed by astrocytes (1:3; 22522; Immunostar); NG2, a marker expressed by immature oligodendrocytes (1:200; AB5320; Millipore Bioscience Research Reagents); microtubule-associated protein 2 (MAP2), a marker for mature neurons (1:500; AB5622; Millipore Bioscience Research Reagents); and CD11b, a surface marker expressed by microglial cells (1:200; MCA74G; Serotec). Cells were subsequently incubated with the appropriate secondary Alexa Fluor 488- or 594-conjugated secondary antibodies (1:200; Invitrogen). To identify proliferating cells that had incorporated 5-bromodeoxyuridine (BrdU), the cultures were fixed with 4% PFA after the cell-specific labeling, rinsed two times in saline, and incubated in 2 M HCl in saline for 30 min at 37°C. The BrdU staining was performed using primary anti-BrdU antibody (1:500; OBT0030; Accurate Chemical and Scientific) and Alexa Fluor 594-conjugated secondary antibody (1:200; Invitrogen). Cell nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (0.5 μ g/ml; Sigma-Aldrich). The immunofluorescence was visualized with an epifluorescence microscope (Zeiss Axiovert 200M) as previously described (Voloboueva et al., 2008).

Live imaging. To monitor changes in mitochondrial membrane potential, cells were incubated with the mitochondrial membrane potential sensitive dye tetramethylrhodamine ethyl ester (TMRE) (50 nM). Cells were illuminated at 535 nm, and fluorescence emission was observed at 590 nm. The location of the collected images was marked on the cell culture wells. The cells were subsequently fixed and stained with anti-Dcx antibody as described above, and the images of Dcx staining were taken in the same areas where TMRE imaging was performed. Subsequently, the red and green channels corresponding to TMRE and Dcx fluorescent signals were overlapped using Adobe Photoshop 6.0. Mitochondrial-produced ROS were evaluated after 1 h of antimycin A treatment using MitoSox staining (Invitrogen) according to the manufacturer's instructions. Staining for apoptotic cells was performed using Magic Red caspase 3 and 7 detection kit (Immunochemistry Technologies) according to the manufacturer's instructions.

Dcx-green fluorescent protein construct and lentiviral preparation. The mouse Dcx promoter (MmDCXp) was cloned from C57BL/6 mouse genomic DNA by PCR using the following primers: CTCGAGATATCTTATCGCCGCACATC and GGATCCTTGGTGAACACAGCAACCTGA. The product was cloned into pCR2.1 using a TOPO-TA kit (Invitrogen) and excised by XhoI and BamHI. It was subcloned into pL_UGIN (Iain Frasier, Caltech, Pasadena, CA), after removing the ubiquitin promoter with the same enzymes. To produce virus, the resulting plasmid, pL_DCXpGIN, was cotransfected with pMD.G and pCMVDR8.91 (Ory et al., 1996; Zufferey et al., 1997) into 293FT cells (Invitrogen) using FuGene6 (Roche Diagnostics) in a T75 flask with 12 ml of 50% DMEM plus 8% FBS, 50% OptiMEM (Invitrogen). Forty-eight hours after transfection, 293 FT cells were fusing and expressing green fluorescent protein (GFP). The culture medium was harvested, filtered through a 0.45 μ m syringe filter (Millipore), and concentrated from 12 to \sim 0.5 ml using a Vivaspin 20 with 100,000 kDa molecular weight cutoff (Sartorius). Viral titers were typically in the range 10^6 cfu/ml after concentration. The entire concentrated viral supernatant was mixed with dissociated neurospheres suspended in 40 ml of the differentiation medium. The cells were plated onto 24-well laminin-coated plates as described above and allowed to differentiate for 7–8 d.

Mitochondrial mtHsp75 transfection. mtHsp75/mortalin is an essential mitochondrial chaperone that has been shown to protect mitochondrial function both *in vitro* and *in vivo* (Voloboueva et al., 2008; Williamson et al., 2008; Xu et al., 2009). For mtHsp75 transfection experiments, the neurospheres were grown, passaged, and dissociated for plating onto laminin-coated 24-well plates to induce differentiation as described above. Immediately before plating, the cells were cotransfected with either LXSN-mtHsp75 (Voloboueva et al., 2008) and pDsRed2-N1 (Clontech) plasmids, or empty LXSN and pDsRed2-N1 plasmids for control conditions, using FuGene 6 (Roche Diagnostics). pDsRed2-N1 plasmid was used as a fluorescent marker to label the transfected cells. After 3 d of differentiation, the cells were again transfected with the same plasmids using FuGene HD (Roche Diagnostics). The cells were allowed to differentiate

for a total of 5–6 d before 24 h exposure to microglial conditioned media. The cells were fixed and immunostained for Dcx as described above. Four $400 \times 300 \mu\text{m}^2$ areas per well, in three wells per transfection, were quantified in three independent experiments. Only the transfected Dcx cells demonstrating DsRed2 fluorescence were counted.

Thiamine treatment and x-ray irradiation. Two-month-old male C57BL/6 mice (~20 g; The Jackson Laboratory) were used in all studies. Mice were housed in a temperature- and light-controlled environment with a 12 h light/dark cycle and provided food and water *ad libitum*. All protocols were approved by the Stanford Panel on Laboratory Animal Care and Use. The mice were split into two groups 5 d before the irradiation procedure, with the thiamine-treated group receiving 1% thiamine in the drinking water for the remainder of the experiment. All mice, including sham-irradiated animals, were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Irradiation was performed with a Phillips orthovoltage x-ray system operated at 200 kVp and 20 mA. A single dose of 5 Gy was limited by lead shielding to a 1 cm diameter column that included left and right hippocampal formations. The remaining body, neck, eyes, ears, and snout were shielded.

Dosimetry and field dimensions were confirmed using TLD dosimeters (K&S Associates) buried in the hippocampi and surrounding brain areas of killed mice. The dose rate was ~78.0 cGy/min. To label dividing cells, animals were injected intraperitoneally with halogenated thymidine analogs 5-iodo-2'-deoxyuridine (IdU) (Sigma-Aldrich) or 5-chloro-2'-deoxyuridine (CldU) (Sigma-Aldrich) at a daily dose of 50 mg/kg using a 10 mg/ml solution in saline. IdU injections were given on days 1–3. CldU injections were given on days 4–7.

Tissue preparation. Animals were anesthetized and killed 8 d after irradiation. Brains were fixed by transcardial perfusion with 4% PFA, removed from the skull, and postfixed overnight in 4% PFA, and then equilibrated in phosphate-buffered 30% sucrose. Free-floating $40 \mu\text{m}$ sections were collected on a freezing sledge microtome and stored in tissue cryoprotectant solution at -20°C until used.

Immunohistochemistry, cell counting, and unbiased stereology. For confocal imaging, the sections were immunostained as described previously (Monje et al., 2003) using the following primary antibodies: anti-NeuN (1:200; MAB377; Millipore Bioscience Research Reagents), anti-Dcx (1:500; sc-8066; Santa Cruz Biotechnology), and anti-Iba-1 (1:1000; Wako). To detect IdU⁺ and CldU⁺ nuclei, the sections were labeled with rat anti-BrdU (1:300; 347580; BD Biosciences) and mouse anti-BrdU (1:500; ab6326-250; Abcam). For the purpose of the study, IdU and CldU labeling was not discriminated by using secondary antibodies with the same detection marker, Cy3-conjugated donkey anti-mouse and Cy3-conjugated donkey anti-rat (1:500; 715-165-151 and 712-165-153, respectively; Jackson ImmunoResearch). The total number of dividing cells was estimated as the sum of IdU⁺ and CldU⁺ cells, and thus dividing cells are denoted as "IdU⁺+CldU⁺" in all experiments. Confocal microscopy was performed using a Zeiss 510 confocal microscope as described previously (Monje et al., 2003). In stereological studies, all counts were limited to the hippocampal granule cell layer proper and a $50 \mu\text{m}$ border along the hilar margin that included the neurogenic subgranular zone. The total number of Dcx- or IdU⁺+CldU⁺-labeled cells per hippocampal granule cell layer and subgranular zone was determined using immunodetection of Dcx or IdU⁺+CldU⁺ followed by HRP-coupled secondary antibodies and diaminobenzadine stain (Vector Laboratories). Stained Dcx- and IdU⁺+CldU⁺-positive cells were scored under light microscopy using MicroBrightField Stereo Investigator software and a modification of the disector method in which random grid placement

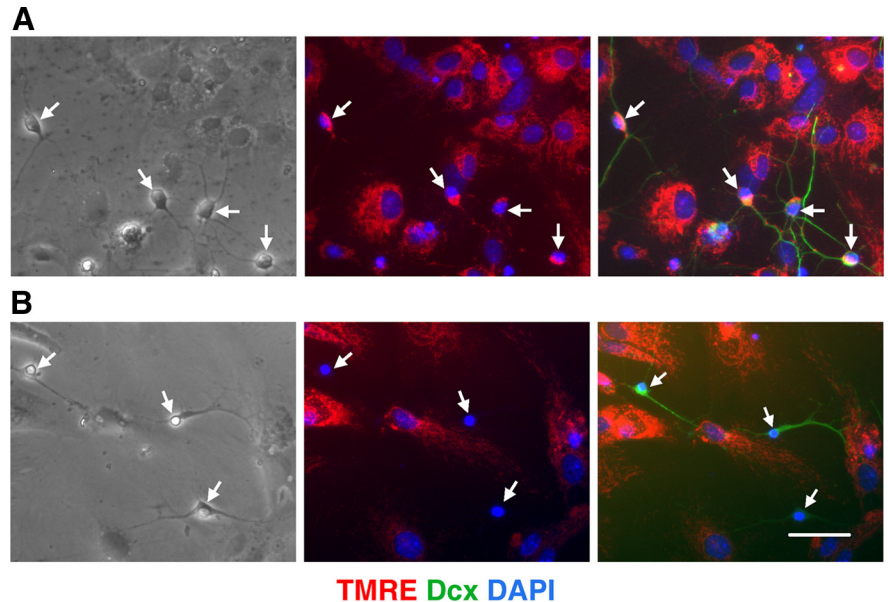


Figure 1. Treatment with the mitochondrial inhibitor antimycin A promotes loss of mitochondrial membrane potential in Dcx⁺ cells. **A**, Dcx⁺ cells (arrows; green staining) in control vehicle-treated cultures demonstrate normal mitochondrial potential by bright mitochondrial TMRE red staining. Cell nuclei are counterstained with DAPI (blue). **B**, Treatment for 4 h with antimycin A promotes strong selective loss of mitochondrial potential in Dcx⁺ cells (arrows), whereas nearby cells retain TMRE staining. Scale bar, 50 μm .

provided systematic and unbiased sampling of Dcx⁺ cell density within the dentate gyrus. All analyses were performed by investigators blinded to sample identity and treatment group.

Statistics. Statistical differences between two groups were determined using unpaired two-tailed Student's *t* test with Welch's correction. Comparisons between multiple groups were performed with ANOVA followed by Bonferroni's test for selected groups. Analysis of protection by different mitochondrial compounds was performed with ANOVA followed by Dunnett's posttest for comparison between CM-treated group versus all other treatment groups.

Results

Mitochondrial inhibition promotes rapid loss of mitochondrial potential in Dcx⁺ cells

The effect of inhibition of mitochondrial electron transport on the mitochondrial membrane potential depends on cell type (Beltrán et al., 2000; Almeida et al., 2001) and cellular energetic demands (Voloboueva et al., 2007). We used the mitochondrial membrane potential-sensitive dye TMRE to investigate changes induced by mitochondrial inhibition in neural cultures after 8–9 d of differentiation. Immature neurons express Dcx, a reliable and specific marker of neurogenesis (Couillard-Despres et al., 2005). Figure 1A shows bright TMRE staining of Dcx⁺ and surrounding cells in control vehicle-treated cultures. Treatment with the mitochondrial inhibitor antimycin A promoted rapid loss of mitochondrial membrane potential in Dcx⁺ cells, as evidenced by loss of TMRE signal, whereas the surrounding cells retained strong TMRE staining, as shown in Figure 1B. We verified that antimycin A treatment inhibited mitochondrial complex III activity and increased mitochondrial-produced ROS, using MitoSox, a mitochondrial superoxide indicator (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Because collapse of the mitochondrial potential is often associated with release of proapoptotic factors and cell death (Liu et al., 1996; Susin et al., 1999), we investigated changes in viability of different cell types with mitochondrial inhibition.

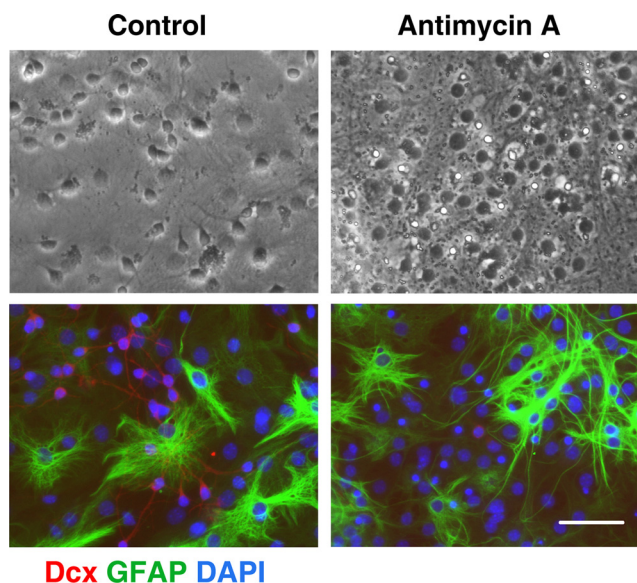


Figure 2. Mitochondrial inhibition does not induce significant changes in the number of astrocytes. Control (left panels) and antimycin A-treated ($2 \mu\text{M}$ for 24 h) (right panels) cultures stained with the astrocyte marker GFAP (green) and the immature neuron marker Dcx (red). Cell nuclei are counterstained with DAPI (blue). Antimycin A treatment promoted marked loss of Dcx⁺ cells but did not significantly change GFAP staining cells. Scale bar, $50 \mu\text{m}$.

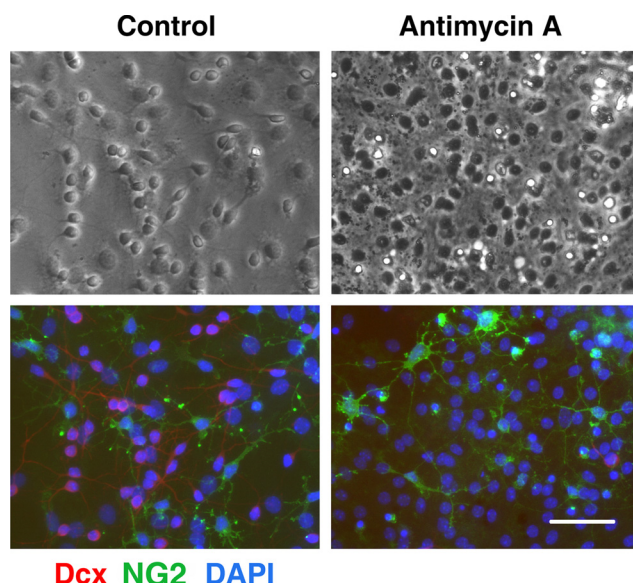


Figure 3. Mitochondrial inhibition does not induce a significant change in the number of immature oligodendrocytes. Control (left panels) and antimycin A-treated ($2 \mu\text{M}$ for 24 h) (right panels) cultures stained with NG2 (green) and Dcx (red) markers. Cell nuclei are counterstained with DAPI (blue). Antimycin A treatment promoted marked loss of Dcx⁺ cells but did not significantly change NG2 staining. Scale bar, $50 \mu\text{m}$.

Effects of mitochondrial inhibition on differentiating cell viability

Neural stem cells allowed to differentiate for 8–9 d were treated with antimycin A for 24 h, and the numbers of surviving cells were assessed. At 8–9 d of differentiation, the majority of cells express cell type-specific markers, allowing us to compare vulnerability of different cell types to mitochondrial inhibition. As shown in the top panels of Figure 2, 24 h mitochondrial inhibition promoted morphological changes in the treated cultures compared with control vehicle-treated cultures. However, the antimycin A treatment promoted no significant change in the fraction of cells expressing the astrocyte-specific marker GFAP (mean \pm SEM, $58 \pm 2.5\%$ GFAP⁺ cells in control vs $56 \pm 3\%$ GFAP⁺ in antimycin A-treated cultures; $p > 0.05$). Comparison of the bright-field and immunostained images of the same field (Fig. 2, control conditions) indicates that the majority of the small process-bearing cells with extended thin processes in bright-field stain for Dcx. These cells either disappeared or demonstrated markedly changed, shrunken morphology after 24 h of mitochondrial inhibition. In parallel, Dcx staining was markedly decreased in the antimycin A-treated cultures. Figure 3 shows that the 24 h antimycin A treatment promoted no significant change in the number of cells expressing immature oligodendrocyte marker NG2 (mean \pm SEM, $12 \pm 1.2\%$ NG⁺ cells in control vs $13 \pm 1\%$ NG⁺ in antimycin A-treated cultures; $p > 0.05$). Again, significant changes were observed in morphology and Dcx staining of the antimycin A-treated cultures. Only a very small fraction of cells ($<0.5\%$) were immunoreactive for the microglial specific marker CD11b. More severe stress caused by chronic antimycin treatment from the beginning of differentiation resulted in complete absence of Dcx cells, and reduced astrocyte differentiation (data not shown), whereas the more limited duration of inhibition only affected Dcx cells, evidence of their greater sensitivity.

Effects of mitochondrial inhibition on immature Dcx⁺ and mature MAP2⁺ neuronal cells

As described above, mitochondrial inhibition for 24 h resulted in a sharp decrease in the number of Dcx⁺ cells. Interestingly, we found that Dcx⁺ cells that also coexpressed MAP2, a marker of more mature neurons, demonstrated better preservation of cell morphology and Dcx⁺ staining. As shown in Figure 4, Dcx⁺-single-positive cells retained very weak Dcx labeling and demonstrated a shrunken cellular morphology after 16 h of antimycin A treatment (Fig. 4A, arrowheads). In contrast, Dcx⁺ cells that colabeled with MAP2 retained a relatively strong Dcx signal and better preserved cell morphology (Fig. 4A, arrows). As demonstrated in Figure 4B, 24 h of mitochondrial inhibition caused a 60% loss of Dcx⁺ cells, whereas MAP2 staining was decreased by only 30%. These results indicate that single-positive Dcx⁺ cells are particularly vulnerable to inhibition of mitochondrial function compared with glial cells and more mature neurons. To investigate whether the high vulnerability of Dcx⁺ cells is related to their proliferative state, we treated cells with $10 \mu\text{M}$ BrdU during the 24 h antimycin A treatment to label proliferating cells. Less than 10% of Dcx⁺ cells were labeled with BrdU after 8–9 d of differentiation (Fig. 4C), and the fraction of BrdU-labeled cells was not changed by antimycin A treatment (mean \pm SEM, $7 \pm 1.3\%$ BrdU⁺ cells in control vs $8 \pm 2\%$ BrdU⁺ in antimycin A-treated cultures, relative to the total number of Dcx⁺ cells; $p > 0.05$).

Mitochondrial inhibition promotes apoptotic death in Dcx⁺ cells

Mitochondrial impairment has been shown to promote apoptotic cell death with depolarization associated with release of apoptosis-inducing factor and cytochrome *c*, and activation of procaspases (Liu et al., 1996; Susin et al., 1999). To investigate the mode of Dcx⁺ cell death induced by mitochondrial inhibition, the cultures were infected with a lentiviral vector in which GFP was placed under the control of the mouse Dcx promoter to label

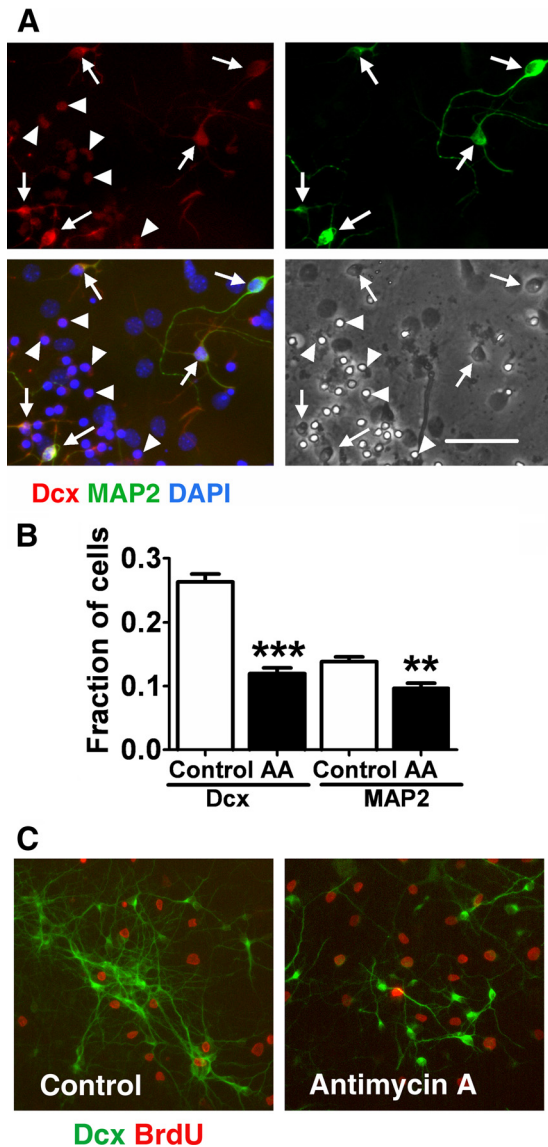


Figure 4. Immature Dcx⁺ neurons are selectively vulnerable to mitochondrial inhibition. **A**, Cultures treated with antimycin A (2 μ M for 16 h) stained with an immature neuron marker Dcx (red) and a marker for more mature neurons MAP2 (green). Cell nuclei are counterstained with DAPI (blue). The cultures were stained after 16 h of antimycin A treatment, compared with the 24 h time point used for quantification to capture the residual Dcx staining that is essentially gone after 24 h. Cells expressing both Dcx and MAP2 cell markers (arrows) showed better preserved morphology and Dcx staining compared with Dcx-only expressing cells (arrowheads). Scale bar, 50 μ m. **B**, Fractions of Dcx⁺ and MAP2⁺ cell (relative to the total cell number) in control- and antimycin A (2 μ M, 24 h)-treated cultures. The data are representative of three independent experiments with at least 700 cells per condition in each experiment. Shown are mean \pm SEM. ** p < 0.01 compared with control MAP2; *** p < 0.001 compared with control Dcx. **C**, Only a small fraction of Dcx⁺ cells (green) were proliferating after 8–9 d of differentiation, as demonstrated by colabeling with BrdU (red). The fraction of proliferating BrdU cells was not significantly changed by 24 h antimycin A treatment.

Dcx⁺ cells (see supplemental Fig. 2, available at www.jneurosci.org as supplemental material). This allowed us to monitor caspase 3 and 7 activity in live cells with a fluorescent caspase substrate. As demonstrated in Figure 5, mitochondrial inhibition induced caspase activation in Dcx⁺ cells.

Protection of mitochondrial function reduces inflammation-associated Dcx⁺ cell death *in vitro*

In our previous study, we demonstrated that treatment with media preconditioned by LPS-activated microglia (CM) signifi-

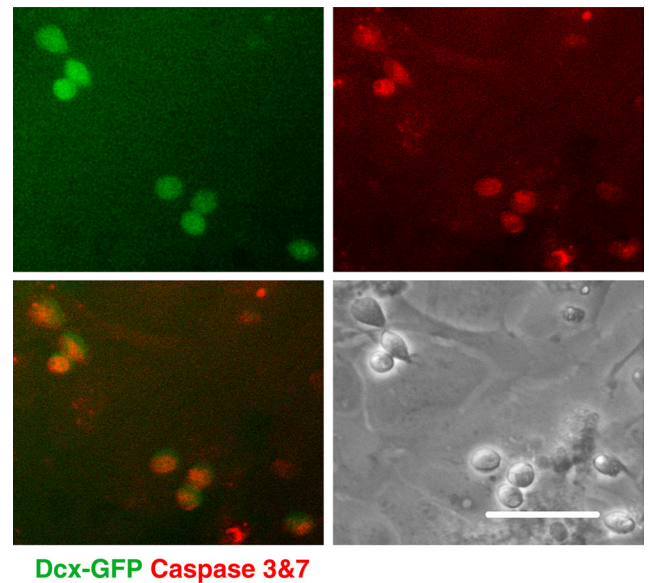


Figure 5. Dcx⁺ cells (green GFP signal) demonstrate signs of apoptotic cell death (MAGIC Red live caspase 3 and 7 staining) after 12 h of antimycin A treatment. Scale bar, 50 μ m.

cantly decreased the number of Dcx⁺ cells *in vitro* (Monje et al., 2003). Activated microglia release a variety of cytokines and reactive species that can impair mitochondrial function, either directly or through cellular pathways. As described above, Dcx⁺ cells are particularly sensitive to mitochondrial impairment. To investigate the effect of inflammatory mediators released by microglia on mitochondrial potential of Dcx⁺ cells, cultures were treated with CM medium for 6 h. Figure 6A shows that CM treatment promoted loss of mitochondrial membrane potential in Dcx⁺ cells, whereas the surrounding cells retained mitochondrial potential, as observed above with direct mitochondrial inhibition with antimycin A (Fig. 1B). We hypothesized that mitochondrial inhibition is at least partially responsible for the inflammation-associated decrease in the number of Dcx⁺ cells. To test this, we studied the effect of different mitochondrial metabolites and substrates that might protect mitochondrial function and thus inhibit the death of Dcx⁺ cells induced by CM exposure. In these experiments, cultures were differentiated for 5–6 d before the CM treatment to reduce the number of more mature MAP2⁺ neuronal cells that were less sensitive to mitochondrial inhibition (Fig. 4).

We investigated whether Dcx⁺ cell death induced by direct mitochondrial inhibition with antimycin A could be alleviated by supplementation with lactate, a major mitochondrial energy substrate (Schurr, 2006; Herrero-Mendez et al., 2009). Because antimycin A is a potent inhibitor of mitochondrial complex III, the antimycin A concentration was titrated down to a concentration that caused only partial impairment of mitochondrial activity. Treatment with 10 nM antimycin A for 24 h resulted in 49% Dcx⁺ cell loss, compared with 67% loss in cultures treated with 2 μ M antimycin A. The loss induced by 10 nM antimycin A treatment was completely prevented by cotreatment with 5 mM lactate (Fig. 6B).

Treatment with CM for 24 h resulted in 60% loss of Dcx cells compared with control cultures treated with media preconditioned by resting microglia (Fig. 6C). Cotreatment with mitochondrial metabolites and substrates that might protect mitochondrial function showed that lactate (5 mM) reduced CM-associated cell death such that 80% of Dcx⁺ cells survived

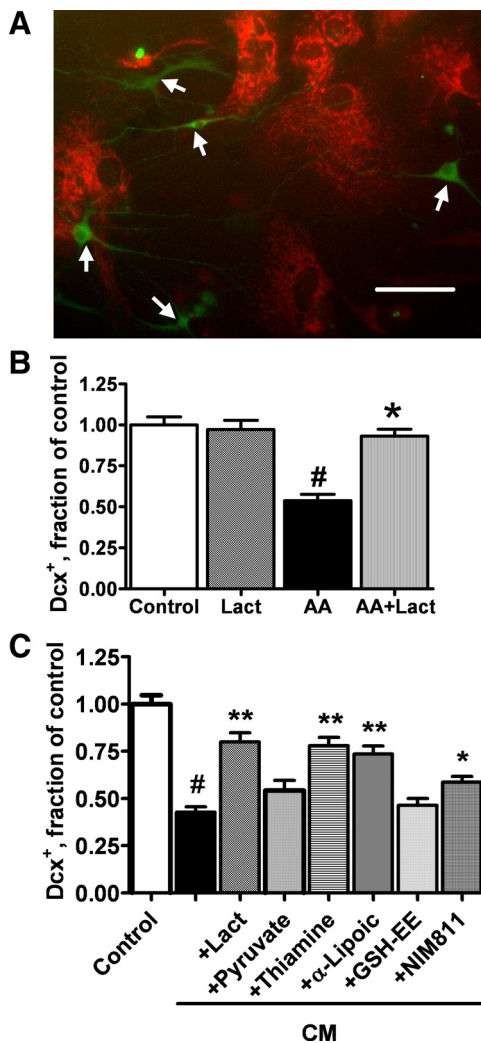


Figure 6. Cotreatment with mitochondrial metabolites and protective compounds attenuate loss of Dcx⁺ cells induced by exposure to LPS-treated microglial CM. **A**, CM treatment promoted rapid loss of mitochondrial membrane potential in Dcx⁺ cells (arrows) as evidenced by selective disappearance of TMRE staining after 6 h of CM treatment. Scale bar, 50 μm. **B**, Changes in the number of Dcx⁺ cells induced by 24 h of 10 nM antimycin A (AA) exposure, with and without 5 mM lactate (Lact). **C**, Changes in the number of Dcx⁺ cells induced by CM treatment alone or with the addition of mitochondrial metabolites and compounds: lactate (5 mM), pyruvate (5 mM), thiamine (4 mM), α-lipoic acid (100 μM), GSH-EE (1 mM), and NIM811 (2 μM). The data are representative of three independent experiments with at least 150 Dcx⁺ cells per control condition in each experiment. Shown are mean ± SEM. #*p* < 0.001 compared with control; **p* < 0.05 compared with CM treatment; ***p* < 0.01 compared with CM treatment.

compared with cultures treated with control CM. Interestingly, cotreatment with pyruvate (5 mM), another direct substrate of mitochondrial metabolism, was less effective, with protection not reaching significance (*p* = 0.06). Thiamine and α-lipoic acid are both cofactors of mitochondrial pyruvate and α-ketoglutarate dehydrogenases that have been shown to be protective against brain mitochondrial damage (Sheline et al., 2002; Liu, 2008). Cotreatment with thiamine (4 mM) or α-lipoic acid (100 μM) partially protected against CM toxicity, resulting in survival of 78 and 74% of Dcx⁺ cells, respectively, compared with control. Because α-lipoic acid is also a potent antioxidant, we investigated the effect of antioxidant treatment on Dcx⁺ viability. Treatment with different concentrations of the antioxidant GSH (0.2–5 mM) (data not shown) or with the membrane-permeable GSH-EE (0.2–5 mM; 1 mM shown) did not provide significant protection against CM injury. Treatment with NIM811 (2 μM), an inhibitor

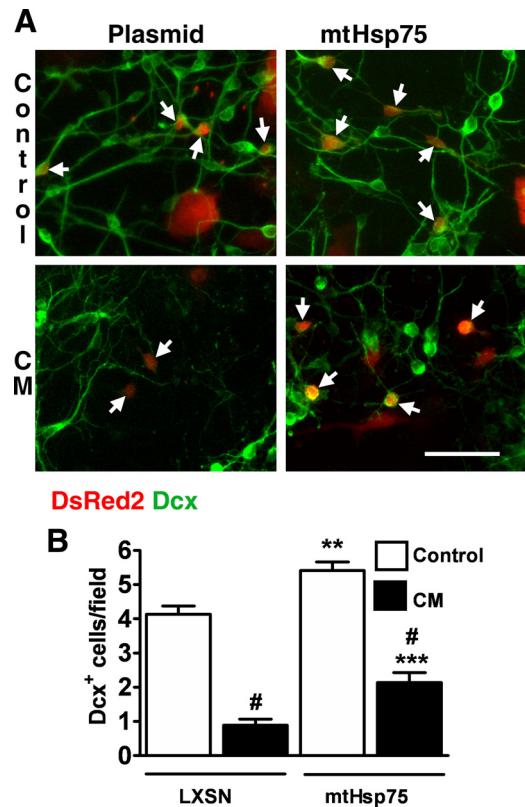


Figure 7. mtHsp75 overexpression attenuates the loss of Dcx⁺ cells induced by CM exposure. **A**, Examples of Dcx staining in control plasmid- and mtHsp75-transfected cultures. Transfected cells were identified by DsRed2 coexpression (see Materials and Methods) and are indicated by arrows. Scale bar, 50 μm. **B**, Changes in the number of transfected Dcx⁺ cells in control (LXSN empty plasmid) and mtHsp75-overexpressing cultures induced by 24 h CM exposure (black columns). The data are representative of three independent experiments with a total of ~150 Dcx⁺ cells per vector control condition. Shown are mean ± SEM. #*p* < 0.001 compared with respective plasmid- or mtHsp75-transfected cultures without CM treatment; ***p* < 0.01 compared with plasmid without CM treatment; ****p* < 0.001 compared with plasmid plus CM.

of mitochondrial permeability transition pore opening that is devoid of calcineurin-inhibiting activity, resulted in survival of 59% of Dcx⁺ cells compared with control. Treatment with CsA, a more common inhibitor of the mitochondrial permeability transition, did not protect Dcx⁺ cells against the CM toxicity (data not shown), likely because of its additional activity as a calcineurin inhibitor (Nazareth et al., 1991).

As an alternative strategy to target mitochondrial protection, we investigated the effect of mitochondrial mtHsp75 overexpression on Dcx⁺ cell viability. Our previous studies demonstrated the protective properties of mtHsp75 overexpression against ischemic stress both *in vitro* and *in vivo* (Voloboueva et al., 2008; Xu et al., 2009). Figure 7 shows Dcx staining in control and mtHsp75-overexpressing cultures. mtHsp75 overexpression resulted in a significant 1.3-fold increase in the number of Dcx⁺ cells in untreated cultures. This suggests that mitochondrial function limits Dcx cell survival under baseline culture conditions. In this series of experiments, CM treatment for 24 h caused an 80% loss of Dcx⁺ cells in control plasmid-transfected cells compared with a 60% Dcx⁺ cell loss in mtHsp75-transfected cultures, significantly better survival.

Protection of Dcx⁺ cells *in vivo*

To test whether this strategy of providing mitochondrial protection would be effective *in vivo*, we used thiamine oral supplementen-

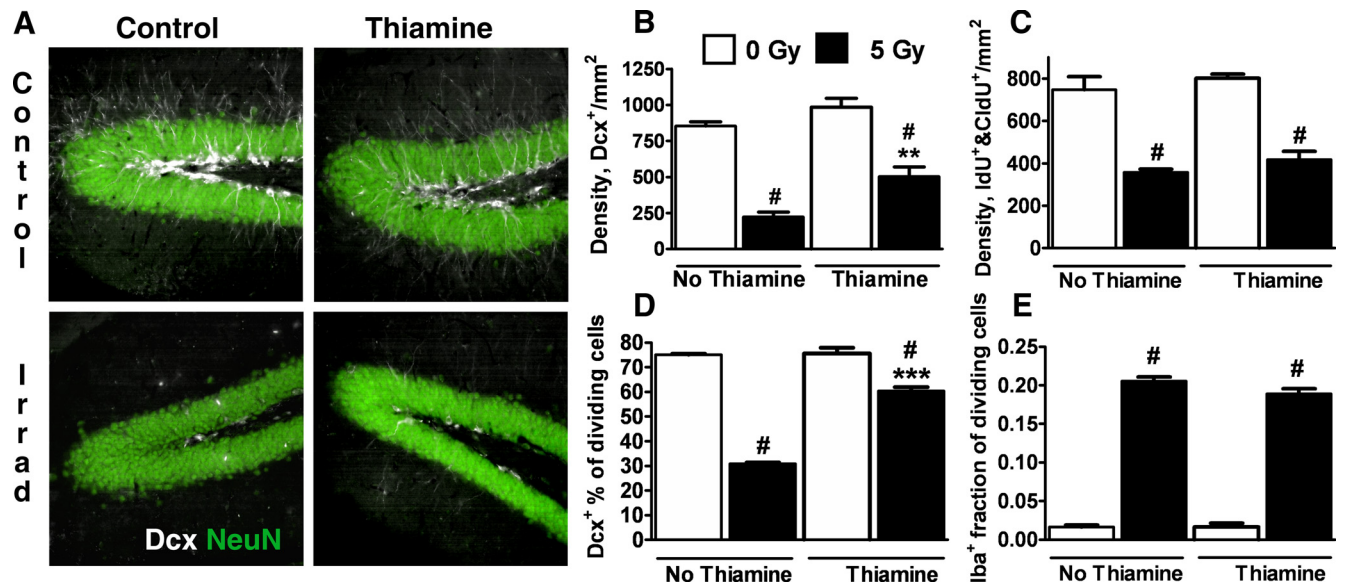


Figure 8. Thiamine treatment attenuates the irradiation-associated loss of hippocampal Dcx⁺ cells. **A**, Representative Dcx staining of control and thiamine-treated hippocampi 8 d after x-irradiation. **B**, Quantification of hippocampal Dcx⁺ cells shows that thiamine treatment significantly reduced irradiation-induced Dcx⁺ cell loss. **C**, Thiamine treatment promotes no significant change in the irradiation-induced loss of proliferating IdU⁺ + CldU⁺ cells in hippocampus. **D**, The percentage of proliferating IdU⁺ + CldU⁺ cells that coexpress Dcx is markedly decreased after irradiation. Thiamine treatment significantly attenuates this decrease in the Dcx⁺ fraction of IdU⁺ + CldU⁺ cells. **E**, Microglial proliferation (fraction of IdU⁺ + CldU⁺ cells coexpressing the microglial marker Iba-1) is strongly increased by irradiation, and thiamine treatment does not change the number of proliferating microglia. The data are representative of five animals per each treatment group. Shown are mean ± SEM. #*p* < 0.001 compared with corresponding nonirradiated group, ***p* < 0.01, ****p* < 0.001 compared with control-irradiated group.

tation in the setting of brain radiation. Thiamine treatment has been considered in several clinical settings (Ozdemir et al., 2002; Haupt et al., 2005; Gibson and Blass, 2007), and studies in animals have shown that thiamine protects against disorders involving mitochondrial dysfunction (Sheline and Wei, 2006). Thiamine is water soluble and absorbed in the intestine by both active and passive transport. Thiamine treatment nearly doubled survival of Dcx⁺ cells in hippocampi after irradiation from 26% in control-irradiated animals, to 51% in animals receiving thiamine (Fig. 8*A, B*). We next investigated the effect of thiamine treatment on proliferating Dcx⁺ cells. As shown in Figure 8*C*, irradiation promoted a 50% decrease in the total number of dividing IdU⁺ and CldU⁺ cells in hippocampus, with thiamine treatment resulting in no significant change in IdU⁺ and CldU⁺ cell number in either control or irradiated animals. The percentage of proliferating IdU⁺ and CldU⁺ cells demonstrating Dcx⁺ colabeling was decreased by 60% after irradiation. Thiamine treatment did not significantly change the Dcx⁺ percentage of proliferating IdU⁺ and CldU⁺ in nonirradiated animals, but doubled the Dcx⁺ fraction of IdU⁺ and CldU⁺ cells in irradiated animals (Fig. 8*D*). The results indicate that thiamine protection doubled the surviving numbers of both total and proliferating Dcx⁺ cells. Figure 8*E* shows that, although irradiation caused marked proliferation of microglia, 20% of dividing IdU⁺ and CldU⁺ cells coexpressed the microglial marker Iba-1 in irradiated animals, thiamine treatment had no significant effect on this aspect of irradiation-induced inflammation (Fig. 8*E*).

Discussion

Different cell types have different sensitivities to interventions targeting mitochondrial function. It has been shown that, although various cell lines, like HeLa, XP30RO, and GM10115, can tolerate mitochondrial DNA (mtDNA) depletion for extended periods of time, neural precursor cells die within a short period of time after mtDNA depletion (Fike et al., 2009). Inhibition of mitochondrial electron transport in astrocytes strongly upregu-

lates glycolysis. Glycolytically generated ATP is used to support mitochondrial membrane potential through the reversed action of mitochondrial F₁F₀-ATPase in astrocytes (Almeida et al., 2001). Inhibition of mitochondrial electron transport in neurons promotes rapid loss of mitochondrial membrane potential (Bolaños and Almeida, 2006), although some studies indicate a limited ability of neurons to maintain mitochondrial potential through F₁F₀-ATPase reversal (Anderson et al., 2002). In our previous study, we demonstrated that changes in energetic demands can affect the ability of the cell to maintain mitochondrial potential in the face of mitochondrial inhibition. In that study, energetic demands imposed on astrocytes by coculture with neurons or by added metabolic load during mitochondrial inhibition prevented maintenance of mitochondrial membrane potential through F₁F₀-ATPase reversal and resulted in loss of mitochondrial potential and astrocytic death (Voloboueva et al., 2007).

Loss of mitochondrial membrane potential and mitochondrial permeability transition can initiate both apoptotic and necrotic cell death (Kroemer et al., 1998; Lemasters et al., 1998). Because of this, the ability of cells to maintain mitochondrial membrane potential closely correlates with their viability under conditions promoting mitochondrial dysfunction. Here, we observed rapid loss of mitochondrial membrane potential in Dcx⁺ cells on inhibition of mitochondrial electron transport. This rapid loss of mitochondrial potential correlated with a significant drop in Dcx⁺ cell survival, in striking contrast to astrocytes and oligodendrocytes, which exhibited no change in viability. Even comparing Dcx single-positive cells to slightly more mature Dcx and MAP2 double-positive cells viability was cut in one-half. This indicates that Dcx⁺ single-positive immature neurons are particularly vulnerable to conditions impairing mitochondrial metabolism, including the presence of inflammatory mediators.

Previous studies demonstrated that microglial CM exposure resulted in decreased neurogenesis *in vitro*, estimated by the number of Dcx⁺ cells (Monje et al., 2003). Activated microglia

release cytokines and reactive species, including TNF- α , IL-1 α , IL-6, ROS, and nitric oxide, that promote mitochondrial dysfunction (Giuffrè et al., 1996; Zell et al., 1997; Halliwell, 2006; Behrens et al., 2008). Here, we found that treatment of differentiating neurons with lactate, a major mitochondrial substrate (Schurr, 2006; Herrero-Mendez et al., 2009), during exposure to CM provided significant protection of Dcx⁺ cells, whereas cotreatment with pyruvate, a direct substrate of the mitochondrial PDH complex, was less effective. These results are consistent with several previous studies that demonstrated that lactate was a more efficient stimulus of mitochondrial metabolism compared with pyruvate (Yang et al., 1999; Ainscow et al., 2002). Conversion of lactate to pyruvate also generates NADH, whereas excessive pyruvate concentrations stimulate lactate dehydrogenase to operate in reverse mode and consume NADH, thus promoting a more oxidized cytosolic environment (Zhao and Rutter, 1998; Ainscow et al., 2002). This suggests that maintenance of a sufficiently reduced cytosol is important in the protection of Dcx⁺ cells by lactate. Also, cytosolic NADH generated from lactate conversion can promote increased mitochondrial NADH levels through the activity of mitochondrial redox shuttles (i.e., the glycerol phosphate shunt or malate–aspartate shunt), resulting in higher mitochondrial electron transport activity.

Thiamine is a coenzyme of mitochondrial PDH and α -keto glutarate dehydrogenase (KGDH). Thiamine treatment has been shown to enhance PDH and KGDH activities (Rokutan et al., 1987; Strumilo and Markiewicz, 1995) and protect mitochondrial function both *in vitro* and *in vivo* (Sheline and Choi, 2004; Depeint et al., 2006). Cotreatment with thiamine during exposure to inflammatory CM resulted in a 1.8-fold increase in the number of Dcx⁺ cells compared with CM-only-treated cultures. α -Lipoic acid is also a coenzyme of mitochondrial PDH and KGDH complexes. Supplementation with α -lipoic acid has been shown to protect against oxidative and ischemic injury *in vitro* (Packer et al., 1997) and attenuate pathology-associated mitochondrial and cognitive dysfunction of the brain (Liu, 2008). In our experiments, cotreatment with α -lipoic acid during the CM exposure resulted in 1.7-fold increase in the number of Dcx⁺ cells compared with CM-only-treated cultures. Lipoic acid is also a potent antioxidant. However, we failed to observe protection of CM-treated Dcx⁺ cells by glutathione. This is in agreement with a recent study demonstrating that overexpression of the antioxidant enzyme glutathione peroxidase did not attenuate the decrease in neurogenesis associated with traumatic brain injury (Potts et al., 2009).

Since mitochondrial damage promotes the permeability transition and can trigger both apoptotic and necrotic cell death (Kroemer and Reed, 2000), we also investigated the protective potential of agents that inhibit mitochondrial permeability transition. One commonly used inhibitor is CsA. In our experiments, CsA treatment during CM exposure exacerbated the loss of Dcx⁺ cells induced by inflammatory CM (data not shown). CsA toxicity has been observed in several studies and might be attributable to CsA inhibition of calcineurin (Nazareth et al., 1991; Qian et al., 1997). NIM811, a CsA analog that does not inhibit calcineurin but inhibits permeability transition without cytotoxic effects (Waldmeier et al., 2002; McEwen et al., 2007), was effective in improving survival of Dcx⁺ cells compared with CM-treated cultures. This suggests that Dcx⁺ cell death induced by inflammatory CM is mediated by opening of the permeability transition pore and is consistent with our observation that caspases are activated in the Dcx⁺ cells.

mtHsp75 is the mitochondrial localized member of the HSP70 family and is an essential mitochondrial chaperone. Several stud-

ies have demonstrated the protective potential of mtHsp75 overexpression against mitochondrial dysfunction and cell death (Liu et al., 2005; Voloboueva et al., 2008; Williamson et al., 2008; Xu et al., 2009). In the present study, mtHsp75 overexpression resulted in improved survival of Dcx⁺ cells in untreated cultures, as well as additional improved survival of inflammatory CM exposed Dcx⁺ cells. This suggests that the survival of Dcx⁺ cells under these *in vitro* differentiation conditions is already limited to some extent by mitochondrial impairment.

Mitochondrial inactivation has been suggested to be a critical mechanism of cellular damage in several neurodegenerative disorders (Fukui and Moraes, 2008; Niizuma et al., 2009). Thiamine supplementation ameliorated mitochondrial dysfunction and reduced neuronal death induced by copper supplementation or transient middle cerebral artery occlusion (Sheline and Choi, 2004; Sheline and Wei, 2006). Thiamine has been demonstrated to show benefits in treating several mitochondrial disorders including mitochondrial encephalomyopathy, West syndrome, lactic acidemia, and Leigh syndrome (Tanaka et al., 1997; Naito et al., 1998, 1999). In our experiments, oral thiamine supplementation resulted in significantly improved survival of Dcx⁺ cells in experimental animals 8 d after irradiation treatment. Cranial radiation is known to be associated with cognitive decline in both children and adults (Crossen et al., 1994; Roman and Sperduto, 1995; Duffner, 2004); thus, the possibility of using thiamine to ameliorate this devastating process deserves consideration, especially in light of the long history of the use of thiamine clinically, and its relative lack of side effects.

The results of recent *in vivo* studies suggest that mitochondrial function might play an important role in the process of neurogenesis both during development and in adulthood. Mice deficient in mitochondrial KGDH complex had fewer Dcx⁺ neuronal progenitor cells (Calingasan et al., 2008), and thiamine deficiency impaired hippocampal neurogenesis and caused cognitive dysfunction in adult mice (Zhao et al., 2008). Thiamine deficiency has long been appreciated to cause Wernicke–Korsakoff syndrome, a form of encephalopathy and psychosis, which is characterized by reduced activity of the KGDH complex in the brain (Gibson et al., 1984). It is plausible that the memory impairment seen in Korsakoff psychosis is related in part to reduced hippocampal neurogenesis.

Neurogenesis is decreased in several inflammation-associated conditions including aging, age-associated pathologies, and irradiation (Mizumatsu et al., 2003; Rola et al., 2004; Rao et al., 2006; Verret et al., 2007). Although inflammation has been shown to downregulate neurogenesis *in vitro* and *in vivo* (Ekdahl et al., 2003; Monje et al., 2003), the mechanisms of this downregulation remain poorly understood. Understanding the mechanisms that suppress neurogenesis during inflammation may help identify therapeutic targets for treatment relevant to multiple neurodegenerative disorders. Our findings shed light on mitochondrial mechanisms involved in neurogenesis, identify the immature Dcx⁺ neuron as exquisitely vulnerable to mitochondrial impairment, and reinforce the concept of targeting mitochondria to prevent death of newborn neurons and enhance neurogenesis under conditions of inflammation.

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