Nicotinamide nucleotide transhydrogenase (Nnt) links the substrate requirement in brain mitochondria for hydrogen peroxide removal to the thioredoxin/peroxiredoxin (Trx/Prx) system*

Pamela Lopert¹ and Manisha Patel²

¹Neuroscience Program, University of Colorado Anschutz Medical Campus, Aurora, Colorado 80045
²Department of Pharmaceutical Sciences, of Colorado Anschutz Medical Campus, Aurora, Colorado 80045

*Running Title: Nnt links mitochondrial respiration to Trx/Prx H₂O₂ removal

To whom correspondence should be addressed: Manisha Patel, Department of Pharmaceutical Sciences, University of Colorado Anschutz Medical Campus, 12850 E. Montview Blvd, Aurora, Colorado 80045 Tel: (303) 724-3604, Fax: (303) 724-7266, E-mail: Manisha.Patel@ucdenver.edu

Keywords: Oxidative stress, Parkinson’s Disease, Thioredoxin Reductase, Mitochondria, Reactive Oxygen Species (ROS), Nicotinamide Nucleotide Transhydrogenase (Nnt)

Background: Actively respiring brain mitochondria can consume H₂O₂ through thioredoxin/peroxiredoxin (Trx/Prx).

Results: Inhibition of Nicotinamide Nucleotide Transhydrogenase (Nnt) decreases NADPH levels, decrease Trx and Trx Reductase activity, and increases toxicity to oxidative stress.

Conclusion: Nnt links mitochondrial respiration and antioxidant activity in brain mitochondria.

Significance: Nnt may be a therapeutic target to increase the antioxidant activity in cells.

ABSTRACT

Mitochondrial reactive oxygen species (ROS) are implicated in the etiology of multiple neurodegenerative diseases including Parkinson’s disease (PD). Mitochondria are known to be net producers of ROS but recently we have shown that brain mitochondria can consume mitochondrial hydrogen peroxide (H₂O₂) in a respiration-dependent manner predominantly by the thioredoxin/peroxiredoxin (Trx/Prx) system. Here we sought to determine the mechanism linking mitochondrial respiration with H₂O₂ catabolism in brain mitochondria and dopaminergic cells. We hypothesized that nicotinamide nucleotide transhydrogenase (Nnt), which utilizes the proton gradient to generate NADPH from NADH and NADP⁺, provides the link between mitochondrial respiration and H₂O₂ detoxification through the Trx/Prx system. Pharmacological inhibition of Nnt in isolated brain mitochondria significantly decreased their ability to consume H₂O₂ in the presence, but not absence, of respiration substrates. Nnt inhibition in liver mitochondria, which do not require substrates to detoxify H₂O₂, had no effect. Pharmacological inhibition or lentiviral knockdown of Nnt in N27 dopaminergic cells a) decreased H₂O₂ catabolism b) decreased NADPH and increased NADP⁺ levels and c) decreased basal, spare and maximal mitochondrial oxygen consumption rates. Nnt deficient cells possessed higher levels of oxidized mitochondrial Prx which rendered them more susceptible to steady-state increases in H₂O₂ and cell death following exposure to subtoxic levels of paraquat. This data implicates Nnt as the critical link between the metabolic and H₂O₂ antioxidant function in brain mitochondria and suggests Nnt as a potential therapeutic target to improve the redox balance in conditions of oxidative stress associated with neurodegenerative diseases.

Mitochondrial reactive oxygen species (ROS) play important roles in physiological cell signaling as well as pathological degeneration associated with neurodegenerative diseases such as...
Parkinson’s disease (PD) (1-3). Brain mitochondria have multiple antioxidant pathways which help maintain the balance between ROS production (for signaling pathways) and consumption/catabolism (to prevent oxidative damage). Mitochondrial superoxide (O$_2^-$) radicals are efficiently converted to hydrogen peroxide (H$_2$O$_2$) by spontaneous dismutation and/or enzymatic reaction by manganese superoxide dismutase (MnSOD) (1,4,5). Additionally, mitochondrial H$_2$O$_2$ can be produced by citric acid cycle (TCA) enzymes such as aconitase and alpha-ketoglutarate dehydrogenase (6-8). H$_2$O$_2$ is detoxified to water by the thioredoxin/peroxiredoxin (Trx/Prx) pathway or catalase (present in liver, but not brain mitochondria) and MnSOD do not (4,15). Approximately 45% of NADPH in the mitochondria is produced by nicotinamide nucleotide transhydrogenase (Nnt) and the remainder comes from NADP$^+$-isocitrate dehydrogenase 2 (IDH) or mitochondrial NAD(P)-malic enzyme (16,17). What separates Nnt from the other mechanisms of NADPH production is it utilizes the proton gradient generated through the TCA cycle and the ETC to convert NADP$^+$ into NADPH (18,19). Nnt’s role in mitochondrial redox regulation has been demonstrated previously in studies which showed that Nnt inhibition decreased GSH redox status and increased sensitivity to oxidative stress however its role in modulating the Trx/Prx system in brain mitochondria remains unknown (16,20-22).

We hypothesized that Nnt activity links the mitochondria’s ability to remove H$_2$O$_2$ in a substrate- and respiration dependent manner to the Trx/Prx antioxidant system. This hypothesis predicts that the presence of Nnt is essential for mitochondrial defense against normal and low-levels of oxidative stress as supported by previous research demonstrating that defects in the Nnt gene could be a potential risk for increased mitochondrial vulnerability (19-21). Here we show that inhibition of Nnt results in decreased 1) mitochondrial H$_2$O$_2$ removal rates, 2) cellular NADPH levels, 3) TrxR and Trx activity in isolated mitochondria and increased 4) mitochondrial Prx oxidation and 5) sensitivity to ROS production and oxidative cell death. This data implicates Nnt as the link between mitochondrial respiration and H$_2$O$_2$ catabolism via the Trx/Prx antioxidant pathway.

**EXPERIMENTAL PROCEDURES**

**Chemical Reagents-**All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

**Isolation of Pure Rat Liver and Brain Mitochondria-**All animal experiments were performed with approval from the Institutional Animal Care and Use Committee at the University of Colorado Denver Anschutz Medical Campus. Mitochondria were isolated from adult male Sprague-Dawley rats as previously described through a Percoll density gradient centrifugation (14,23,24). For liver mitochondria, a crude isolation was conducted first as previously described by Salvi et al then subjected to the Percoll gradient similar to brain mitochondria (25). Purity of mitochondrial and cytosolic
Nnt links mitochondrial respiration to Trx/Prx H$_2$O$_2$ removal

fractions was confirmed by Western blotting for Actin, Lamin B and Complex IV (Fig. 1A). Protein levels were determined by the Bradford assay.

**Isolation of Crude Rat Brain Mitochondria**
Adult male Sprague-Dawley rat brains were homogenized in isolation buffer and the homogenate centrifuged at 3,000 RPM at 4°C for 10 min. Supernatant was removed and centrifuged at 13,000 RPM at 4°C for 10 min. Supernatant was removed and saved as the cytosolic fraction and mitochondrial pellet was re-suspended in desired assay buffer and protein levels were determined by Bradford protein assay.

**Cell Culture**
N27 immortalized rat dopaminergic (DA) cells were a kind gift from Drs. Curt Freed and Kedar Prasad at the University of Colorado, Anschutz Medical Campus (26). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). N27 cells were grown and plated as previously reported (26,27). All experiments were conducted in cells passage #3-10.

**Nnt Knockdown**
Nnt levels were inhibited in N27 cells using SMART vector 2.0 lentiviral shRNA particles according to manufacturer’s protocol (Thermo Scientific Dharmacon, Lafayette, CO, USA) and as previously outlined in Lopert et al (28). Dharmacon provided 3 predesigned gene specific shRNA lentiviral particles and all 3 were screened for transfection efficiency. The best lentiviral particle was used for the remainder of the experiments (GGAGTATCCACATTTGCCA).

**Real-time PCR**
According to manufacturer’s protocol, RNA from transfected N27 cells was isolated using the RNeasy kit® (Qiagen, Valencia, CA, USA). RNA was quantified through 260/280 wavelength measurement by a Nanodrop 2000c spectrophotometer (ThermoScientific, Waltham, MA, USA). Pure RNA was reverse transcribed using the high capacity cDNA reverse transcription kit according to manufacturer’s protocol (Applied Biosystems, Foster City, CA, USA). Real time PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR system. Primers and probes for rat 18S, Nnt, TrxR2 and TrxR1 were purchased from Applied Biosystems.

**Isocitrate Dehydrogenase (IDH) Activity Assay**
IDH activity was determined as previously described (29,30). Cells were collected and lysed in 0.01% (v/v) Triton-X and protein levels were determined by Bradford protein assay. 500 µg of cell solution was used to initiate the reaction and the reduction of NADP$^+$ to NADPH was measured in a 1 mL cuvette spectrophotometrically at 25°C for 3 min.

**TrxR and Trx Activity Assay**
TrxR and Trx activity was measured in isolated pure rat brain mitochondria using an insulin-reduction assay in the presence of E. coli Trx or rat TrxR as previously described by Arnér et al (31) with slight modification. 25 µg of isolated mitochondria were plated and exposed to respiration substrates, palmitoyl coenzyme A (Palm CoA) or water for 15 min. After incubation, mitochondria were lysed with 0.01% (v/v) Triton-X added to the assay buffer without exogenously added NADPH. After 1 h incubation, the number of reduced thiols was determined on a Versamax micro plate reader (Molecular Devices, Sunnyvale, CA, USA).

**Polarographic Measurement of Exogenous H$_2$O$_2$ removal**
H$_2$O$_2$ removal rates were measured in 1x10$^6$ cells per sample or 100 µg of isolated pure rat brain or liver mitochondria (as determined by Bradford assay) using a 100 µM Clark-type electrode with an Apollo 4000 Free Radical Analyzer (World Precision Instruments, Inc., Sarasota FL, USA). Measurements were conducted as previously described by Drechsel et al (14). Briefly, respiration substrates and/or pharmacological inhibitors were added and a stable H$_2$O$_2$ removal rate was measured. Next, mitochondria or cells were added to the chamber and H$_2$O$_2$ removal rates were calculated based on the linear signal decay after the addition of mitochondria or cells compared to rates with respiration substrates or pharmacological inhibitors.

**Nnt Activity Assay**
Nnt activity was measured on Shimadzu UV-2401PC UV-Vis Recording Spectrophotometer (Kyoto, Japan) as previously described by J. Rydström and Shimomura K et al (32,33). Briefly isolated mitochondria or N27 cells were suspended in an activity buffer and 300 µM APAD and NADPH plus pharmacological inhibitors were added. Nnt activity was measured over a 3 min linear decay period with a dual wavelength of 375 nm and 400 nm (reference) in a 1 mL cuvette at 37°C.
HPLC to measure NADH, NAD+, NADPH and NADP+. The concentration of NADP+, NAD+, NADH and NADPH in cells was measured using a HPLC system with spectrophotometer detection (Elite LaChrom System; Hitachi) following the method previous described (34) with a slight modification. The reduced forms were extracted by 0.25 M KOH (basic condition), then passed through a Millipore ultrafree Eppendorf filtration system (Millipore, Bedford, MA, USA,) to eliminate proteins then 1 M KH2PO4 (1/4 volume) was added to neutralize pH. The oxidized forms were extracted by 0.1 N PCA (acidic condition) and centrifuged at 16000 g for 10 min at 4°C to precipitate protein. The pyridine dinucleotides were then separated with a YMC-Pack ODS-A column (4.6 × 250 mM, 5 µM, Waters, Milford, MA, USA) and the mobile phase was 100 mM potassium phosphate (pH 5.0) and 5% (v/v) methanol for the reduced forms, and 100 mM potassium phosphate (pH 6.0) and 5% (v/v) methanol for the oxidized forms. NADPH and NADH were measured by absorbance at 340 nm and NADP+ and NAD+ at 254 nm.

HPLC to determine glutathione (GSH) and glutathione disulfide (GSSG) levels-GSH assay was performed with an ESA 5600 CoulArray HPLC equipped with eight electrochemical cells as previously described by Liang and Patel (35). The potentials of the electrochemical cells were set and analyte separation was conducted on a TOSOHAAS (Montgomeryville, PA) reverse-phase ODS 80-TM C-18 analytical column (4.6mm × 250cm; 5 µm particle size). A two-component gradient elution system was used with component A of the mobile phase composed of 50mM NaH2PO4, pH 3.2, and component B composed of 50mM NaH2PO4 and 40% methanol, pH 3.2. An aliquot (40 µl) of the supernatant was injected into the HPLC.

Western Blot—Total protein levels were measured using standard SDS-Page Western blots with 15 µg of mitochondria, cytosol or cell extract. Actin, was purchased from Abcam (Cambridge, MA, USA) and used at 1:10,000. Lamin B was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Complex IV was purchased from Mitosciences (Eugene, OR, USA) and diluted to 1:1,000. Goat anti-rabbit secondary was purchased from Abcam and used at 1:10,000. Antibodies were detected using enhanced chemiluminescence (Thermo Scientific, Pittsburgh PA, USA) and bands were imaged on a Storm Optical Scanner (Molecular Dynamics Inc, Sunnyvale, CA, USA).

Peroxiredoxin 3 (Prx3) Redox Blot-The redox state of Prx3 was conducted as previously described by Cox et al (36) and optimized in rat N27 cells. Prx3 primary antibody was purchased from Abcam and secondary anti-mouse antibody was purchased from Jackson ImmunoReasearch (West Grove, PA, USA) and used at 1:2,500 and 1:10,000, respectively. Bands were quantified using densitometry with Image J software (NIH, Bethesda, MD, USA).

Measurement of Oxygen Consumption Rate (OCR)-Oxygen consumption rates were determined using a Seahorse XF24 analyzer (Seahorse Biosciences, North Billerica, MA, USA) and conducted as previously outlined in Cantu, 2011 and Lopert, 2012 (27,28).

Mitochondrial Membrane Potential Measurement-TMRE (tetramethylrhodamine, ethyl ester) (Invitrogen) was conducted as outlined in the manufactures protocol (Abcam) with a slight modification. 2.0x10^4 cells/well were seeded and allowed to grow overnight. Media was removed and replaced with RPMI along with 20 µM FCCP as positive control. After 10 min incubation, 1 µM TMRE was added and incubated for 20 min. After incubation cells were washed 3 times with warmed PBS supplemented with 0.2% (w/v) BSA and immediately read on Synergy™ multi-mode microplate reader. All values were normalized to protein as determined by a Bradford assay.

Detection of H2O2-H2O2 levels were measured using the Amplex Red fluorescence assay (Invitrogen) (27,28) using a Synergy™ multi-mode microplate reader (Biotek).

Cell Death Assessment-Cells were plated in 96-well plated at 2.0x10^4 cells/well. Media and cell lysis (Tris/NaCl + 0.1% (v/v) Triton) samples were collected and lactate dehydrogenase (LDH) levels was measured by measuring the release of LDH enzyme activity as described by Bergmeyer et al and published previously (28,37).

Statistical Methods-Data were analyzed in GraphPad Prism software Version 5 (San Diego, CA, USA). One-way ANOVA was used to test differences between multiple groups with a Bonferroni post-test. To determine statistical
significance between two groups a two-tailed student’s t-test was utilized.

RESULTS

H2O2 removal is linked to Nnt activity in isolated brain mitochondria - To implicate Nnt in mitochondrial H2O2 catabolism, we asked whether pharmacological inhibition of Nnt directly affected the rates of H2O2 removal in isolated mitochondria and whether this occurred in a respiration dependent manner. A polarographic method using a Clark-type electrode was used to measure real-time H2O2 levels in isolated brain mitochondria. Isolated pure brain mitochondria (Fig 1A) had Nnt inhibited with the pharmacological inhibitor Palm CoA (38) and exposed to exogenous H2O2 with and without respiration substrates present. As outlined in Table 1, isolated brain mitochondria with no addition of respiration substrates (state 1) had very little H2O2 removal. However, stimulation with the respiration substrates malate and glutamate (state 2) resulted in a significant increase in the mitochondria’s ability to remove exogenous H2O2. When 100 µM Palm CoA was added to actively respiring mitochondria, there was a significant decrease in the rate of H2O2 removal back to the rates of non-stimulated mitochondria. Addition of Palm CoA had no additional effect on respiration when mitochondria were in state 1. These data demonstrate that Nnt activity controls respiration-dependent H2O2 catabolism by isolated brain mitochondria.

Nnt activity is not required for H2O2 removal in liver mitochondria - We have previously shown that in contrast to brain mitochondria, isolated liver mitochondria remove H2O2 in a non-respiration dependent manner (14). To further establish Nnt activity as a mechanistic link between respiration-dependent H2O2 removal in brain mitochondria, we sought to determine the consequence of Nnt inhibition on H2O2 removal by liver mitochondria. We hypothesized that since liver mitochondria remove H2O2 in a respiration or substrate-independent manner, Nnt inhibition would not alter the rate of removal. As outlined in Table 1, isolated liver mitochondria alone consumed H2O2 at the same rate as when malate and glutamate were present. Additionally, addition of Palm CoA did not decrease H2O2 removal. As a control aminotriazole, an inhibitor of catalase (which is not NADPH dependent), was added and there was a significant decrease (~45%) in activity (data not shown). The inability of Nnt inhibition to alter H2O2 removal rates in liver mitochondria confirms the substrate independence of this process and suggests that Nnt activity selectively plays a critical role in actively respiring brain mitochondria’s ability to remove exogenous H2O2.

Pharmacological inhibition of Nnt results in respiration-dependent decreases in Trx and TrxR activity - As indicated previously isolated pure brain mitochondria primarily consume H2O2 by the Trx/Prx system (14). To determine if the decrease in H2O2 removal in isolated mitochondria following pharmacological inhibition of Nnt was due to a decrease in Trx/TrxR activity, Trx and TrxR activity was measured in isolated brain mitochondria incubated with malate, glutamate, and Palm CoA. As indicated in Figure 1, addition of malate and glutamate caused a significant increase in TrxR activity (~300%). When 100 µM Palm CoA was added, there was no change in Trx or TrxR activity when mitochondria were in state 1. However, when malate and glutamate were present and Nnt was inhibited there was a significant decrease in Trx and TrxR activity (~83% and ~95% respectively) indicating Trx and TrxR activity is linked to Nnt activity in actively respiring mitochondria. Additionally, the purity of mitochondrial was confirmed by Western blot for Complex IV (mitochondrial), Lamin B (nuclear) and Actin (cytosol) as depicted as a representative blot in Figure 1A.

Pharmacological inhibition of Nnt in N27 cells results in decreased H2O2 removal rates - Based on the results obtained in isolated mitochondria, the next step was to determine the effects of pharmacological inhibition of Nnt in intact cells. N27 DA cells exposed to 100 µM Palm CoA had a ~48% decrease in Nnt activity (Fig. 2A). The decrease in Nnt activity resulted in a significant decrease in the cell’s ability to remove exogenous H2O2 by 22 ± 2% (Fig. 2B). This indicates that pharmacological inhibition of Nnt results in a DA cellular model also results in a decrease in H2O2 catabolism.

Constitutive knockdown of Nnt in N27 cells - To confirm the role of Nnt without the use of pharmacological agents with potential off-target effects in a cell model, N27 cells were transfected with a shRNA construct knocking down Nnt
expression (Nnt deficient) or a non-targeting control (mock). As indicated in Figure 3, transfection with a shRNA targeting vector for Nnt resulted in a 94.1 ± 0.4% decrease in Nnt mRNA levels (Fig. 3A) corresponding to a 74.4 ± 5.7% decrease in Nnt activity (Fig. 3B).

Nnt deficient cells have decreased NADPH and increased NADP⁺ levels resulting in decreased H₂O₂ removal rates and GSH levels - To determine if decreased Nnt activity resulted in a decrease in NADPH levels (the reducing equivalent used by TrxR to help detoxify H₂O₂ and generated by Nnt) NADPH and NADP⁺ levels were measured in cells via a HPLC method. Figure 4 shows that Nnt deficient cells had a significant decrease in NADPH levels (~25%) which correlated with a significant increase in NADP⁺ levels (~30%) compared to mock control cells. The increase in NADP⁺ levels paired with the decrease in NADPH levels resulted in a significant increase in NADP⁺/NADPH ratios by ~75% indicating the Nnt deficient cells have a more oxidized NADP⁺/NADPH intracellular environment (Fig. 4C). To determine if the effects observed with pharmacological inhibition in N27 cells was specific to Nnt inhibition, and that the decrease in NADPH levels in Nnt deficient cells resulted in changes in H₂O₂ catabolism, Nnt deficient cells were tested for exogenous H₂O₂ removal rates compared to mock controls. As depicted in Figure 4D, Nnt deficient cells showed significant decreases (~40%) in their ability to remove H₂O₂ compared to the mock controls. The magnitude of the decrease was similar to that observed in isolated mitochondria and N27 cells treated with Palm CoA. Additionally, GSH and GSSG levels were measured by an HPLC method to determine if the NADPH deficiency in Nnt KO cells resulted in changes in the GSH system (another NADPH utilizing pathway). Indeed Nnt deficient cells had significantly higher basal GSSG and concomitantly decreased GSH levels compared to mock controls (Fig. 4E-F) consistent with the decrease in NADPH levels.

To determine if Nnt knockdown and resultant decrease of NADPH levels caused alterations in enzymatic activities of other NADPH-producing enzymes, we measured IDH activity. No changes in IDH activity was observed in Nnt knockdown compared to mock controls (Fig. 4G).

Nnt deficient cells have more oxidized mitochondrial Prx3 than mock controls - To determine if the decrease in NADPH levels in Nnt deficient cells resulted in a more oxidized Trx/Prx system, a redox blot for Prx3 was conducted in mock and Nnt deficient cells. As shown in Figure 5, Nnt deficient cells had significantly more (~40%) oxidation of Prx3 compared to mock controls. This indicates that the decrease in TrxR activity in the Nnt deficient cells results in more oxidized Prx3. Total Prx3 levels was measured in Nnt deficient and mock cells to determine if the increased oxidation was due to a decrease in total protein levels. As shown in Figure 5D total Prx3 levels did not differ significantly between control and Nnt deficient cells.

Decreased mitochondrial Oxygen Consumption Rates (OCR) occurs in Nnt deficient cells - To examine if Nnt inhibition which results in decreased NADPH levels and more oxidized Prx3 leads to mitochondrial dysfunction, we measured mitochondrial OCR as well as other bioenergetic parameters in Nnt deficient cells and mock controls using an XF24 analyzer (Seahorse Biosciences). As outlined in the summary trace in Figure 6 and detailed in Table 2, Nnt deficient cells showed significant decreases in baseline respiration, ATP turnover, respiratory capacity, reserve respiratory capacity and glycolysis. This data suggests that Nnt knockdown per se results in mitochondrial dysfunction based on decreased baseline OCR as well as maximal and reserve respiratory capacity.

Nnt deficient cells are more susceptible to oxidative stress - To determine if the changes in mitochondrial antioxidant activity and resulting mitochondrial function leads to increased susceptibility to oxidative stress, the mitochondrial membrane potential was measured. Additionally, to test Nnt’s role in an in vitro model of PD, cells were exposed to various levels of the known PD toxicant paraquat (PQ). Nnt deficient cells had a significantly altered mitochondrial membrane potential (Fig. 7A) which indicates theses cells have increased mitochondrial dysfunction. Indeed, as indicated in Figure 7B, baseline H₂O₂ production in Nnt deficient cells was increased compared to control (~35% more). When incubated with 100, 600 and 1000 µM PQ for 24 h, Nnt deficient cells produced significantly more H₂O₂ compared to mock controls (~40, 35, and
40% increase respectively). After 48 h of PQ incubation (Fig. 7C), Nnt deficient cells had significantly increased LDH release at 300, 600 and 1,000 µM compared to controls (~12, 16 and 18% increased release, respectively). Cell death was not observed following 24 h of PQ exposure indicating the increase in H$_2$O$_2$ production at this time point occurred prior to overt increases in cell death (data not shown).

**DISCUSSION**

Here we identify Nnt activity as a critical link between respiration- and substrate-dependent H$_2$O$_2$ catabolism by the Trx/Prx antioxidant system in isolated brain mitochondria and N27 DA cells. Three principal observations support the role of Nnt as the link between respiration and H$_2$O$_2$ detoxification by the Trx/Prx antioxidant pathway. First, pharmacological inhibition of Nnt in actively respiring brain mitochondria significantly decreased H$_2$O$_2$ removal rates and Trx/TrxR activity. Furthermore, Nnt inhibition failed to alter H$_2$O$_2$ removal in liver mitochondria consistent with the ability of brain but not liver mitochondria to utilize respiration substrates for H$_2$O$_2$ catabolism. Secondly, deficiency of Nnt either pharmacologically or by shRNA in N27 DA cells, resulted in a significant decrease in NADPH levels along with a decrease in H$_2$O$_2$ removal rates and an increase in oxidized Prx3, with no change in Prx3 protein expression. Finally, Nnt deficient cells showed decreased mitochondrial reserve and maximal respiratory capacity, increased steady-state H$_2$O$_2$ levels, and increased cell death when exposed to low levels of the redox cycling agent PQ. Together, these results highlight the importance of Nnt as a link between mitochondrial respiration and H$_2$O$_2$ catabolism in isolated brain mitochondria and DA cells (Fig. 8).

Nnt activity provides a novel mechanistic explanation to our previous observation that isolated brain mitochondria catabolize H$_2$O$_2$ by the Trx/Prx system when actively respiring (14). Interestingly, liver mitochondria do not need to be actively respiring to catabolize H$_2$O$_2$ as they primarily use the antioxidant catalase which is not present in isolated brain mitochondria (5,13,14,39,40). Catalase does not require the reducing equivalent NADPH to detoxify H$_2$O$_2$ and can work independently of the activity of the ETC (1). Although liver mitochondria have Nnt as well as other sources of NADPH (IDH, malic enzyme etc.), H$_2$O$_2$ catabolism may be favored by mitochondrial catalase rather than dependence on respiration substrates.

The main antioxidant systems in the brain mitochondria (Trx/Prx and GSH) require the presence of NADPH as the reducing equivalents (41). The requirement of mitochondrial respiration for antioxidant activity in the brain is likely due to the unique function of neurons to generate neuronal action potentials. Neurons have activity-dependent ion fluxes which require a significant demand for ATP (generated by the mitochondria) to maintain the membrane potential through ATP dependent sodium/potassium pumps whereas liver mitochondria are not subjected to such dramatic ion fluxes. Indeed, Nnt inhibition in actively respiring brain mitochondria resulted in a significant decrease in TrxR, H$_2$O$_2$ removal. However, there was no effect of substrates or Nnt inhibition in isolated liver mitochondria indicating that Nnt activity is critical in coupling substrate-dependent respiration and H$_2$O$_2$ removal in isolated brain but not liver mitochondria by the Trx/Prx pathway.

Of the different types of neuronal cells, DA cells have an increased risk for ROS production presumably due to increased iron levels, monoamine oxidase activity, DA forming quinones and semi-quinones, a high metabolic activity and the pacemaking ability of DA neurons utilizing Ca$^{2+}$ channels (42-44). Trx/Prx activity is critically important in mitochondria to maintain the delicate balance of ROS production for signaling activities vs. catabolism to prevent detrimental oxidative stress. The Trx/Prx and GSH systems require NADPH which can be generated through multiple pathways in neuronal cells (45-47) and therefore it was important to determine the link between Nnt activity to mitochondrial function and antioxidant activity in intact ROS sensitive DA cells. Previous work in cell and animal models suggests that Nnt inhibition by siRNA transfection results in a more oxidized NADP$^+$/NADPH ratio accompanied by a more oxidized GSH/GSSG ratio (20,22,48). Indeed Nnt inhibition by shRNA transfection resulted in a more oxidized NADP$^+$/NADPH ratio and an increase in GSSG and decrease in GSH levels (Fig 4). Additionally, the decrease in NADPH production in Nnt deficient cells was not
compensated by altering IDH activity. Thus, Nnt deficiency appears to decrease the activities of NADPH-utilizing antioxidant enzymatic pathways rather than NADPH-producing enzymes such as NADP⁺-dependent IDH. However, our data do not rule out the role of additional enzymes that may compensate to replenish mitochondrial NADPH in Nnt deficient cells.

Notably, the role of Nnt activity and the Trx/Prx system in DA cells has not been studied in depth. Results from N27 DA cells in this study confirmed that Nnt inhibition via shRNA transfection resulted in significant decreases in NADPH levels and consequently a decrease in the H₂O₂ removal rates and more oxidized Prx3 (Figs. 4 & 5). This suggests a critical role of Nnt in NADPH production in brain mitochondria and further validates Nnt’s link between substrate-driven H₂O₂ catabolism and NADPH dependent Trx/Prx activity in N27 DA cells as depicted in Figure 9. Given the critical role of oxidative stress in neurodegenerative diseases, such as PD, these results also suggest that alterations in Nnt activity may be an important checkpoint or control mechanism for changes in steady-state H₂O₂ levels during disease pathogenesis. As stated previously, DA cells have an increased risk for detrimental ROS production and postmortem studies have shown increased oxidative stress in the substantia nigra pars compacta in patients with PD (49,50). Additionally pesticide exposure, decreased complex I activity and aging have been implicated in the pathogenesis of sporadic PD (44,51-53). The finding that Nnt inhibition resulted in decreased OCR and mitochondrial membrane potential resulting in increased ROS production and cell death validates the idea that Nnt activity in DA cells plays a critical role in the cells ability to detoxify ROS through the Trx/Prx system when cells are exposed to subtoxic levels of oxidative stress. This is consistent with our previous work showing increased vulnerability of N27 cells to inhibition of TrxR activity in the presence of subtoxic levels PQ or 6-hydroxydopamine (28).

The role of Nnt activity as a risk factor for rendering DA neurons vulnerable to age-related neurodegeneration is novel and has not been noted in the literature to date. However, the precedence for Nnt’s ability to control disease pathogenesis exists. Nnt mutations have been linked to multiple disease states including diabetes, obesity, failing myocardium and most recently homozygous Nnt knockout in a family showed a link between Nnt knockout and glucocorticoid deficiency (16,33,54-57). Additionally, the Nnt gene has been identified as a modifier gene in MnSOD deficient mice which show increased mitochondrial oxidative stress (58). Specifically it was demonstrated that MnSOD deficient mice without Nnt had a shorter lifespan than MnSOD deficient mice with the Nnt gene present (58).

Improving mitochondrial antioxidant function has been an area of interest for the treatment of PD, however to date no pharmacological drugs targeting this area have been FDA approved (59,60). Thus, increasing Nnt activity or levels may improve the redox status of a critical antioxidant system such as Trx/Prx and potentially lead to new therapies in PD.
REFERENCES

1. Halliwell, B., and Gutteridge, J. (2007) Free Radicals in Biology and Medicine, 4 ed., Oxford University Press, New York
2. Murphy, M. P. (2009) How Mitochondria produce reactive oxygen species. Biochem. J. 417, 1-13
3. Tait, S. W., and Green, D. R. Mitochondria and cell signalling. J. Cell. Sci. 125, 807-815
4. Fukai, T., and Ushio-Fukai, M. (2011) Superoxide dismutases: role in redox signaling, vascular function, and diseases. Antioxid. Redox. Signal 15, 1583-1606
5. Mavelli, I., Rigo, A., Federico, R., Ciriolo, M. R., and Rotilio, G. (1982) Superoxide dismutase, glutathione peroxidase and catalase in developing rat brain. Biochem. J. 204, 535-540
6. Gardner, P. R., and Fridovich, I. (1991) Superoxide sensitivity of the Escherichia coli aconitase. J Biol. Chem. 266, 19328-19333
7. Tretter, L., and Adam-Vizi, V. (2000) Inhibition of Krebs cycle enzymes by hydrogen peroxide: A key role of [alpha]-ketoglutarate dehydrogenase in limiting NADH production under oxidative stress. J. Neurosci. 20, 8972-8979
8. Starkov, A. A., Fiskum, G., Chinopoulos, C., Lorenzo, B. J., Browne, S. E., Patel, M. S., and Beal, M. F. (2004) Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species. J. Neurosci. 24, 7779-7788
9. Boveris, A., and Chance, B. (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem. J. 134, 707-716
10. Nohl, H., and Hegner, D. (1978) Do mitochondria produce oxygen radicals in vivo? Eur. J. Biochem. 82, 563-567
11. Giulivi, C., Poderoso, J. J., and Boveris, A. (1998) Production of nitric oxide by mitochondria. J. Biol. Chem. 273, 11038-11043
12. Ghafourifar, P., and Cadenas, E. (2005) Mitochondrial nitric oxide synthase. Trends Pharmacol. Sci. 26, 190-195
13. Zoccarato, F., Cavallini, L., and Alexandre, A. (2004) Respiration-dependent removal of exogenous H2O2 in brain mitochondria: inhibition by Ca2+. J. Biol. Chem. 279, 4166-4174
14. Drehsel, D. A., and Patel, M. (2010) Respiration-dependent H2O2 removal in brain mitochondria via the thioredoxin/peroxiredoxin system. J. Biol. Chem. 285, 27850-27858
15. Mishra, S., and Imlay, J. (2012) Why do bacteria use so many enzymes to scavenge hydrogen peroxide? Arch. Biochem. Biophys. 525, 145-160
16. Sheeran, F. L., Rydstrom, J., Skahparonov, M. I., Pestov, N. B., and Pepe, S. (2010) Diminished NADPH transhydrogenase activity and mitochondrial redox regulation in human failing myocardium. Biochim. Biophys. Acta. 1797, 1138-1148
17. Rydstrom, J. (2006) Mitochondrial NADPH, transhydrogenase and disease. Biochim. Biophys. Acta. 1757, 721-726
18. Bizouarn, T., Fjellstrom, O., Meuller, J., Axelsson, M., Bergkvist, A., Johansson, C., Goran Karlsson, B., and Rydstrom, J. (2000) Proton translocating nicotinamide nucleotide transhydrogenase from E. coli. Mechanism of action deduced from its structural and catalytic properties. Biochim. Biophys. Acta. 1457, 211-228
19. Yap, L. P., Garcia, J. V., Han, D., and Cadenas, E. (2009) The energy-redox axis in aging and age-related neurodegeneration. Adv. Drug Deliv. Rev. 61, 1283-1298
20. Arkblad, E. L., Tuck, S., Pestov, N. B., Dmitriev, R. I., Kostina, M. B., Stenvall, J., Tranberg, M., and Rydstrom, J. (2005) A Caenorhabditis elegans mutant lacking functional nicotinamide nucleotide transhydrogenase displays increased sensitivity to oxidative stress. Free Radic. Biol. Med. 38, 1518-1525
21. Huang, T. T., Naeemuddin, M., Elchuri, S., Yamaguchi, M., Kozy, H. M., Carlson, E. J., and Epstein, C. J. (2006) Genetic modifiers of the phenotype of mice deficient in mitochondrial superoxide dismutase. Hum. Mol. Genet. 15, 1187-1194
Nnt links mitochondrial respiration to Trx/Prx H₂O₂ removal

22. Yin, F., Sancheti, H., and Cadenas, E. (2012) Silencing of nicotinamide nucleotide transhydrogenase impairs cellular redox homeostasis and energy metabolism in PC12 cells. Biochim. Biophys. Acta. 1817, 401-409

23. Sims, N. R., and Anderson, M. F. (2008) Isolation of mitochondria from rat brain using Percoll density gradient centrifugation. Nat. Protoc. 3, 1228-1239

24. Castello, P. R., Drechsel, D. A., and Patel, M. (2007) Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. J. Biol. Chem. 282, 14186-14193

25. Salvi, M., Battaglia, V., Brunati, A. M., La Rocca, N., Tibaldi, E., Pietrangeli, P., Marcocci, L., Mondovi, B., Rossi, C. A., and Toninello, A. (2007) Catalase takes part in rat liver mitochondria oxidative stress defense. J. Biol. Chem. 282, 24407-24415

26. Prasad, K. N., Carvalho, E., Kentroti, S., Edwards-Prasad, J., La Rosa, F. G., Kumar, S., Freed, C. R., and Vernadakis, A. (1994) Production of terminally differentiated neuroblastoma cells in culture. Restor. Neurol. Neurosci. 7, 13-19

27. Cantu, D., Fulton, R. E., Drechsel, D. A., and Patel, M. (2011) Mitochondrial aconitase knockdown attenuates paraquat-induced dopaminergic cell death via decreased cellular metabolism and release of iron and H₂O₂. J. Neurochem. 118, 79-92

28. Lopert, P., Day, B. J., and Patel, M. (2012) Thioredoxin reductase deficiency potentiates oxidative stress, mitochondrial dysfunction and cell death in dopaminergic cells. PLoS One 7, e50683

29. Ronchi, J. A., Figueira, T. R., Ravagnani, F. G., Oliveira, H. C., Vercesi, A. E., and Castilho, R. F. (2013) A spontaneous mutation in the nicotinamide nucleotide transhydrogenase gene of C57BL/6J mice results in mitochondrial redox abnormalities. Free Radic. Biol. Med. 63, 446-456

30. Yan, H., Parsons, D. W., Jin, G., McLendon, R., Rasheed, B. A., Yuan, W., Kos, I., Batinic-Haberle, I., Jones, S., Riggins, G. J., Friedman, H., Friedman, A., Reardon, D., Herndon, J., Kinzler, K. W., Velculescu, V. E., Vogelstein, B., and Bigner, D. D. (2009) IDH1 and IDH2 mutations in gliomas. N. Engl. J. Med. 360, 765-773

31. Arner, E. S., and Holmgren, A. (2001) Measurement of thioredoxin and thioredoxin reductase. Curr. Protoc. Toxicol. Chapter 7, Unit 7 4

32. Rydstrom, J. (1979) Assay of nicotinamide nucleotide transhydrogenases in mammalian, bacterial, and reconstituted systems. Methods Enzymol. 55, 261-275

33. Shimomura, K., Galvanovskis, J., Goldsworthy, M., Hugill, A., Kaizak, S., Lee, A., Meadows, N., Quwailid, M. M., Rydstrom, J., Teboul, L., Ashcroft, F., and Cox, R. D. (2009) Insulin secretion from beta-cells is affected by deletion of nicotinamide nucleotide transhydrogenase. Methods Enzymol. 457, 451-480

34. Kalhorn, T. F., Thummel, K. E., Nelson, S. D., and Slattery, J. T. (1985) Analysis of oxidized and reduced pyridine dinucleotides in rat liver by high-performance liquid chromatography. Anal. Biochem. 151, 343-347

35. Liang, L. P., and Patel, M. (2006) Seizure-induced changes in mitochondrial redox status. Free Radic. Biol. Med. 40, 316-322

36. Cox, A. G., Winterbourn, C. C., and Hampton, M. B. Measuring the redox state of cellular peroxiredoxins by immunoblotting. Methods Enzymol. 474, 51-66

37. Bergmeyer, H. U., and Bernt, E. (1983) Chapter 2.2: Lactate Dehydrogenase. UV-Assay with Pyruvate and NADH. Methods of Enzymatic Anal. 3rd Edition, 118-125

38. Rydstrom, J. (1972) Site-specific inhibitors of mitochondrial nicotinamide-nucleotide transhydrogenase. Eur. J. Biochem. 31, 496-504

39. Radi, R., Turrens, J. F., Chang, L. Y., Bush, K. M., Crapo, J. D., and Freeman, B. A. (1991) Detection of catalase in rat heart mitochondria. J. Biol. Chem. 266, 22028-22034

40. Sinet, P. M., Heikkila, R. E., and Cohen, G. (1980) Hydrogen peroxide production by rat brain in vivo. J. Neurochem. 34, 1421-1428
41. Arner, E. S., and Holmgren, A. (2000) Physiological functions of thioredoxin and thioredoxin reductase. *Eur. J. Biochem.* **267**, 6102-6109
42. Surmeier, D. J., and Schumacker, P. T. (2013) Calcium, bioenergetics, and neuronal vulnerability in Parkinson's disease. *J. Biol. Chem.* **288**, 10736-10741
43. Guzman, J. N., Sanchez-Padilla, J., Chan, C. S., and Surmeier, D. J. (2009) Robust pacemaking in substantia nigra dopaminergic neurons. *J. Neurosci.* **29**, 11011-11019
44. Jenner, P. (2003) Oxidative stress in Parkinson's disease. *Ann. Neurol.* **53** Suppl 3, S26-36; discussion S36-28
45. Ying, W. (2008) NAD+/NADH and NADP+/NADPH in cellular functions and cell death: regulation and biological consequences. *Antioxid. Redox Signal* **10**, 179-206
46. Yankner, B. A., Lu, T., and Loerch, P. (2008) The aging brain. *Annu. Rev. Pathol.* **3**, 41-66
47. Lam, P. Y., Yin, F., Hamilton, R. T., Boveris, A., and Cadenas, E. (2009) Elevated neuronal nitric oxide synthase expression during aging and mitochondrial energy production. *Free Radic. Res.* **43**, 431-439
48. Vogel, R., Wiesinger, H., Hamprecht, B., and Dringen, R. (1999) The regeneration of reduced glutathione in rat forebrain mitochondria identifies metabolic pathways providing the NADPH required. *Neurosci. Lett.* **275**, 97-100
49. Jenner, P., Schapira, A. H., and Marsden, C. D. (1992) New insights into the cause of Parkinson's disease. *Neurology* **42**, 2241-2250
50. Jenner, P., and Olanow, C. W. (1996) Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology* **47**, S161-170
51. Dinis-Oliveira, R. J., Remiao, F., Carmo, H., Duarte, J. A., Navarro, A. S., Bastos, M. L., and Carvalho, F. (2006) Paraquat exposure as an etiological factor of Parkinson's disease. *Neurotoxicology* **27**, 1110-1122
52. Jenner, P., and Olanow, C. W. (1998) Understanding cell death in Parkinson's disease. *Ann. Neurol.* **44**, S72-84
53. Zhang, Y., Dawson, V. L., and Dawson, T. M. (2000) Oxidative stress and genetics in the pathogenesis of Parkinson's disease. *Neurobiol. Dis.* **7**, 240-250
54. Heiker, J. T., Kern, M., Kosacka, J., Flehmig, G., Stumvoll, M., Shang, E., Lohmann, T., Dressler, M., Kovacs, P., Bluher, M., and Kloting, N. (2013) Nicotinamide nucleotide transhydrogenase mRNA expression is related to human obesity. *Obesity (Silver Spring)* **21**, 529-534
55. Wong, N., Blair, A. R., Morahan, G., and Andrikopoulos, S. (2010) The deletion variant of nicotinamide nucleotide transhydrogenase (Nnt) does not affect insulin secretion or glucose tolerance. *Endocrinology* **151**, 96-102
56. Meimaridou, E., Kowalczyk, J., Guasti, L., Hughes, C. R., Wagner, F., Frommolt, P., Nurnberg, P., Mann, N. P., Banerjee, R., Saka, H. N., Chapple, J. P., King, P. J., Clark, A. J., and Metherell, L. A. (2012) Mutations in NNT encoding nicotinamide nucleotide transhydrogenase cause familial glucocorticoid deficiency. *Nat. Genet.* **44**, 740-742
57. Yamaguchi, R., Kato, F., Hasegawa, T., Katsumata, N., Fukami, M., Matsui, T., Negasaki, K., and Ogata, T. (2013) A novel homozygous mutation of the nicotinamide nucleotide transhydrogenase gene in a Japanese patient with familial glucocorticoid deficiency. *Endocr. J.* **60**, 855-859
58. Kim, A., Chen, C. H., Uressl, P., and Huang, T. T. (2010) Genetic modifier of mitochondrial superoxide dismutase-deficient mice delays heart failure and prolongs survival. *Mamm. Genome* **21**, 534-542
59. Smith, R. A., and Murphy, M. P. (2010) Animal and human studies with the mitochondria-targeted antioxidant MitoQ. *Ann. N. Y. Acad. Sci.* **1201**, 96-103
60. Gilgun-Sherki, Y., Melamed, E., and Offen, D. (2001) Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. *Neuropharmacology* **40**, 959-975
Acknowledgments – We thank Li-Ping Liang for his help with the HPLC methodology to measure NADP+/NADPH levels and GSH and GSSG levels. Additionally we would like to thank James Roede for his critical help, reagents and equipment in regards to the Prx3 redox blot.

Footnotes:

* This work was supported by the National Institutes of Health grant RO1NS04748 (MP)
1 Neuroscience Program, University of Colorado Anschutz Medical Campus, Aurora, Colorado 80045
2 Department of Pharmaceutical Sciences, of Colorado Anschutz Medical Campus, Aurora, Colorado 80045
3 The abbreviations used are: Nnt, nicotinamide nucleotide transhydrogenase; Trx, thioredoxin; Prx, peroxiredoxin; H2O2, hydrogen peroxide; TrxR, thioredoxin reductase; ROS, reactive oxygen species; PD, Parkinson’s disease; O2•-, superoxide; MnSOD, manganese superoxide; TCA, citric acid cycle; Gpx, glutathione peroxidases; GSH, Glutathione; GSSG, Glutathione disulfide; ETC, electron transport chain; DA, dopaminergic; IDH, isocitrate dehydrogenase; GR, glutathione reductase; GSGG, oxidized glutathione; Prx3, peroxiredoxin 3; OCR, oxygen consumption rates; TMRE, tetramethylrhodamine ethyl ester; LDH, lactate dehydrogenase; Palm CoA, palmitoyl coenzyme A; PQ, paraquat; Nnt deficient, N27 cells transfected with shRNA targeting Nnt; Mock, N27 cells transfected with non-targeting control; H+, proton.

FIGURE LEGENDS

FIGURE 1: Pharmacological inhibition of Nnt results in substrate dependent decreases in TrxR and Trx activity in isolated brain mitochondria. (A) A representative Western blot to indicate the purity of isolated mitochondria conducted in these studies. Trx (B) or TrxR (C) activity assay was conducted as described in ‘Experimental Procedure’ section. There was a significant decrease in TrxR and Trx activity in actively respiring mitochondria with Nnt pharmacologically inhibited. Data is represented as mean ± SEM (n = 4-11). * = p<0.05; ** = p<0.005; *** = p<0.001 as determined by 1way ANOVA.

FIGURE 2: N27 cells with Nnt pharmacologically inhibited results in a decrease in H2O2 catabolism and TrxR activity. N27 cells were exposed to 100 µM Palm CoA and H2O2 removal rates and TrxR activity was measured. There was a significant decrease in Nnt activity (A) n = 7-9. N27 cells exposed to 100 µM Palm CoA had a significant decrease in the ability to consume 3 µM exogenous H2O2 (B) n = 5-6. Data is represented as mean ± SEM; * = p<0.05 *** = p<0.001 as determined by students t-test.

FIGURE 3: Generation of Nnt deficient cell line. N27 cells were transfected with Nnt shRNA (Nnt deficient) and compared to mock-transfected cells (mock). (A) Nnt mRNA expression in Nnt deficient cells had a ~95% decrease in Nnt mRNA compared to mock transfected cells (n = 3). (B) Nnt activity was measured in mock control and Nnt deficient cells and there was a ~75% decrease in activity (n = 9). All bars represent mean ± SEM; *** = p<0.0001 (students t-test).

FIGURE 4: Nnt deficient N27 cells have increased NADP+ and decreased NADPH levels, H2O2 removal rates and GSH levels (A) NADP+ levels and (B) NADPH levels were determined in Nnt deficient cells and compared to mock control via HPLC. There was a significant increase in NADP+ levels coupled with a decrease in NADPH levels. Each individual NADP+ value was divided by the average NADPH value to determine the ratio (C) which was significantly oxidized in the Nnt deficient cells (n = 4-9). (D) Nnt deficient cells showed a ~40% decrease in the ability to remove exogenous H2O2 (n = 10). Additionally Nnt deficient cells showed (E) a decrease in GSH levels and an (F) increase in GSSG level (n= 3). (G)
There was no change in IDH activity (n = 5-8). * = p<0.05, ** = p<0.005 *** = p<0.0005 (students t-test). Data is represented as mean ± SEM.

**FIGURE 5:** Nnt deficient N27 cells had a significant increase in oxidation of Prx3 compared to mock controls. (A) A representative oxidized vs. reduced Western blot (B) Quantification of the redox blot for mitochondrial Prx3 from 5 separate samples. (C) A representative SDS-page blot for total Prx3 levels in mock and Nnt deficient cells. (D) Quantification from 4-5 separate samples normalized to β-actin levels for total levels of Prx3. * = p<0.05 as determined by students t-test. Graph is represented as mean ± SEM.

**FIGURE 6:** Summarized trace of decreased OCR in Nnt deficient cells compared to mock cells. Using a Seahorse 24XF analyzer, mitochondrial OCR was measured in Nnt deficient and mock control N27 cells under different respiratory parameters. Data is represented as mean ± SEM (n= 18-19)

**FIGURE 7:** Decreased mitochondrial membrane potential leading to increased susceptibility to PQ toxicity in Nnt deficient compared to mock control N27 cells. (A) Mitochondrial membrane potential was measured via TRME assay and there was a significant decrease in the membrane potential in the Nnt deficient compared to mock control N27 cells (n = 35-36). (B) Nnt deficient and mock control cells were exposed to various concentrations of PQ for 24 h and the amount of H$_2$O$_2$ produced was measured via Amplex Red assay. Nnt cells produced significantly more H$_2$O$_2$ alone and when exposed to various concentrations of PQ (n = 10). Indicated in (C), there was a significant shift in LDH released in Nnt deficient cells exposed to various concentrations of PQ compared to mock after 48 h exposure (n = 5-10). * = p<0.05; ** = p<0.01; *** = p<0.001 as determined by 2-way ANOVA. Data is represented as mean ± SEM.

**FIGURE 8:** A proposed model of Nnt’s activity in linking respiration-dependent H$_2$O$_2$ removal by the Trx/Prx antioxidant system in isolated brain mitochondria. When respiration substrates are present the TCA cycle and ETC can actively generate NADH and maintain the proton (H$^+$) gradient. Nnt will then utilize the H$^+$ gradient and NADH to create NADPH which will then be utilized by TrxR to keep the Trx/Prx antioxidant system in a reduced state to detoxify H$_2$O$_2$. Thus Nnt links the energy side of the schematic to the redox side.
TABLES

Table 1: Decreased H\textsubscript{2}O\textsubscript{2} removal rates in isolated brain mitochondria but not liver mitochondria with Nnt pharmacologically inhibited. H\textsubscript{2}O\textsubscript{2} removal/catabolism rates were determined with a H\textsubscript{2}O\textsubscript{2} sensitive Clark-type electrode. Isolated brain mitochondria required respiration substrates to consume H\textsubscript{2}O\textsubscript{2} while liver mitochondria did not. Addition of Palm CoA affected brain, but did not affect liver mitochondrial H\textsubscript{2}O\textsubscript{2} removal. Data is represented as mean ± SEM (n = 3-11) α = p<0.0001 vs. no substrates, β = p<0.0001 vs. malate + glutamate only as determined by 1way ANOVA.

| Substrate/Condition | Rate (nmol/min) | Substrate/Condition | Rate (nmol/min) |
|---------------------|-----------------|---------------------|-----------------|
| No Substrates       | 3.6 ± 0.6       | No Substrates       | 128 ± 4         |
| Malate/Glutamate    | 19.8 ± 0.9\textsuperscript{a} | Malate/Glutamate    | 121 ± 4         |
| 100µM Palmitoyl CoA | 2.3 ± 0.7       | 100µM Palmitoyl CoA | 124 ± 4         |
| Malate/Glutamate + 100µM Palmitoyl CoA | 2.6 ± 1.0\textsuperscript{b} | Malate/Glutamate + 100µM Palmitoyl CoA | 119 ± 4 |
Table 2: Summarized table of OCR parameters in Nnt deficient cells and mock controls. Nnt deficient cells had significant decreases in baseline OCR rates along with ATP turnover, reserve respiratory capacity and maximal respiratory capacity compared to mock controls (n = 18-19). * = p<0.05; *** = p<0.0001 as determined by students t-test. Data is represented as mean ± SEM.

| Parameter            | [OCR (pmol/min)/(mg/mL)] | Mock       | Nnt Deficient |
|----------------------|---------------------------|------------|---------------|
| Baseline             | 399 ± 22                  | 228 ± 22***|               |
| ATP Turnover         | 345 ± 17                  | 154 ± 17***|               |
| Proton Leak          | 52 ± 9                    | 75 ± 11    |               |
| Reserve Capacity     | 443 ± 20                  | 343 ± 37*  |               |
| Respiratory Capacity | 841 ± 36                  | 558 ± 56***|               |
Figure 1:

A

B

Trx Activity

C

TrxR Activity

Complex IV
Lamin B
Actin

-Cytosol- -Mitochondria-

No Substrate
100 μM Palm
Malate + Glutamate
100 μM Palm + M&G

Trx Activity per mg protein (Abs 412)

TrxR Activity per mg protein (Abs 413)

No Substrate
100 μM Palm
Malate + Glutamate
100 μM Palm + M&G

* ** ***
Figure 2

A  

**NNT Activity**

| [Palmitoyl CoA] (μM) | % Control |
|----------------------|-----------|
| 0                    | 100       |
| 100                  | 25        |

***

B  

**H₂O₂ Removal Rate**

| [Palmitoyl CoA] (μM) | H₂O₂ Removal Rate (nM/min) |
|----------------------|----------------------------|
| 0                    | 0.03                       |
| 100                  | 0.04                       |

*
Figure 3

A

**Nnt mRNA Levels**

| Relative Quantification | Mock | Nnt Deficient |
|-------------------------|------|--------------|
| 1.0                     |      |              |

B

**Nnt Activity**

| % Control | Mock  | Nnt Deficient |
|-----------|-------|---------------|
| 100       |       |               |
| 75        | ***   | ***           |
| 25        |       |               |
| 0         |       |               |
Figure 4

A) NADP⁺

B) NADPH

C) NADP⁺/NADPH Ratio

D) H₂O₂ Removal Rate

E) GSH

F) GSSG

G) Isocitrate Dehydrogenase
Figure 5:

A

B

Oxidized

Reduced

Mock  Nnt Deficient

Prx3 oxidation (% control)

Mock  Nnt Deficient

C

D

Prx3 Total Levels

Mock  Nnt Deficient

Prx3 (Arbitrary Units)

Mock  Nnt Deficient

Prx3

B-Actin

Mock  Nnt Deficient

Nnt links mitochondrial respiration to Trx/Prx $H_2O_2$ removal
Nnt links mitochondrial respiration to Trx/Prx H$_2$O$_2$ removal

Figure 6

Oxygen Consumption Rates

ROC (pMol/min)/protein(mg/ml)

Time (min)

Oligo  FCCP  Anti-A

Mock  NNT Deficient
Figure 7

A. Mitochondrial Membrane Potential

\[ \text{% Control} \]

Mock | Nnt Deficient

---

B. \( \text{H}_2\text{O}_2 \) Production

\[ \text{Fluorescence (RFU)} \]

Mock \( \bullet \)
Nnt Deficient \( \square \)

---

C. Cell Death

\[ \text{LDH Released (U/mg)} \]

Mock \( \bullet \)
Nnt Deficient \( \square \)
Nnt links mitochondrial respiration to Trx/Prx \( \text{H}_2\text{O}_2 \) removal
Nicotinamide nucleotide transhydrogenase (Nnt) links the substrate requirement in brain mitochondria for hydrogen peroxide removal to the thioredoxin/peroxiredoxin (Trx/Prx) system
Pamela Lopert and Manisha Patel

J. Biol. Chem. published online April 10, 2014

Access the most updated version of this article at doi: 10.1074/jbc.M113.533653

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts