Mitochondrial Bioenergetics in Stem Cell Models of Neurodegeneration

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P6 — 2012 Buck Symposium, Stem Cell Research and Aging, March 1-2, 2012 • Novato, California

Introduction

Parkinson disease (PD) is a multi-factorial progressive neuro-degenerative disorder affecting primarily dopaminergic neurons in the substantia nigra (1). Genetic predisposition, such as a triplication of the α-synuclein gene (SNCA-Tri), can accelerate disease progression and result in earlier onset of the disorder (2). Environmental factors, such as toxicants and free radical generators targeting cellular bioenergetics have been associated with PD epidemiology (3). Dopaminergic neurons are an extreme high energy demanding cell type , and mitochondria are essential for their function. Consequently, mitochondrial dysfunction and a higher susceptibility to programmed cell death of these neurons have been suggested to play a significant role in disease progression (4).

Induced pluripotent stem cells (iPSCs) derived neuronal precursor cells (NPCs) generated from PD patients have opened new possibilities for *in vitro* modeling of PD disease mechanisms and progression (5). Neuronal degeneration of adult dopaminergic neurons is a primary target of research, but equally interesting are the stability and developmental potential of neuronal stem cells.

Hypothesis

Neuronal stem cells (NPCs) derived from PD patients with mutations for PD are more susceptible to cellular stress and bioenergetic challenges. The accumulated stress then contributes to the predisposition of NPC-derive differentiated dopaminergic neurons to premature cell death.

Objective

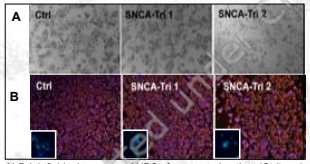
To investigate cellular and mitochondrial bioenergetics in iPSC-derived NPCs with the α-synuclein gene triplication and study the impact of environmental and toxicant challenges.

Conclusions

- iPSC derived NPCs can be used to investigate neuronal stem cell bioenergetics.
- NPCs with the α-synuclein triplication mutation (SNCA-Tri) show normal morphology under standard growth conditions, but over-express the α-synuclein protein.
- SNCA-Tri NPCs are highly sensitive to reactive oxygen species and have a reduced capacity to mitigate the impact of oxidative stress.
- SNCA-Tri NPCs have bioenergetic deficiencies in both cytoplasmic and mitochondrial energy metabolism that are magnified by nutritional stress or toxicant exposure.
- SNCA-Tri NPCs show altered cellular quality control, indicative of reduced cellular plasticity.
- SNCA-Tri NPCs are more sensitive to mitochondrial permeability transition opening and apoptotic cell death.
- The observed early changes in cellular bioenergetics and mitochondrial function of SNCA-Tri NPCs may set the stage for decreased fitness, accelerated aging and increased cell death of NPC-derived neurons later in life.

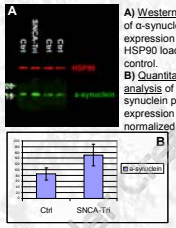
1. iPSC-derived SNCA-Tri NPC Morphology and α-Synuclein Expression

Stem Cell Markers



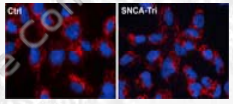
A) Brightfield microscopy of NPCs from control patient (Ctrl) and two patients with the α-synuclein gene triplication (SNCA-Tri 1/2) (100x magnification). **B)** Nestin Immunocytochemistry with nuclear counter stain by H33342 (blue) (200x). Inserts in B): Immuno-cytochemistry for the nuclear stem cell marker SOX1 (green) (400x).

α- Synuclein Expression



A) Western Analysis of α-synuclein protein expression in NPCs. HSP90 loading control.
B) Quantitative analysis of α-synuclein protein expression levels normalized to HSP90.

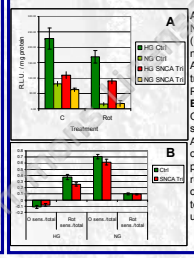
Cellular & Mitochondrial Morphology



Mitochondrial and nuclear content in NPCs assayed by fluorescence microscopy with an mitochondria-specific accumulated dye (Mitotracker Red). Nuclei are counterstained with H33342 (blue). (400x).

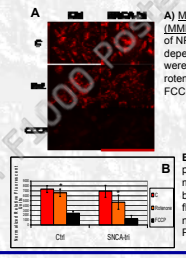
2. SNCA-Tri NPCs display bioenergetic changes

Cellular and Mitochondrial ATP Production



A) Cellular ATP content in SNCA-Tri NPCs under normal growth conditions (high glucose=HG) and under nutritional stress (no glucose=NG). ATP content of untreated (C) and cells treated with the mitochondrial inhibitor Rotenone (Rot).
B) Mitochondrial ATP synthesis. Oligomycin- & rotenone-sensitive (O sens and Rot sens) contribution to total ATP production levels either under HG or NG conditions. Cells were either pretreated with Complex I inhibitor rotenone or ATP-synthase inhibitor oligomycin. ATP levels were normalized to cellular protein (relative luminescent units, R.L.U./mg protein).

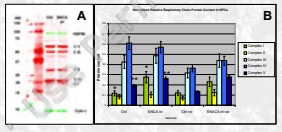
Mitochondrial Membrane Potential



A) Mitochondrial Membrane Potential (MMP). Live fluorescent microscopy of NPCs stained with MMP-dependent fluorescent TMRM. NPCs were left untreated (C) or treated with rotenone (Rot) or the ionophore FCCP as negative control (640x).

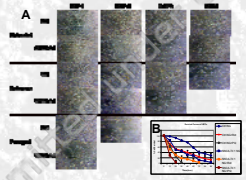
B) Semi-quantitative analysis of panel A). NPC fluorescence was measured using a micor-plate based assay. Average fluorescence intensities were normalized to cell number (*: P≤0.01).

Respiratory Chain Protein Content



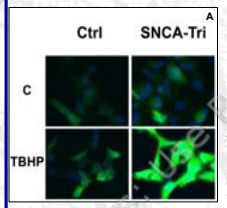
A) Western analysis of mitochondrial respiratory chain subunit proteins from complex I-V (red, CI-V). Loading controls (green) were HSP90 and cytochrome c (Cyto c).
B) Quantitative analysis of complex I-V proteins from untreated and rotenone (rot) pretreated cell lines normalized to HSP90 from three independent experiments. (*: P≤0.001, **: P ≤0.01).

Response to Nutritional Stress

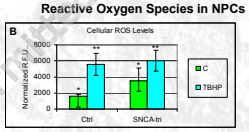


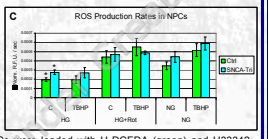
A) Brightfield microscopy of NPC survival in medium without glucose (NG). Cultures were untreated or treated with Rotenone (Rot) or Paraquat (PQ) (25x).
B) Survival Curves for NPCs as under A).

3. SNCA-Tri NPCs are more sensitive to Oxidative Stress



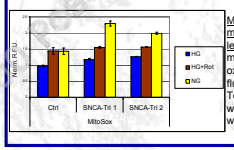
Reactive Oxygen Species in NPCs





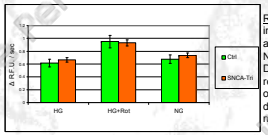
A) ROS detection in NPCs by fluorescence microscopy. NPCs were loaded with H_2DCFDA (green) and H33342 (blue) (640x). Cells were either untreated (C) or treated with tert-butyl-hydroxyperoxide (TBHP).
B) Semi-quantitative analysis of cellular ROS levels in confluent NPCs by microplate-based fluorescence spectroscopy. Fluorescence intensity from cells under A) was normalized to cell number.
C) Semi-quantitative analysis of ROS production rates measured and normalized as under B). Rates were measured with normal medium (HG) with or without rotenone (Rot) and in medium without glucose (NG).

Mitochondrial Superoxide Levels in NPCs



Micorplate-based analysis of mitochondrial superoxide levels in NPCs detecting the mitochondrial targeted and oxygen radical activated fluorescent dye (MitoSox: Life Techn.) under high glucose, with rotenone (HG+Rot), or without glucose (NG)

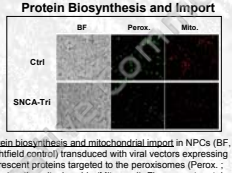
Nitric Oxide Production in NPCs



Rate of cellular NO production in NPCs by microplate-based analysis of the fluorescent Nitric Oxide indicator DAF-DA. Depicted are changes in relative fluorescence intensity of the intracellular indicator dye over time (Δ R.F.U. / sec) normalized to cell number.

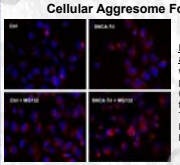
4. SNCA-Tri NPCs have altered protein quality control

Protein Biosynthesis and Import



Protein biosynthesis and mitochondrial import in NPCs (BF, brightfield control) transduced with viral vectors expressing fluorescent proteins targeted to the peroxisomes (Perox., green) or the mitochondria (Mito.; red). Fluorescent protein expression was then evaluated by live cell microscopy. Above images are taken 20hrs after transduction (100x).

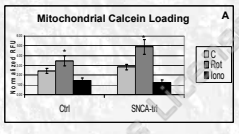
Cellular Aggresome Formation

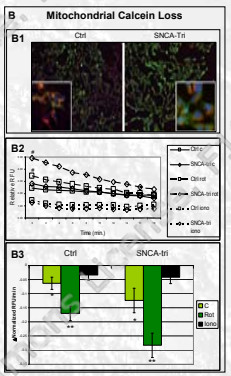


Detection of cellular aggresomes in NPCs with or without proteasome inhibitor (MG132) by fluorescent Bodipy TMR-AHX$_{(L)}$-VS (red). Nuclear counter stain by H33342 (400x).

5. SNCA-Tri NPCs are more sensitive to Apoptosis

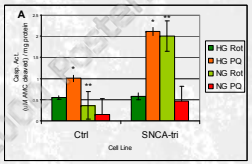
Mitochondrial Permeability Transition

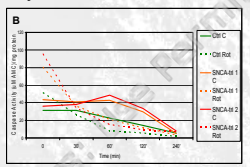




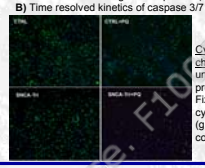
A) Semi-quantitative analysis of mitochondrial calcein uptake in untreated (C) and rotenone-(Rot) and ionomycin-treated NPCs (Iono). *p≤0.001.
B1) Mitochondrial calcein loss in NPCs. NPCs were loaded with fluorescent Mitotracker Red™ (red) and mitochondrial calcein (green) were exposed to phototoxic stress (60x/ Inserts 640x). H33342 was used as counter stain (blue).
B2) Kinetics of mitochondrial calcein loss after induction of the mitochondrial permeability transition pore; #: R^2≥0.95; ##: R^2≥0.4.
B3) Rates of mitochondrial calcein loss as depicted under C). Loss of cellular calcein signal over time was recorded in cells pretreated with a cytoplasmic calcein quencher. Ionomycin (Iono) was used as positive control.*p ≤0.01; **p≤0.001.

Caspase 3/7 Activation and Cytochrome c Release





A) Caspase 3/7 activity in NPCs challenged with rotenone (Rot) or paraquat (PQ) 15min. before cell lysis and analysis. Caspase activity was detected by a fluorimetric assay* for the fluorescent caspase substrate 7-amino-4-methylcoumarin (AMC) using a fluorescent plate reader. Activity is shown as μM AMC fluorescence / mg cellular protein (detected by Bradford protein assay). .*p ≤0.01; **p≤0.001
B) Time resolved kinetics of caspase 3/7 activity in paraquat (PQ)-challenged NPCs as under A).



Cytochrome c Immunocyto-chemistry in adherent NPCs either untreated (CTRL) or short term pretreated with paraquat (PQ). Fixed NPCs were stained with a cytochrome c specific antibody (green). H33342 was used as a counterstain (blue) (200x).

References:
1) Selkoe DJ. Folding proteins in fatal ways. Nature. 2003 Dec 18;426(6968):900-4.
2) Singleton AB. et al. alpha-Synuclein locus triplication causes Parkinson's disease. Science 302, 841 (2003)
3a) Tanner, CM. Rotenone, Paraquat and Parkinson's Disease. Environmental Health Perspectives Volume 119 | number 6 | June 2011.
4) Mak, S.K. Mitochondrial dysfunction in fibroblasts from a patient with an alpha-synuclein triplication. Journal Parkinson's disease 1 (2011)175-183.
5) Mak, S.K. Small molecules greatly improve conversion of human induced pluripotent stem cells to the neuronal lineage. Stem Cells International, in press.