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2,4-dinitrophenol induces neural differentiation of murine embryonic stem cells

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Abstract 2,4-Dinitrophenol (DNP) is a neuroprotective compound previously shown to promote neuronal differentiation in a neuroblastoma cell line and neurite outgrowth in primary neurons. Here, we tested the hypothesis that DNP could induce neurogenesis in embryonic stem cells (ESCs). Murine ESCs, grown as embryoid bodies (EBs), were exposed to 20 μM DNP (or vehicle) for 4 days. Significant increases in the proportion of nestin- and β-tubulin III-positive cells were detected after EB exposure to DNP, accompanied by enhanced glial fibrillary acidic protein (GFAP), phosphorylated extracellular signal-regulated kinase (p-ERK) and ATP-linked oxygen consumption, thought to mediate DNP-induced neural differentiation. DNP further protected ESCs from cell death, as indicated by reduced caspase-3 positive cells, and increased proliferation. Cell migration from EBs was significantly higher in DNP-treated EBs, and migrating cells were positive for nestin, ß-tubulin III and MAP2, similar to that observed with retinoic acid (RA)-treated EBs. Compared to RA, however, DNP exerted a marked neuritogenic effect on differentiating ESCs, increasing the average length and number of neurites per cell. Results establish that DNP induces neural differentiation of ESCs, accompanied by cell proliferation, migration and neuritogenesis, suggesting that DNP may be a novel tool to induce neurogenesis in embryonic stem cells.
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Introduction

Neuronal differentiation of embryonic stem cells (ESCs) has emerged as a potential approach to study and overcome neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases (Tomaskovic-Crook and Crook, 2011; Nikoletopoulou and Tavernarakis, 2012; Visan et al., 2012). Retinoic acid (RA), a natural morphogen, is extensively used to induce differentiation of stem cells into a neuronal phenotype, but it is also well known to trigger apoptosis and cell toxicity (Sarkar and Sharma, 2002). On the other hand, viral-mediated approaches have been employed to deliver

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differentiation factors (Dottori et al., 2011) but they induce striking transcriptional reprogramming and might not be safe for human therapy.

2,4-Dinitrophenol (DNP) is classically known as a mitochondrial uncoupling agent that is toxic at high concentrations (De Felice and Ferreira, 2006). However, recent studies have shown that, at low micromolar concentrations, DNP is neuroprotective. Low-dose DNP protects against central and peripheral neurodegeneration (Maragos et al., 2003; Pandya et al., 2007; da Costa et al., 2010), and blocks the aggregation and neurotoxicity of Alzheimer's amyloid-beta peptide (De Felice et al., 2001; De Felice, 2004). In addition, DNP has been shown to promote neuronal differentiation in a neural cell line and neurite outgrowth in primary cultured neurons, an effect that is accompanied by stimulation of cAMP signaling (Wasilewska-Sampaio et al., 2005; Sebollela et al., 2009). Collectively, these studies suggest that DNP could be a useful tool in the development of novel therapeutics against neurodegeneration and brain injury (De Felice et al., 2007).

Here, we asked whether DNP would induce neural differentiation in murine ESCs and investigated the mechanisms underlying this effect. We found that DNP triggered neuronal differentiation and cell migration from embryoid bodies, reduced apoptosis and caused an increase in neurite density and length in differentiated cells. We further demonstrate that DNP increases the mitochondrial ATP turnover coupled to O_2 consumption and ERK activation, mechanisms likely involved in neuronal differentiation. Results indicate that, at low concentrations, DNP may be a novel, effective and safe drug to integrate disease modeling and cell-based therapies against neurodegenerative diseases.

Methods

2,4-dinitrophenol (DNP)

DNP stock solution was freshly prepared immediately before each experiment at 2 mM. The solution was filtered using a 0.22 μm sterile filter and kept protected from light under sterile conditions.

Cell culture

Murine ESCs (cell line USP1) were kindly provided by Dr. Lygia Pereira (University of São Paulo-USP) and by Dr. Irina Kerkis (Federal University of São Paulo). Murine ESCs were grown as previously described (Marinho et al., 2010; Nones et al., 2010). Briefly, cell colonies were grown on a mouse embryonic fibroblast (MEF) feeder layer previously arrested with mitomycin C (Sigma), in 15% knockout serum replacement (KSR; Gibco) — DMEM/F12 (Gibco) supplemented with 1% non-essential amino acid solution (Gibco), 2 mM L-glutamine (Gibco), 0.1 mM β-mercaptoethanol (Gibco), 40 mg/mL gentamicin sulfate (Schering-Plough), and 0.2% of conditioned medium from Chinese hamster ovary (CHO) cells producing leukemia inhibitory factor (LIF).

Formation, treatment and cell migration from embryoid bodies (EBs)

Murine ESCs (3×10^6 cells) were passed to a 6-well plate coated with 0.1% gelatin (Sigma) on the first day of culture, and were cultured for 4 days in 15% KSR-DMEM/F12 media. At 4 days in vitro, colonies were treated with TrypLE-Express (Invitrogen) for 5 min at 37 °C, transferred to non-adherent plate dishes with DMEM/F12 medium with 15% FBS (Gibco) and cultured for an additional 4 days. Spherical cell aggregates, denoted embryoid bodies (EBs), assembled between 4 and 8 days in vitro in DMEM/F12 with 15% FBS. Experimental treatments of EBs were performed from 8 to 12 days in vitro in LIF-free medium and 15% FBS. Retinoic acid (RA; Sigma) was used as a positive control to induce neuronal differentiation. At 8 days in vitro, EBs were divided in 3 groups: (a) control, receiving 0.1% DMSO (RA vehicle) or Milli-Q H_2O (DNP vehicle), (b) treated with 2 μM RA, and (c) treated with 20 μM DNP. Culture medium was changed every other day. To allow cell migration, at 12 days in vitro EBs were plated onto 1 mg/mL laminin- and 1 mg/mL fibronectin-coated dishes and cultured in DMEM/F12 basal medium with N2 supplement and 20 ng/mL of fibroblast growth factor-2 for an additional 6 days.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium) reduction measures the generation of NADH- or NADPH-dependent chemical potentials within living cells, providing an assessment of cell viability (Giordano et al., 2011). MTT reduction was assayed as described (Vieira et al., 2007). After treatment with DNP or vehicle, EBs were dissociated with TrypLE-Express (Invitrogen), plated at a density of 1.0×10^4 cells/well (in 96-well plates) and incubated for 4 h with 100 μg/mL MTT (Sigma). Cells were disrupted and formazan blue crystals were dissolved by addition of 100 μL 10% SDS solution in 10 mM HCl. Absorption was measured at 540 nm in a plate reader after incubation at 25 °C for 16 h.

Immunofluorescence

Prior to immunofluorescence, frozen EB sections (10 μm) or whole-mount migrating cells from EBs were permeabilized with 0.3% Triton X-100/PBS for 9 min and washed with saline for 5 min. After blocking in 5% bovine serum albumin (Invitrogen) for 30 min, samples were incubated with the respective primary antibody overnight. Cells were further incubated in secondary antibodies (goat anti-mouse IgG conjugated to Alexa Fluor 546 (Molecular Probes, 1:400)) for 1 h. The samples were then washed and nuclear counterstaining was performed with 1.0 mg/mL 4,6-diamidino-2-phenylindole (DAPI) for 7 min. EB sections were imaged on a Nikon Eclipse TE 300 fluorescence microscope. For cell migration assays, immunofluorescence was imaged on a Zeiss AxioObserver Z1 microscope equipped with an Apotome module (Zeiss). For caspase-3 and Ki67 staining, images were acquired on a Nikon confocal microscopy and are presented as maximal projections.

Primary antibodies anti-nestin (Chemicon; mouse, 1:100), anti-β-tubulin III (Covance; mouse, 1:100) and anti-MAP2 (Covance; mouse, 1:100) were used to label neural progenitor

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