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## Mitochondria-targeted antioxidants protect against Abeta toxicity in Alzheimer's disease neurons

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### Abstract

Alzheimer's disease (AD) is a progressive, neurodegenerative disease characterized by progressive decline of memory and cognitive functions. Despite tremendous progress that has been made in understanding disease progression and therapeutics of AD, we still do not have drugs that are capable of slowing its progression. The purpose of our study was to investigate the effects of the mitochondria-targeted antioxidants (MTAs) MitoQ and SS31, and the anti-aging agent resveratrol on neurons from a mouse model of Alzheimer's disease (AD) (Tg2576 line) and on mouse neuroblastoma (N2a) cells incubated with the amyloid beta (A $\beta$ ) peptide. Using electron and confocal microscopy, gene expression analysis, and biochemical methods, we studied mitochondrial structure and function, and neurite outgrowth in N2a cells treated with MitoQ, SS31, and resveratrol, and then incubated with A $\beta$ . In N2a cells only incubated with the A $\beta$ , we found increased expressions of mitochondrial fission genes and decreased expression of fusion genes, and also decreased expression of peroxiredoxins, endogenous cytoprotective antioxidant enzymes. Electron microscopy of the N2a cells incubated with A $\beta$  revealed a significantly increased number of mitochondria, indicating that A $\beta$  fragments mitochondria. Biochemical analysis revealed that function is defective in mitochondria. Neurite outgrowth was significantly decreased in A $\beta$ -incubated N2a cells, indicating that A $\beta$  affects neurite outgrowth. However, in N2a cells treated with MitoQ, SS31, and resveratrol, and then incubated with A $\beta$ , abnormal expression of peroxiredoxins and mitochondrial structural genes were prevented and mitochondrial function was normal; intact mitochondria were present and neurite outgrowth was significantly increased. In primary neurons from amyloid beta precursor protein (A $\beta$ PP) transgenic mice that were treated with MitoQ and SS31, neurite outgrowth was significantly increased and

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### CONFLICT OF INTEREST STATEMENT

Patent applications have been filed by the Cornell Research Foundation Inc (CRF) for the technology (SS peptides) described in this article. Hazel H Szeto is the inventor. CRF, on behalf of Cornell University, has licensed the technology for further research and development to a commercial enterprise in which CRF and Dr. Szeto have financial interests. Patents in the MitoQ technology are being commercialized by Antipodean Pharmaceuticals in which M. Murphy has a financial interest.

cyclophilin D expression was significantly decreased. These findings suggest that the MTAs, MitoQ and SS31 prevent A $\beta$  toxicity in mitochondria, which would warrant the study of MitoQ and SS31 as potential drugs to treat patients with AD.

### Keywords

Amyloid beta; Amyloid precursor protein 1; Cyclophilin D; Mitochondrial-targeted antioxidants; Peroxiredoxins

## INTRODUCTION

Alzheimer's disease (AD) is a late-onset neurodegenerative disorder that is characterized by the progressive decline of memory, cognitive functions, and changes in behavior and personality [1,2]. In addition to the presence of extracellular amyloid beta (A $\beta$ ) protein deposits and intracellular neurofibrillary tangles (3). AD is also associated with the loss of synapses, loss of synaptic function [4,5], mitochondrial structural and functional abnormalities [6–16], inflammatory responses [17,18], and neuronal loss [2,3].

In a previous study, to determine cellular changes associated with the mutant amyloid precursor protein (A $\beta$ PP) and A $\beta$  in AD progression, we conducted a global, time-course gene expression study using an AD transgenic mouse model (APP transgenic mice; Tg2576 mouse line) [7]. We found an up-regulation of mitochondrial genes in the 2-, 5-, and 18-month-old A $\beta$ PP mice, suggesting that mitochondrial function is impaired by mutant APP and A $\beta$  and that the up-regulation of mitochondrial genes may be a compensatory response to mutant A $\beta$ PP and mutant A $\beta$ . Further, recently, we [10] and several other researchers [19,20] have recently found A $\beta$  monomers and oligomers in mitochondria in the cortex and hippocampus of A $\beta$ PP transgenic mouse lines and mouse neuroblastoma (N2a) neurons expressing mutant APP [10], and postmortem AD brains. Further, using A $\beta$ PP transgenic mice [10,19,21], A $\beta$ PP/PS1 transgenic mice [22] and A $\beta$ PP/PS1/Tau mice [16,20] to study mitochondrial function, researchers have reported that mitochondrial A $\beta$  decreases cytochrome oxidase activity, and increases H<sub>2</sub>O<sub>2</sub> production and protein carbonyls [10,16,19,20]. These findings suggest that early interventions targeted at mitochondria may be effective in delaying AD development and slowing its progression.

Given the significant involvement of mitochondrial A $\beta$  in synaptic damage, a diet of antioxidants in AD patients may decrease mitochondrial toxicity and boost cognitive function. However, recent studies in which AD patients consumed antioxidants gave mixed results. Some studies found a reduced risk of AD in elderly persons treated with natural antioxidants, such as vitamins C and E [23–26]. Other studies found that a diet of antioxidants in elderly persons with and without AD did not delay AD onset [27–30]. One possible reason for these mixed findings is that vitamins C and E, which are naturally occurring antioxidants, might not cross the blood-brain barrier effectively and so cannot reach the brain. Therefore, they cannot reach neuronal mitochondria in order to decrease mitochondrial toxicity.

Recently, several MTAs – including MitoQ, MitoVitE, and cell-permeable, small peptide antioxidants – have been developed [31,32]. Reports suggest that these MTAs are capable of entering mitochondria: MitoQ reaches the mitochondrial matrix [31] and SS31 concentrates in the inner mitochondrial membrane [32] several hundred folds more than do natural antioxidants, and decrease mitochondrial toxicity [31,32].

Further, studies of SS31 treatment using experimental MPTP mice for Parkinson's disease found that SS31 decreased mitochondrial swelling and toxicity in these mice, and prevented dopaminergic cell death [33]. However, SS31 has not been tested in neurons and in AD transgenic mouse models. Recent studies have demonstrated that resveratrol, an anti-aging agent with anti-aging properties, reduces mitochondrial dysfunction, suggesting that resveratrol may have neuroprotection properties for AD [34,35].

In the study reported here, we sought to determine whether MitoQ and SS31 can prevent or reduce mitochondrial damage in N2a cells incubated with A $\beta$  peptide 25–35, and primary neurons in brains from APP mice. We also studied resveratrol to determine its efficacy in reducing mitochondrial dysfunction induced by A $\beta$  in N2a cells and in the primary neurons of APP mice and non-transgenic, wild-type mice [34,35]. We studied: 1) mRNA expression and protein levels of the neuroprotective genes PGC1 $\alpha$  and FOXO1, and the NMDA receptor; a family of cytoprotective, antioxidant enzyme proteins - peroxiredoxins 1–6, mitochondrial-encoded electron transport chain (ETC) genes, and mitochondrial structural genes; the fission genes Drp1 and Fis1 and the fusion genes Mfn1, Mfn2, Opa1, and Tomm40; and the mitochondrial matrix proteins HSP60, Bcl2, and CypD; 2) ultrastructural changes in N2a cells, including changes in the number of mitochondria and their morphology; 3) neurite outgrowth, 4) mitochondrial function (H<sub>2</sub>O<sub>2</sub> production, cytochrome oxidase activity, lipid peroxidation, ATP production, cell viability, and mitochondrial membrane potential), and 5) neurite outgrowth and CypD levels in primary neurons from APP mice and wild-type mice treated with MitoQ, SS31, and resveratrol.

## MATERIALS AND METHODS

### Tissue culture work

N2a cells (ATCC, Manassas, VA) were grown for 7 days in a serum-free medium (1:1 mixture of DMEM and OptiMEM, plus penicillin and streptomycin [Invitrogen, Carlsbad, CA]) or until the cells developed neuronal processes. The N2a cells that developed neuronal processes were used for 7 experimental treatments and one control: 1) N2a cells were incubated for 48 hrs with the A $\beta$  peptide 25–35 (in-frame and reverse 35-25 – 30  $\mu$ M); 2) N2a cells were treated with MitoQ for 48 hrs (0.1  $\mu$ M; the original MitoQ was dissolved in DMSO and further diluted in PBS); 3) N2a cells were treated with SS31 for 48 hrs (0.1 nM; the original SS31 was dissolved in DMSO and further diluted in PBS); 4) N2a cells were treated with resveratrol for 48 hrs (5  $\mu$ M dissolved in PBS; Sigma-Aldrich, St. Louis, MO); 5) N2a cells were treated with MitoQ (0.1  $\mu$ M) for 6 hrs and then incubated with A $\beta$  25–35 for 48 hrs, 6) N2a cells were treated with SS31 (1 nM) for 6 hrs and then incubated with A $\beta$  25–35 for 48 hrs, 7) N2a cells were treated with resveratrol (5  $\mu$ M) for 6 hrs and then incubated with A $\beta$  25–35 for 48 hrs, and 8) N2a cells were untreated and not incubated (the negative control). The cells from all the treatments were then harvested, and the cell pellet was collected and used for quantitative real-time RT-PCR; assays for ATP production, lipid peroxidation, and MTT determination. Isolated mitochondria from cells of all the treatments were used to determine cytochrome oxidase activity and H<sub>2</sub>O<sub>2</sub>.

### Quantification of mRNA expression of peroxiredoxins, ETC, and neuroprotective genes using real-time RT-PCR

Using the reagent TriZol (Invitrogen), we isolated total RNA from 3 independent treatments of N2a cells (n=3) from control (untreated N2a cells) and experimental treatments (N2a cells treated with A $\beta$ ; N2a cells treated with MitoQ, SS31, and resveratrol; N2a cells pretreated with MitoQ and then incubated with A $\beta$ ; N2a cells pretreated with SS31 and then incubated with A $\beta$ ; and N2a cells pretreated with resveratrol and then incubated with A $\beta$ ). Using primer express Software (Applied Biosystems), we designed the oligonucleotide primers for

the housekeeping genes  $\beta$ -actin; GAPDH; PGC1 $\alpha$  [36], FOXO1 [37] and NMDA receptor [38], a family of cytoprotective, antioxidant enzyme proteins (peroxiredoxins 1, 2, 3, 4, 5 and 6) [39,40], mitochondrially encoded ETC genes (Complex I – subunit 1 & 5, Complex III – CytB, Complex IV - Cox1, and Complex V – ATPase); mitochondrial structural genes; fission (Drp1 and Fis1); fusion genes (MFN1, MFN2, Opa1, Tomm40); and mitochondrial matrix proteins (HSP60, Bcl2 and CypD). The primer sequences and amplicon sizes are listed in Table 1. With SYBR-Green chemistry-based quantitative real-time RT-PCR, we measured mRNA expression of the genes mentioned above as described by Manczak et al. [41]. Briefly, 2  $\mu$ g of DNase-treated total RNA was used as starting material, to which we added 1  $\mu$ l of oligo (dT), 1  $\mu$ l of 10 mM dNTPs, 4  $\mu$ l of 5 $\times$  first strand buffer, 2  $\mu$ l of 0.1 M DTT, and 1  $\mu$ l RNase outout. The reagents RNA, dT), and dNTPs were mixed first, then heated at 65°C for 5 min, and finally chilled on ice until the remaining components were added. The samples were incubated at 42°C for 2 min, and then 1  $\mu$ l of Superscript II (40 U/ $\mu$ l) was added. The samples were then incubated at 42°C for 50 min, at which time the reaction was inactivated by heating at 70°C for 15 min.

Quantitative real-time PCR amplification reactions were performed in N2a cells in an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA) in a 25- $\mu$ l volume of total reaction mixture. The reaction mixture consisted of 1 $\times$  PCR buffer containing SYBR-Green; 3 mM MgCl<sub>2</sub>; 100 nm of each primer; 200 nm of dATP, dGTP, and dCTP each; 400 nm of dUTP; 0.01 U/ $\mu$ l of AmpErase UNG; and 0.05 U/ $\mu$ l of AmpliTaq Gold. A 20 ng cDNA template was added to each reaction mixture.

The C<sub>T</sub>-values of  $\beta$ -actin and the GAPDH were tested to determine the unregulated endogenous reference gene in N2a cells that were treated with A $\beta$  and protective molecules, and in untreated N2a cells. In the latter case, the C<sub>T</sub>-value was similar in untreated and N2a cells treated with MTAs and A $\beta$ . The C<sub>T</sub>-value is an important quantitative parameter in real-time PCR analysis as described in Manczak et al [41], and Gutala & Reddy [42]. All RT-PCR reactions were carried out in triplicate, with no template control. The PCR conditions were: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The fluorescent spectra were recorded during the elongation phase of each PCR cycle. To distinguish specific amplicons from non-specific amplifications, a dissociation curve was generated. The C<sub>T</sub>-values were calculated with sequence-detection system (SDS) software V1.7 (Applied Biosystems) and an automatic setting of base line, which was the average value of PCR, cycles 3–15, plus C<sub>T</sub> generated 10 times its standard deviation. The amplification plots and C<sub>T</sub>-values were exported from the exponential phase of PCR directly into a Microsoft Excel worksheet for further analysis.

The mRNA transcript level was normalized against  $\beta$ -actin and the GAPDH at each dilution. The standard curve was the normalized mRNA transcript level, plotted against the log-value of the input cDNA concentration at each dilution. To compare  $\beta$ -actin, GAPDH, and neuroprotective markers, relative quantification was performed according to the C<sub>T</sub> method (Applied Biosystems; [41,42]). Briefly, the comparative CT method involved averaging triplicate samples, which were taken as the C<sub>T</sub> values for  $\beta$ -actin, GAPDH, and neuroprotective markers.  $\beta$ -actin normalization was used in the present study because  $\beta$ -actin C<sub>T</sub> values were similar for the N2a cells treated with A $\beta$ , for the neuroprotective molecules, mitochondrial ETC genes, mitochondrial structural genes and for the untreated N2a cells. The  $\Delta$ C<sub>T</sub>-value was obtained by subtracting the average  $\beta$ -actin C<sub>T</sub> value from the average C<sub>T</sub>-value of for the neuroprotective genes, peroxiredoxins, mitochondrial ETC genes, mitochondrial structural genes. The  $\Delta$ C<sub>T</sub> of N2a cells was used as the calibrator. The fold change was calculated according to the formula  $2^{-(\Delta \Delta C_T)}$ , where  $\Delta \Delta C_T$  is the difference between  $\Delta C_T$  and the  $\Delta C_T$  calibrator value.

To determine the statistical significance of mRNA expression in untreated N2a cells and N2a cells treated with A $\beta$ , MitoQ, Resveratrol, SS31, and/or a combination of MitoQ+A $\beta$ , resveratrol+A $\beta$ , and SS31+A $\beta$ , the CT value difference between untreated N2a cells and treated N2a cells was used in relation to  $\beta$ -actin normalization, and statistical significance was calculated using one-way ANOVA.

### Immunoblotting analysis

To determine whether MitoQ, SS31, resveratrol, or A $\beta$  alters the protein levels of mitochondrial structural and neuroprotective genes that showed a decreased in mRNA expression, in our real-time RT-PCR, we performed immunoblotting analyses of protein lysates from N2a neurons of control and experimental treatments in independent N2a neurons treatments (n=3). Fifty  $\mu$ g protein lysates from control N2a neurons and N2a neurons that were treated with MitoQ, SS31, or resveratrol, or that were incubated with A $\beta$  were resolved on a 4–12% Nu-PAGE gel (Invitrogen). The resolved proteins were transferred to nylon membranes (Novax Inc., San Diego, CA) and then were incubated for 1 hr at room temperature with a blocking buffer (5% dry milk dissolved in a TBST buffer). The nylon membranes were incubated overnight with the primary antibodies Prx1 (1:200; rabbit polyclonal Abcam, Cambridge, MA), Prx2 (1:200, rabbit polyclonal Abcam), Prx3 (1:300; mouse-monoclonal Abcam), Prx4 (1:500, rabbit polyclonal), Prx5 (1:500; mouse-monoclonal Abcam), Prx6 (1:500, rabbit polyclonal Abcam, NMDAR (1:400 rabbit polyclonal Abcam), PGC1 $\alpha$  (1:500 rabbit polyclonal Abcam, FOXO1 (1:1000 rabbit polyclonal Abcam), Drp1 (1:200 rabbit polyclonal Novus Biological. Inc, Littleton, CO), Fis1 (1:200, Protein Tech Group Inc., Chicago, IL), Mfn1 (1:300, rabbit polyclonal Santa Cruz Biotechnology Inc. Santa Cruz, CA, Mfn2 (1:300, rabbit polyclonal Abcam), Opa1 (1:500; mouse-monoclonal BD Biosciences, San Jose, CA), and  $\beta$ -actin (1:500; mouse-monoclonal Chemicon-Millipore, Temecula, CA).

The membranes were washed with a TBST buffer 3 times at 10-min intervals and then incubated for 2 hr with appropriate secondary antibodies, followed by 3 additional washes at 10-min intervals. Microglial proteins were detected with chemiluminescent reagents (Pierce Biotechnology, Rockford, IL), and the bands from immunoblots were quantified on a Kodak Scanner (ID Image Analysis Software, Kodak Digital Science, Kennesaw, GA). Briefly, image analysis was used to analyze gel images captured with a Kodak Digital Science CD camera. The lanes were marked to define the positions and specific regions of the bands. An ID fine-band command was used to locate and scan the bands in each lane and to record the readings.

### H<sub>2</sub>O<sub>2</sub> production

Using an Amplex® Red H<sub>2</sub>O<sub>2</sub> Assay Kit (Molecular Probes, Eugene, OR), we measured the production of H<sub>2</sub>O<sub>2</sub> in independent experiments (n=4) of N2a cells treated with MitoQ and SS31, and then incubated with A $\beta$ , as described in Manczak et al. [10]. Briefly, the production of H<sub>2</sub>O<sub>2</sub> was measured in the mitochondria isolated from N2a cells, from the control and the experimental treatments. A BCA Protein Assay Kit (Pierce Biotechnology) was used to estimate protein concentration. The reaction mixture contained mitochondrial proteins ( $\mu$ g/ $\mu$ l), Amplex Red reagents (50  $\mu$ M), horseradish peroxidase (0.1 U/ml), and a reaction buffer (1X). The mixture was incubated at room temperature for 30 min, followed by spectrophotometer readings of fluorescence (570 nm). Finally, H<sub>2</sub>O<sub>2</sub> production was determined, using a standard curve equation expressed in nmol/ $\mu$ g mitochondrial protein.

### Cytochrome oxidase activity

Cytochrome oxidase activity was measured in the mitochondria isolated from N2a cells of control and experimental treatments (n=4), as described in Manczak et al. [10]. Enzyme

activity was assayed spectrophotometrically using a Sigma Kit (Sigma–Aldrich) following manufacturer’s instructions. Briefly, 2 µg mitochondrial protein was added to 1.1 ml of a reaction solution containing 50 µl 0.22 mM ferricytochrome *c* fully reduced by sodium hydrosulphide, Tris–HCl (pH 7.0), and 120 mM potassium chloride. The decrease in absorbance at 550 nm was recorded for 1-min reactions at 10-sec intervals. Cytochrome *c* oxidase activity was measured according to the following formula: mU/mg total mitochondrial protein = (A/min sample – A/min blank) × 1.1 mg protein × 21.84). The protein concentrations were determined following the BCA method.

### ATP levels

ATP levels were measured in mitochondria isolated from N2a cells of control and experimental treatments (n=4) using the ATP determination kit (Molecular Probes) [43]. The bioluminescence assay is based on the reaction of ATP with recombinant firefly luciferase and its substrate luciferin. Luciferase catalyzes the formation of light from ATP and luciferin. It is the emitted light that is linearly related to the ATP concentration, which was measured using a luminometer. We measured ATP from mitochondrial pellets using a standard curve method.

### Lipid peroxidation assay

Lipid peroxidates are unstable indicators of oxidative stress in neurons [44]. 4-hydroxy-2-nonenol (HNE) is the final product of lipid peroxidation that was measured in the cell lysates from N2a cells of control and experimental treatments (n=4), using an HNE-His ELISA Kit (Cell BioLabs, Inc., San Diego, CA). Briefly, freshly prepared protein was added to a 96-well protein binding plate and incubated overnight at 4°C. It was then washed 3 times with wash buffer. After the last wash, the anti-HNE-His antibody was added to the wells and incubated for 2 hr at room temperature, and then washed the wells 3 times. Next, the samples were incubated with a secondary antibody conjugated with peroxidase for 2 hr at room temperature, followed by an incubation with enzyme substrate. Optical density was measured to quantify the level of HNE.

### Cell viability test (MTT assay)

Mitochondrial respiration, an indicator of cell viability, was assessed in the N2a cells from control and experimental treatments (n=4), using the mitochondrial-dependent reduction of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan [45]. Briefly, in this reduction, N2a cells were seeded in 12-well plates at a density of ~10<sup>5</sup> neurons per well. After treatments, MTT (5 mg/ml in PBS) was added to the plates, and the cells (control and experimental) were incubated for 3 hrs. The medium was then replaced with an SDS/DMSO lysis buffer, and MTT absorption was measured at 570 nm. Results were expressed as the percentage of MTT reduction, assuming that the absorbance of the control N2a cells was 100%.

### Mitochondrial membrane potential

To determine the effect of Aβ on the mitochondrial membrane potential, the potential was measured in N2a cells from control and experimental treatments (n=4) using live-cell imaging and JC-1 (fluorochrome (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) staining, as described in Wagner et al. [46]. In JC-1 staining, the JC1 dye accumulates in the mitochondria of healthy cells as aggregates and are fluorescent red. When cells are under stress or exposed to toxic chemicals, such as Aβ, the mitochondrial potential collapses, and the JC1 dye can no longer accumulate in the mitochondria. Consequently, the JC1 aggregates remain in the cytoplasm, in a monomeric form, which is green. In this experiment, the N2a cells were stained for 10 min at 37°C, until

a final concentration of 5  $\mu\text{g/ml}$  JC1 was reached. The cells were then washed 3 times with PBS and analyzed for mitochondrial membrane potential by measuring the red:green ratio with confocal microscopy.

### Electron microscopy

To determine the effects of  $\text{A}\beta$  on the number and morphology of mitochondria and the rescue effects of MitoQ, SS31, and resveratrol on mitochondria, we conducted transmission electron microscopy (TEM) of N2a cells from control and experimental treatments ( $n=4$ ). Treated and untreated N2a cells were fixed in 100 mM sodium cacodylate (pH 7.2), 2.5% glutaraldehyde, 1.6% paraformaldehyde, 0.064% picric acid, and 0.1% ruthenium red. They were gently washed and post-fixed for 1 hr in 1% osmium tetroxide plus 0.8% potassium ferricyanide, in 100 mM sodium cacodylate, pH 7.2. After a thorough rinsing in water, the N2a cells were dehydrated, infiltrated overnight in 1:1 acetone:Epon 812, infiltrated for 1 hr with 100% Epon 812 resin, and embedded in the resin. After polymerization, 60- to 80-nm thin sections were cut on a Reichert ultramicrotome and stained for 5 min in lead citrate. They were then rinsed and post-stained for 30 min in uranyl acetate, and then rinsed again and dried. EM was performed at 60 kV on a Philips Morgagne TEM, equipped with a CCD, and images were collected at original magnifications of 1,000–37,000x. The numbers of mitochondria were counted in the control N2a cells (number of cells = 50) and experimental groups (number of cells per group=40), and statistical significance was determined, using one-way ANOVA.

### Immunofluorescence analysis and quantification of neurite outgrowth

To study neurite outgrowth, N2a cells were grown in a serum-free medium for 7 days or until the neurons developed neuronal processes on gridded microscopic slides (BD Biosciences). We performed immunofluorescence analysis using the Drp1 antibody (a synaptic branching protein) in control N2a cells and N2a cells from the 7 experimental treatments.

N2a cells were washed with warm PBS, fixed in freshly prepared 4% paraformaldehyde in PBS for 10 min, and then washed with PBS and permeabilized with 0.1% Triton-X100 in PBS. They were blocked with 1% blocking solution (Invitrogen) for 1 hr at room temperature. All neurons were incubated overnight with a rabbit polyclonal anti-Drp1 primary antibody (Novus Biologicals) at a concentration of 1:400, at 4°C. After being incubated with the primary antibody, the cells were washed 3 times with PBS, for 10 min each. The neurons were incubated with a secondary antibody conjugated with Fluor 488 (Invitrogen) for 1 hr at room temperature. The cells were washed neurons 3 times with PBS, and slides were mounted. Photographs were taken with a multiphoton laser scanning microscope system (ZeissMeta LSM510).

To quantify the immunoreactivity of the Drp1 antibody, for each treatment 10–15 photographs were taken at  $\times 20$  magnification. For the measurement of neurite outgrowth, the immunoreactivity of Drp1 and neurite length were quantified using the ‘neurite tracer’ method published by Pool et al. [47]. The signal intensity indicating the immunoreactivity of the cell body and neurite length was quantified for several randomly selected images, and statistical significance was assessed, using one-way ANOVA for Drp1 in N2a cells from control N2a cells versus N2a cells treated with MitoQ, SS31, and resveratrol, and then incubated with  $\text{A}\beta$ .

### $\text{A}\beta$ PP mice and primary neuronal culture

The  $\text{A}\beta$ PP transgenic mice (Tg2576 line) and age-matched wild-type littermates were housed at the Oregon National Primate Research Center of Oregon Health & Science

University (OHSU). The mice were a gift from Dr Karen Ashe, University of Minnesota [48]. The OHSU Institutional Animal Care and Use Committee approved all procedures for animal care according to guidelines set forth by the National Institutes of Health.

Primary neuronal cultures of APP mice and wild-type mice were prepared using methods described by Brewer and Torricelli [49], with slight modifications. Briefly, mice postnatal day 1 were decapitated and the brains removed into a room-temperature Hibernate®-A medium (Brain Bits, Springfield IL) supplemented with B-27 (Invitrogen) and 0.5mM GlutaMAX™ (Invitrogen). The hippocampus was then dissected and reserved for culturing, and the cerebellum was removed for genotyping. Hippocampus pairs from individual mice were minced into pieces less than 1 mm<sup>3</sup> and transferred to a solution of papain (2 mg/ml; Worthington Biochemical Corp, Lakewood, NJ) that was dissolved in Hibernate®-A without calcium, but supplemented with 0.5mM GlutaMAX™. The tissue was digested for 30 min in a shaking water bath at 30°C. Digested tissue was then removed to 2 ml HABG and triturated 10 times with a fire-polished, siliconized (Sigmacote; Sigma, St. Louis MO), 9" glass pipette. Samples were allowed to settle by gravity for about 1 min. Then the supernatant containing dissociated neurons was removed to a fresh tube. An additional 2 ml HABG was added to the pellet, and the process was repeated until 6 ml dissociated neurons were collected. Neurons were centrifuged at 200×g for 2 min and then washed with 2 ml HABG. The pellets were resuspended in 2 ml Neurobasal™ (Invitrogen) supplemented with B-27 and 0.5 mM GlutaMAX™ (growth medium). The neurons were counted, plated at 500 neurons/mm<sup>2</sup> on poly-D-lysine-coated coverslips, and placed into a 37°C incubator at 5% CO<sub>2</sub>. One hour after plating, the growth medium was completely replaced. After 3 days *in vitro* and every 2 days thereafter for 10 days, one-half of the growth medium was changed. The *in vitro* hippocampal neurons were treated with MitoQ (20 nM), SS31 (1 nM), and resveratrol (5 μM), and were used for experiments.

### **Treatment with mitochondrial-targeted antioxidants and quantification of neurite outgrowth**

One group of postnatal day 1 hippocampal neurons from AβPP transgenic mice and wild-type mice remained untreated, and one group was treated with MitoQ, SS31, and resveratrol for 48 hrs. All neurons were washed with warm PBS, then fixed in a freshly prepared 4% paraformaldehyde for 10–15 min, and washed with PBS and permeabilized with 0.1% Triton-X100 in PBS. All incubations were performed in a humidified environment. All neurons were blocked with 1% blocking solution (Invitrogen) for 1 hr at room temperature. Primary antibodies were diluted in the blocking solution and used under the following 2 conditions: CypD mouse monoclonal (EMD, Gibbstown, NJ), 1:200, room temperature, 1 hr; and Drp1 rabbit polyclonal (Novus Biologicals, Littleton, CO), 1:500, 4°C, overnight. After being incubated with an appropriate primary antibody, the neurons were washed 3 times for 10 min with PBS. A fluorophore-labeled appropriate secondary antibody was then applied for 1 hr at room temperature. The secondary antibody (1:500 in blocking solution) was selected from Alexa Fluor® 488 goat anti-rabbit or Alexa Fluor® 568 goat anti-mouse antibodies (Invitrogen) as appropriate. For double-labeling studies, antigens were labeled sequentially, meaning that the primary antibody was applied singly and then the appropriate secondary antibody was applied. Then the second primary antibody was applied, followed by the appropriate secondary antibody.

Ten to 15 pictures of hippocampal neurons from AβPP transgenic mice and wild-type mice treated with MitoQ, SS31, and resveratrol each were taken at 20× the original magnification. For the measurement of neurite outgrowth (Drp1) and CypD expression, the immunoreactivities of Drp1 and CypD were quantified using the method published by Pool et al. [47].

## Statistical considerations

Statistical analyses were conducted for mitochondrial structural and functional parameters in N2a cells from control and experimental treatments using one-way ANOVA with Dunnett correction. The parameters included H<sub>2</sub>O<sub>2</sub>, cytochrome oxidase activity, lipid peroxidation, ATP production, cell viability, mitochondrial membrane potential, Drp1 levels (neurite outgrowth), and electron microscopy values. To determine the effect of MitoQ, SS31 and resveratrol in the absence and presence of A $\beta$  we analyzed/compared the data 2 ways: 1. untreated N2a cells (control) versus N2a cells treated with MitoQ, SS31, resveratrol and incubated with A $\beta$ ; 2. N2a cells incubated with A $\beta$  versus N2a cells pretreated with MitoQ and then incubated with A $\beta$ , N2a cells pretreated with SS31 and then incubated with A $\beta$  and N2a cells pretreated with resveratrol and then incubated with A $\beta$ .

We also assessed the statistical significance for Drp1 and CypD immunoreactivities in primary neurons from APP mice and wild-type mice between untreated primary neurons and primary neurons treated with MitoQ, untreated primary neurons and primary neurons treated with SS31, and untreated primary neurons and primary neurons treated with resveratrol using one-way ANOVA.

## RESULTS

### mRNA expression of mitochondrial structural genes

We determined the effects of A $\beta$  on mitochondrial structural genes and the protective effects of MTAs on mRNA expression of the mitochondrial genes Drp1 and Fis1 (fission); Mfn1, Mfn2, Opa1 and Tomm40 (fusion); and CypD, Hsp60, and Bcl2 (matrix genes) (Table 2A). We analyzed the data 2 ways: 1) to determine the effect of A $\beta$ , MitoQ, SS31, and resveratrol (Table 2A) in N2a cells, and 2) to determine the preventive effects of MitoQ, SS31 and resveratrol, if any, in the presence of A $\beta$  (Table 2B).

In N2a cells treated with A $\beta$  compared to untreated N2a cells, mRNA expression levels significantly increased in Drp1, by 1.7 fold (P<0.05); and in Fis1, by 3.4 fold (P<0.05). In contrast, the levels of mRNA expression of mitochondrial fusion genes were significantly decreased (Table 2A), indicating that the presence of abnormal mitochondrial dynamics in N2a cells treated with A $\beta$ . Interestingly, mRNA expression of matrix genes were significantly increased (CypD by a 2.2-fold, P<0.005; Bcl2 by a 5.1-fold, P<0.005; and HSP by a 5.7 fold, P<0.05) in N2a cells treated with A $\beta$ , indicating that mitochondrial matrix genes were activated.

mRNA levels were significantly decreased for Drp1 (1.2 fold decrease, P<0.05 in MitoQ treated cells; 1.2 fold decrease, P<0.05 in SS31; 1.4 fold decrease for resveratrol treated cells) and Fis1 (1.4 fold decrease, P<0.05 in MitoQ; 2.7 fold decrease, P<0.05 in SS31 and 2.9 fold in resveratrol treated) in cells pretreated with MitoQ, SS31, and resveratrol and then treated with A $\beta$  compared with cells treated with A $\beta$  alone (Table 2B). These observations indicate that MTAs and resveratrol prevent abnormal fission and fusion gene expressions.

### mRNA expression of ETC genes

To determine the effects of A $\beta$ , MitoQ, SS31, and resveratrol on mitochondrial oxidative phosphorylation, mRNA expression levels of ETC genes were measured. Significantly increased mRNA expressions were found in all ETC genes except NADH subunit3 studied in the N2a cells treated with A $\beta$  (Table 2A), indicating a compensatory response by mitochondrial-encoded genes. Interestingly, in N2a cells treated with SS31 relative to untreated N2a cells, significantly increased levels of mRNA expression were found in ETC genes (NADH subunit1 increased by 7.4 fold, P<0.005; subunit3, by 8.2 fold, P<0.005;

subunit5, by 6.6 fold,  $P<0.005$ ; CytB increased by 9.5 fold,  $P<0.005$ ; COX1 increased by 9.4 fold,  $P<0.005$ ; COX3, by 8.9 fold,  $P<0.005$ ; and ATPase6 increased by 8.2 fold ( $P<0.005$ ) (Table 2A). MitoQ and resveratrol treated N2a cells showed increased of ETC genes but mRNA levels were not significant for all genes studied (Table 2A).

As shown Table 2B, mRNA expression levels were increased for ETC all genes but not significant in N2a cells pretreated with MitoQ, SS31 and incubated with A $\beta$  compared to N2a cells treated with A $\beta$  alone. Overall, these findings suggest that SS31 enhance mitochondrial gene expressions in the presence and absence of A $\beta$ .

### mRNA expression of peroxiredoxins

To determine the effects of A $\beta$ , MitoQ, SS31 and resveratrol in the antioxidant enzymes peroxiredoxins (Prxs 1–6), mRNA expression levels of Prxs were measured. As shown in Table 2A, levels of mRNA expression were found significantly decreased in all Prxs (1–6) in N2a cells treated with A $\beta$ . A 5-fold decrease was found for Prx1 ( $P<0.005$ ); for Prx6, a 4.5-fold decrease ( $P<0.005$ ); for Prx5, a 2.8-fold decrease ( $P<0.005$ ); for Prx2, a 2.3-fold decrease ( $P<0.005$ ); for Prx4, a 1.7-fold decrease ( $P<0.05$ ); and for Prx3, a 1.3-fold decrease ( $P<0.05$ ). Interestingly, Prxs were significantly increased in N2a cells treated with SS31 (a 4.5-fold increase for Prx3 with  $P<0.005$ ; a 3.4-fold increase for Prx4 with  $P<0.005$ ; a 2.6-fold increase for Prx6 with  $P<0.005$ ), resveratrol (a 6-fold increase for Prx6 with  $P<0.005$ ; a 5.5-fold increase for Prx3 with  $P<0.005$ ) and MitoQ (a 4.4-fold increase for Prx6 with  $P<0.005$ ).

As shown in Table 2B, the levels of mRNA expression of Prxs were significantly increased in N2a cells pretreated with SS31, MitoQ and resveratrol and then incubated with A $\beta$  compared to cells treated with A $\beta$  alone, indicating that MTAs and resveratrol prevent the decreased mRNA expressions of antioxidant enzymes (Prxs) caused by A $\beta$ .

### mRNA expression in neuroprotective genes

mRNA expressions of neuroprotective genes PGC1 $\alpha$  and FOXO1, and the NMDA receptor (NMDAR) were measured in N2a cells treated with A $\beta$ , MitoQ, SS31 and resveratrol (Table 2A). The levels of mRNA expression were significantly increased for PGC1 $\alpha$  (2.4 fold with  $P<0.05$ ); for FOXO1 (7.5 fold with  $P<0.05$ ); and for NMDAR (3.1 fold with  $P<0.05$ ) in N2a cells treated with A $\beta$ . Interestingly, neuroprotective genes were significantly increased in N2a cells treated with SS31 (a 14.9-fold increase for FOXO1 with  $P<0.005$ ; a 8.8 fold increase for NMDAR with  $P<0.005$ ; a 2.5 fold increase for PGC1 $\alpha$  with  $P<0.05$ ), MitoQ (a 8.2-fold increase for NMDAR with  $P<0.005$ ; a 4.8-fold increase for PGC1 $\alpha$  with  $P<0.005$ ; a 3.1-fold increase for FOXO1 and resveratrol (a 9.4-fold increase for NMDAR with  $P<0.005$ ; a 3.7-fold increase for FOXO1 (Table 2A). These increased expressions of neuroprotective genes, indicate MitoQ, SS31 and resveratrol are protective to N2a cells.

The levels of mRNA expression of NMDAR, FOXO1 and PGC1 $\alpha$  were significantly increased in N2a cells pretreated with SS31, MitoQ and then incubated with A $\beta$  compared to N2a cells treated with A $\beta$  alone (Table 2B) but not for resveratrol pretreated cells, indicating that MTAs enhance mRNA expressions of neuroprotective genes in the presence of A $\beta$ .

### Immunoblotting analysis

**Peroxiredoxins**—In N2a cells treated with A $\beta$ , protein levels were significantly decreased for Prx1 ( $P<0.004$ ), Prx2 ( $P<0.04$ ), Prx3 ( $P<0.001$ ) (Figure 1A – western blot, and Figure 2A - quantitative densitometry values). These findings agreed with our real-time RT-PCR data. However, protein levels were significantly increased for Prx1 ( $P<0.03$ ), Prx3 ( $P<0.02$ ), Prx4 ( $P<0.01$ ) and Prx6 ( $P<0.01$ ) for SS31 treated cells; followed by significantly increased

for Prx1 ( $P<0.001$ ), Prx4 ( $P<0.01$ ) and Prx6 ( $P<0.01$ ) in MitoQ treated cells. However, in resveratrol treated cells, significantly increased protein levels were found only Prx1. These observations indicate that SS31 increased peroxiredoxins at RNA and protein levels.

The protein levels of Prxs were significantly increased in N2a cells pretreated with SS31, MitoQ and then incubated with A $\beta$  compared to cells treated with A $\beta$  alone (Figure 1A – western blot, and Figure 2A -quantitative densitometry values), indicating that MTAs enhance protein levels of Prxs in the presence of A $\beta$ . No significant changes were found in cells pretreated with resveratrol and incubated with A $\beta$ .

**Mitochondrial structural proteins**—To determine the effects of A $\beta$  on mitochondrial structural proteins and the useful effects of MitoQ, SS31 and resveratrol at protein level, we quantified mitochondrial proteins in 3 independent treatments of N2a cells with A $\beta$ , MitoQ, SS31 and resveratrol. In N2a cells treated with A $\beta$  compared to untreated N2a cells, significantly increased proteins for Drp1 ( $P<0.02$ ) and Fis1 ( $P<0.001$ ) (Figure 1B – western blot, and Figure 2B - quantitative densitometry values). In contrast, we found decreased levels of mitochondrial fusion proteins, Mfn1 ( $P<0.02$ ) Mfn2 ( $P<0.004$ ) and Opa1 ( $P<0.04$ ) (Figure 1B – western blot, and Figure 2B quantitative densitometry values). in N2a cells incubated with A $\beta$  compared to compared untreated cells, indicating the presence of abnormal mitochondrial dynamics even at protein level in N2a cells treated with A $\beta$ .

**Neuroprotective proteins**—Significantly increased levels of proteins in NMDAR ( $P<0.01$ ) and FOXO1 ( $P<0.01$ ) were found in N2a cells treated with A $\beta$ . However, in N2a cells treated with MitoQ and SS31, protein levels significantly increased for NMDAR (MitoQ,  $P<0.04$ ; SS31,  $P<0.01$ ), FOXO1 (MitoQ,  $P<0.03$ ; SS31,  $P<0.001$ ) and PGC1 $\alpha$  (MitoQ,  $P<0.02$ ; SS31,  $P<0.03$ ). However, in those cells treated with only resveratrol, protein levels did not increase (Figure 1C – western blot and Figure 2C - quantitative densitometry values).

### Electron microscopy

To determine the effects of A $\beta$  on mitochondrial morphology and number, N2a cells were incubated with A $\beta$ , and the number and morphology of mitochondria were studied using electron microscopy (EM). The number of mitochondria significantly increased ( $P<0.05$ ) (Figure 3C, image a, and Figure 4, image b) in A $\beta$ -incubated N2a cells compared to N2a cells not incubated with A $\beta$ . Most mitochondria were swollen, most of the inner mitochondrial membrane and cristae were not visible, and those that were visible were damaged (Figure 4A, image b). Mitochondrial fragmentation was evident in most of the A $\beta$ -treated N2a cells (Figure 4, image b).

In the untreated N2a cells, elongated mitochondria were found in the neuronal processes, and even at the terminals (Figure 5, image a) of the N2a cells, indicating that mitochondria travel along neuronal processes.

Intact mitochondria were found in the neuronal processes of N2a neurons treated with MitoQ (Figure 5, image b) and SS31 (Figure 5, image c) but not then incubated with A $\beta$ , indicating that mitochondrial trafficking is not affected in MitoQ- and SS31-treated N2a cells. However, in N2a cells treated with MitoQ, SS31 and resveratrol, and then incubated with A $\beta$ , mitochondrial swelling and fragmentation was not found, indicating protective effects of MitoQ, SS31, and resveratrol.

## Neurite outgrowth

Neurite outgrowth was measured in N2a cells incubated with A $\beta$  to determine whether A $\beta$  affects neurite length. We found neurite growth significantly decreased (marked by immunoreactivity of Drp1) in the N2a cells incubated with A $\beta$  compared to the N2a cells not incubated with A $\beta$  ( $P<0.01$ ) (Figure 6B, image a – quantitative data; and Figure 6A, image b – cells), indicating that A $\beta$  does affect neurite length. Significantly increased neurite outgrowth was found in N2a cells treated with MitoQ ( $P<0.01$ ) and SS31 ( $P<0.03$ ) (Figure 6B, image a – quantitative data) compared to N2a cells not treated with MitoQ or SS31 and also not incubated with A $\beta$ . However, neurite outgrowth did not significantly change in the resveratrol-treated N2a cells that were then incubated with A $\beta$ .

Significant neurite outgrowth was found in N2a cells treated MitoQ and then incubated with A $\beta$  ( $P<0.01$ ; Figure 6B, image b – quantitative data), and in N2a cells treated with SS31 and then incubated with A $\beta$  ( $P<0.03$ ; Figure 6B, image b – quantitative data), compared to the N2a cells that were only incubated with A $\beta$ , indicating that MitoQ and SS31 prevent neurite outgrowth from A $\beta$  toxicity.

## Mitochondrial dysfunction

To determine whether A $\beta$  affects mitochondrial function and the preventive effects of MitoQ and SS31, parameters of mitochondrial function were studied in N2a cells ( $n=4$ ). The parameters included H<sub>2</sub>O<sub>2</sub> production, cytochrome oxidase activity, lipid peroxidation, ATP production, cell viability, and mitochondrial membrane potential.

**H<sub>2</sub>O<sub>2</sub> production**—Significantly increased levels of H<sub>2</sub>O<sub>2</sub> were found in mitochondria from N2a cells incubated with A $\beta$  ( $P<0.002$ ) but no changes in H<sub>2</sub>O<sub>2</sub> were found in N2a cells incubated with A $\beta$  35-25 (data not shown). In measurements taken of H<sub>2</sub>O<sub>2</sub> from mitochondria isolated from cells treated with SS31, MitoQ, and resveratrol, but not incubated with A $\beta$ , significantly decreased levels of H<sub>2</sub>O<sub>2</sub> were found in N2a cells treated with MitoQ ( $P<0.01$ ) and SS31 ( $P<0.04$ ) (Figure 7A, image a). These findings suggest that antioxidants that are targeted to mitochondria decrease H<sub>2</sub>O<sub>2</sub> more effectively than do antioxidants that are not targeted to mitochondria, such as resveratrol. Significantly decreased levels of H<sub>2</sub>O<sub>2</sub> were found in the N2a cells treated with MitoQ and then incubated with A $\beta$  ( $P<0.01$ ; Figure 7A, image b).

**Cytochrome oxidase activity**—Significantly decreased levels of cytochrome oxidase activity were found in N2a cells that were incubated with A $\beta$  ( $P<0.001$ ; Figure 7B, image a). However, significantly increased levels were found in the MitoQ-treated N2a cells ( $P<0.04$ ; Figure 7B, image a) and in the SS31-treated N2a cells ( $P<0.02$ ) that were not then incubated with A $\beta$ , whereas resveratrol-treated cells that were not then incubated with A $\beta$  exhibited significantly decreased cytochrome oxidase activity ( $P<0.02$ ). As shown in Figure 7B, image b, significantly increased activity was found in the N2a cells treated with MitoQ and incubated with A $\beta$  ( $P<0.03$ ) and in those treated with SS31 and then incubated with A $\beta$  ( $P<0.02$ ), compared cells incubated with A $\beta$  alone, indicating that MitoQ and SS31 prevent toxic effects of A $\beta$  on cytochrome oxidase activity.

**ATP production**—Significantly decreased levels of ATP were found in N2a cells that were incubated with A $\beta$  ( $P<0.0005$ ; Figure 7C, image a) but were not first treated with MitoQ, SS31, or resveratrol. As shown in Figure 7C, image a, significantly increased levels of ATP were found in N2a cells treated with MitoQ ( $P<0.01$ ) and SS31 ( $P<0.02$ ) but not then incubated with A $\beta$ , compared to untreated N2a cells. Increased ATP levels were found at significant levels in the N2a cells treated MitoQ, SS31, and resveratrol, and then

incubated with A $\beta$  (P<0.02, P<0.04, and P<0.04, respectively), indicating that MitoQ, SS31, and resveratrol enhance ATP levels in the presence of A $\beta$  (Figure 7C, image b).

**Lipid peroxidation**—To determine whether A $\beta$  affects lipid peroxidation in N2a cells that underwent A $\beta$  incubation, we measured 4-hydroxy-2-nonenol, an indicator of lipid peroxidation. Increased levels of lipid peroxidation were found, but not in significant amounts (P=0.54; Figure 7D, image a). However, significantly decreased levels were found in the resveratrol-treated N2a cells (P<0.001) relative to cells that were not incubated with A $\beta$ , and in those treated with resveratrol and then incubated with A $\beta$  (P<0.002). Also, significantly decreased levels were found in N2a cells that were treated with MitoQ and then incubated with A $\beta$  (P<0.005; Figure 7D, image b).

**Cell viability**—Significantly decreased levels of cell viability were found in A $\beta$ -treated N2a cells (P<0.001; Figure 7E, image a). Cell viability was also significantly increased in N2a cells treated with MitoQ and SS31 and then incubated with A $\beta$ , compared to N2a cells only incubated with A $\beta$ , indicating that MitoQ and SS31 prevent a decrease in cell viability that A $\beta$  causes.

**Mitochondrial membrane potential**—Significantly decreased mitochondrial membrane potential was found in N2a cells that underwent incubation with A $\beta$  (P<0.001; Figure 8B, image a). Increased levels of the mitochondrial membrane potential were found in the N2a cells treated with SS31 (P<0.002; Figure 8B, image a) but not then incubated with A $\beta$ . In N2a cells treated with MitoQ, SS31, and resveratrol, and then incubated with A $\beta$ , the membrane potential significantly increased in all 3 treatments (MitoQ+A $\beta$ , P<0.04; SS31+A $\beta$ -P<0.02; and resveratrol+A $\beta$ , P<0.001; Figure 8B, image b), compared to N2a cells only incubated with A $\beta$ . These results indicate that MitoQ, SS31, and resveratrol rescued membrane potential from A $\beta$  toxicity.

### **A $\beta$ PP transgenic mice primary neurons and mitochondrial-targeted antioxidants treatments**

Using Drp1 immunostaining, neurite outgrowth was measured in untreated primary neurons from A $\beta$ PP transgenic mice and non-transgenic littermates and in those treated with MitoQ, SS31 and resveratrol. Significantly increased immunoreactivity of Drp1 was found in cultured hippocampal neurons from A $\beta$ PP transgenic mice treated with SS31 (P<0.044; Figure 9C-image a) and MitoQ (P<0.024; Figure 9C-image a), but not in those treated with resveratrol (P=0.1). Enhanced synaptic branching was found in hippocampal neurons treated with MitoQ (Figure 9A-image c) and SS31 (Figure 9A-image d) compared to untreated primary neurons, indicating that MitoQ and SS31 are capable of increasing synaptic connectivity in AD neurons. In the hippocampal neurons from wild-type mice, immunoreactivity of Drp1 was significantly increased in the neurons treated with SS31 (P<0.03) (Figure 9C-image b).

In the hippocampal neurons from the APP mice, immunoreactivity of CypD was found significantly decreased in the neurons treated with SS31 (P<0.0004; Figure 9D-image a), MitoQ (P<0.006; Figure 9D-image a), and resveratrol (P<0.013; Figure 9D-image a).

Our double labeling analysis of Drp1 and CypD in primary neurons concur with Drp1 and CypD quantification data (Figure 10). Overall, these findings indicate that MTAs can decrease CypD levels, inhibit mitochondrial pore openings, and protect mitochondria from A $\beta$ PP transgenic mice carrying mutant APP and A $\beta$ .

## DISCUSSION

We investigated the toxicity of the A $\beta$  peptide and the protective effects of the MTAs, MitoQ, and SS31, and resveratrol on mitochondrial structure and function, and neurite outgrowth in AD neurons. Our study concludes that A $\beta$  damages mitochondrial structural and function in cells, and MTAs can prevent this damage caused by A $\beta$ .

### mRNA expression

Decreased mRNA levels of mitochondrial fusion genes and increased expression of fission genes were found in N2a cells incubated with A $\beta$ . These results suggest that an imbalance in mitochondrial dynamics in these A $\beta$ -incubated N2a cells. Our findings are in agreement with earlier studies by Wang and colleagues [13,50,51] who found abnormal mitochondrial dynamics in neurons affected by A $\beta$ .

Interestingly, in our study, increased expression of CypD was also found in the N2a cells incubated with A $\beta$ , suggesting that an increase in CypD damages mitochondria, possibly by inducing the opening of mitochondrial permeability pores [12,52], making them susceptible to structural damage. In contrast, mRNA expression of CypD was found decreased in N2a cells treated with MitoQ and SS31. CypD is a mitochondrial member of the cyclophilin family of peptidyl prolyl-*cis, trans*-isomerases and has a crucial role in protein folding [12,52]. CypD is located in the matrix of mitochondria and is involved in regulating the mitochondrial permeable transition pore. The pore opening raises the permeability of the mitochondrial inner membrane, allows an influx of cytosolic molecules into the mitochondrial matrix, increases the matrix volume, disrupts the mitochondrial outer membrane, and ultimately damages mitochondrial structure and function. Overall, findings from this study, together with others [12,52], indicate that A $\beta$  increases CypD expression, may promote the opening of mitochondrial inner membrane, and may be involved in damaging mitochondrial structure.

In our study, increased levels of mRNA expression in the mitochondrial fission genes did not occur in N2a cells treated with either MitoQ or SS31, and then incubated with A $\beta$ , suggesting that MitoQ and SS31 may prevent mitochondrial structural genes from expressing abnormally. Overall, MitoQ and SS31 appear to protect mitochondrial structure by regulating mitochondrial fission, fusion, and matrix genes.

In N2a cells not treated with MitoQ, SS31, or resveratrol, but incubated with A $\beta$ , mRNA expressions significantly decreased in all peroxiredoxins (1–6), particularly Prx1 (with a 5-fold decrease) and Prx2 (a 4.5-fold decrease). Our immunoblotting findings agreed with these mRNA expression results. The decrease of Prxs in N2a cells incubated with A $\beta$  was prevented in those cells that were first treated with MitoQ and SS31, suggesting that these MTAs preserve endogenous antioxidant enzymes in cells. Further, Prxs increased in N2a cells treated with MitoQ, SS31, and resveratrol. These findings suggest that A $\beta$  decreases antioxidant enzyme activities in N2a cells, and MitoQ, SS31, increase Prxs expression in N2a cells.

Peroxiredoxins are a group of antioxidant enzymes present in all organisms. They protect neurons by regulating redox, phosphorylation, and oligomerization in cells [39]. Peroxiredoxins neutralize H<sub>2</sub>O<sub>2</sub> and participate in decreasing oxidative damage, which protects cells from various toxic insults, including A $\beta$  toxicity. The expression of Prxs is a strong indicator of antioxidant activity in the brain. The literature on Prxs expression and activity in the brains from AD patients is limited [53–55]. In postmortem AD brains, Prx1 and Prx6 were found to be greater than control brains, and this increase may be a protective compensatory response against A $\beta$  toxicity [53,55]. Our study is the first to investigate the

preventive effects of MTAs against A $\beta$  toxicity in N2a cells, and the rescue effects of Prxs in protecting AD mice neurons against A $\beta$  toxicity.

The increase of the neuroprotective genes PGC1 $\alpha$ , FOXO1, and NMDAR may be a compensatory response against A $\beta$  toxicity in N2a cells treated with A $\beta$ . Recent literature suggests that increased mRNA expression of genes PGC1 $\alpha$ , FOXO1 and NMDA receptor reported to decrease oxidative damage in brain tissues in patients with neurodegenerative diseases and oxidative insults [36,56–63]. Interestingly, in N2a cells pretreated with MitoQ and SS31, and then incubated with A $\beta$ , the expression of mRNA further increased, indicating that MTAs protects cells from A $\beta$  toxicity. However, the protective effect of resveratrol was lower than that of SS31 or MitoQ, suggesting that targeted antioxidants protect cells against A $\beta$  much more effectively than do untargeted antioxidants, such as resveratrol.

PGC1 $\alpha$  is a transcription co-activator that interacts with a range of transcription factors involved in a wide variety of biological responses, including adaptive thermogenesis and mitochondrial biogenesis of several tissues, such as brain tissues [36]. The overexpression of PGC1 $\alpha$  was found to protect mammalian cells, including neurons, from mitochondrial oxidative damage by mediating gene expressions of transcription factors. Recently, lower-than-normal levels of PGC-1 $\alpha$  expression were found in elderly persons and elderly persons with neurodegenerative diseases such as Alzheimer's [56] and Huntington's [57–59]. Our present findings of increased PGC1 $\alpha$  expression in cells treated with MitoQ and SS31 suggest that these MTAs can play a key role in decreasing oxidative damage.

FOXO1, a fork head transcription factor of the FOXO subfamily, which plays an important role in cellular differentiation, proliferation, and energy metabolism [60–62]. FOXO1 has been implicated with oxidative stress and elevated glucose concentrations. The chronic exposure of cells to elevated glucose concentrations causes a deterioration of  $\beta$  cell function. Glucose toxicity is thought to arise as a consequence of chronic oxidative stress, when intracellular glucose concentrations exceed the glycolytic capacity of the  $\beta$  cell. The overexpression of FOXO1 suppresses oxidative stress under hyperglycemia or abnormal glucose metabolism [62]. The increase in FOXO1 expression that we found in N2a cells treated with MitoQ and SS31 indicate that these MTAs may play a protective role via the FOXO1 pathway in cells.

Recently, NMDAR activity in synapses was found to have protective effects by stimulating the antioxidant enzymes peroxiredoxins [40,63]. In the present study, we measured mRNA expression of PGC1 $\alpha$ , FOXO1, and NMDAR to determine the toxic effects of A $\beta$  and the rescue effects of MitoQ, SS31, and resveratrol in relation to peroxiredoxins.

### Electron microscopy

Given the greater number of damaged mitochondria that we found in N2a cells incubated with A $\beta$  than in N2a cells not incubated with A $\beta$ , A $\beta$  may likely increase mitochondrial fragmentation and damage. Altered and damaged mitochondria have been identified as extensive in AD patients, and they may be responsible for cognitive changes in these patients [6,7,10,15,<sup>19</sup>,20,51,52,64]. In our study, MitoQ and SS31 reduced A $\beta$  toxicity. N2a cells that were treated with these MTAs – whether or not the treatment was followed by A $\beta$  incubation – exhibited less mitochondrial damage than N2a cells that were treated with A $\beta$ . Further, MitoQ- and SS31-treated N2a neurons exhibited a more intact mitochondrial structure than N2a cells incubated with A $\beta$ , suggesting that the MTAs balance fission and fusion machinery. These findings agree with real-time RT-PCR and protein data of fission and fusion genes and with mitochondrial functional data. Elongated mitochondria were found in the soma, axons, neuronal processes, and terminals of untreated N2a cells that were

not incubated with A $\beta$ , indicating that healthy elongated mitochondria travel all along neuronal processes to supply ATP at nerve terminals. The mitochondria found in the neuronal processes of the MitoQ- and SS31-treated N2a cells were much healthier than the elongated mitochondria in the untreated cells, suggesting that the MTAs may also enhance the intact structure of mitochondria and may facilitate the supply of ATP at nerve terminals.

### Neurite outgrowth

Recent studies revealed that the movement of mitochondria into dendritic spines correlates with development and morphological plasticity of the spines. Drp1 is a member of the dynamin superfamily of GTPases. Drp1 is primarily localized in the cell body, axons, and dendrites. Drp1 regulates mitochondrial morphology and distribution [65] and is good marker for synaptic branching. A decrease in the expression of Drp1 correlates with a reduction in dendritic mitochondrial content and the loss of synapses and dendritic spines, an indication that Drp1 is critical for synaptic growth in neurons [65]. In the present study, we found significantly decreased neurite outgrowth (marked by Drp1 immunostaining) in N2a cells that were incubated with A $\beta$ . However, in N2a cells treated with MitoQ and SS31, neurite outgrowth significantly increased in comparison to N2a cells incubated with A $\beta$ , indicating that these MTAs enhance neurite outgrowth. This association of MitoQ and SS31 with an increase in neurite outgrowth has implications for treating AD patients, particularly when the disease is actively progressing and fewer neurons remain in the AD brains. We also found increased neurite outgrowth in cells treated with MitoQ and SS31, in comparison to untreated N2a cells or those incubated with A $\beta$ , indicating that these MTAs may be capable of increasing neurite outgrowth. The outcome from this increase in neurite outgrowth, in N2a cells treated with MitoQ and SS31 may have implications in terms of enhancing synaptic branching of neurons and cognitive functions of normal people.

### Neurite outgrowth in AD transgenic primary neurons

In postnatal primary neurons from AD transgenic mice carrying human mutant APP, treatment of N2a neurons with MitoQ and SS31 increased neurite outgrowth, also indicating that these enhance synaptic branching in neurons with over-expressed mutant APP and A $\beta$ . Further, antioxidants targeted to mitochondria, such as MitoQ and SS31, have been reported to cross the blood brain barrier, ultimately reaching neurons and mitochondria, decreasing mitochondrial dysfunction, and increasing ATP production [31,32]. This study is the first to demonstrate that MitoQ and SS31 increase synaptic branching in primary neurons from AD mice and wild-type mice, suggesting that antioxidants targeted to mitochondria boost neurite outgrowth. These results also suggest that MitoQ and SS31 play an important role in enhancing neuronal branching in neurons that carry APP mutations, and also in wild-type neurons.

### Mitochondrial function

N2a cells that were treated with MitoQ and SS31 exhibited less abnormal mitochondrial function than did N2a cells incubated with A $\beta$  (Figure 7). Similar to other researchers' findings [66,67], our data indicated that mitochondrial function is abnormal in N2a cells incubated with A $\beta$ , more specifically, A $\beta$  negatively affected N2a cells in terms of increasing free radicals and lipid peroxidation; decreasing cytochrome oxidase activity; reducing the mitochondrial membrane potential and ATP production; and reducing cell viability. In N2a cells treated with MitoQ and SS31, and then incubated with A $\beta$ , the neurons exhibited decreased mitochondrial dysfunction. Mitochondrial function – in terms of ATP production, cytochrome oxidase activity, and mitochondrial membrane potential – was significantly improved in N2a cells only incubated with the MTAs. These findings are consistent with gene-expression data and data indicating the reduction of neurite outgrowth in these neurons. In the presence of A $\beta$ , MitoQ and SS31 prevented mitochondrial

depolarization, a major parameter of mitochondrial function. Thus, all of our data point to MitoQ and SS31 protecting N2a cells from A $\beta$  toxicity. Resveratrol, in particular, may protect N2a cells from lipid peroxidation generated by oxidative stress and may enhance the mitochondrial membrane potential. In our study, MitoQ- and SS31-treated N2a cells significantly scavenged free radicals and increased cytochrome oxidase activity (Figure 7), but it was the resveratrol-treated N2a cells that exhibited a significant decrease in lipid peroxidation levels and a significant increase in the mitochondrial membrane potential.

## CONCLUSIONS

The purpose of our study was to investigate the effects of the MTAs MitoQ and SS31, and the anti-aging agent resveratrol on N2a cells from an AD mouse model and on N2a cells from these mice, that were incubated with A $\beta$ .

In N2a cells only incubated with the A $\beta$ , we found increased mRNA levels of fission genes, decreased mRNA levels of fusion genes, and decreased mRNA expression of peroxiredoxins. Electron microscopy revealed a significantly increased number of fragmented mitochondria in these cells, indicating that A $\beta$  damages mitochondria by activating fission genes. Our extensive biochemical analysis of mitochondrial function (H<sub>2</sub>O<sub>2</sub> production, cytochrome oxidase activity, lipid peroxidation, ATP production, cell viability and mitochondrial membrane potential), revealed the dominance of dysfunctional mitochondria and a significant decrease in neurite outgrowth, also indicating negative effects from A $\beta$  on mitochondria.

However, in N2a cells treated with MitoQ, SS31, and resveratrol, and then incubated with A $\beta$ , abnormal expression of peroxiredoxins and mitochondrial with abnormal structure were prevented. Mitochondrial functional changes – including H<sub>2</sub>O<sub>2</sub> production, cytochrome oxidase, ATP production, lipid peroxidation, mitochondrial membrane potential, and cell viability – were not affected in the N2a cells that were treated with the MitoQ, SS31 but were not incubated with A $\beta$ . Neurite outgrowth significantly increased in these cells. This is the first study to report the positive effects of MitoQ, and SS31 against A $\beta$  peptide toxicity.

In primary neurons from A $\beta$ PP transgenic mice, which were treated with MitoQ and SS31, neurite outgrowth significantly increased and cyclophilin D expression significantly decreased. This is the first study to report that MitoQ and SS31 significantly increased neurite outgrowth and synaptic connectivity in neurons affected by AD. These findings indicate that MitoQ and SS31 prevent A $\beta$  toxicity in mitochondria and that the study of these MitoQ, SS31 as potential drugs to treat AD patients is warranted.

## Acknowledgments

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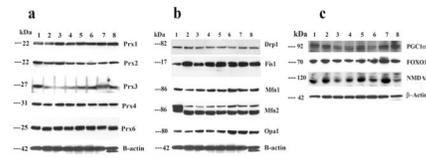
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**Figure 1.**

Representative immunoblots of peroxiredoxins, mitochondrial proteins, and neuroprotective proteins in control N2a neurons, A $\beta$ -incubated N2a cells, and N2a neurons treated with MitoQ and SS31 and then incubated with A $\beta$  (n=3). **Image A.** Fifty  $\mu$ g of protein lysate was used from each cell sample; immunoblotting analysis was performed using antibodies of peroxiredoxins (1, 2, 3, 4, and 6). **Image B.** Drp1, Fis1, Mfn1, Mfn2, and Opa1. **Image C.** PGC1 $\alpha$ , FOXO1, and NMDAR. Bottom panel shows immunoblotting of  $\beta$ -actin for equal loading. Lane 1 represents control N2a cells; lane 2, A $\beta$ -incubated N2a cells; lane 3, resveratrol-treated N2a neurons; lane 4, N2a cells treated with resveratrol and then incubated with A $\beta$ ; lane 5, MitoQ-treated N2a cells; lane 6, N2a cells treated with MitoQ and then incubated with A $\beta$ ; lane 7, SS31-treated N2a cells; and lane 8, N2a cells treated with SS31 and then incubated with A $\beta$ .

Figure 2A

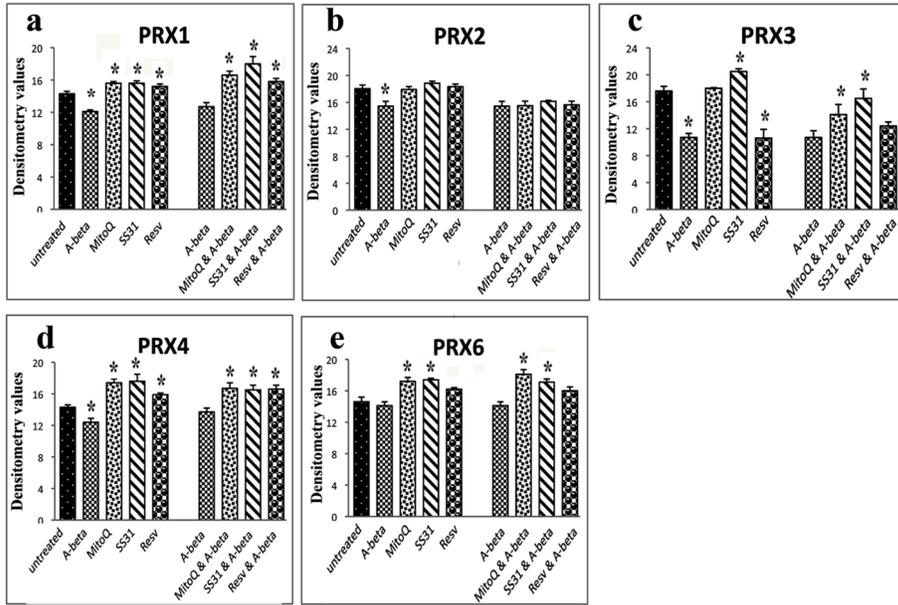


Figure 2B

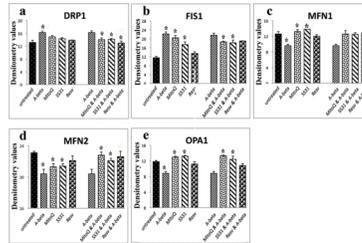


Figure 2C

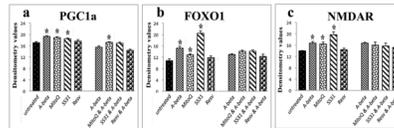
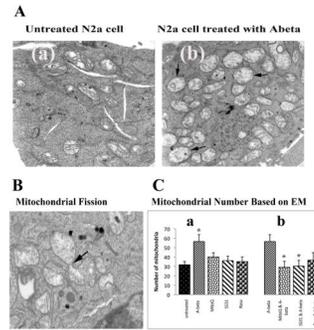


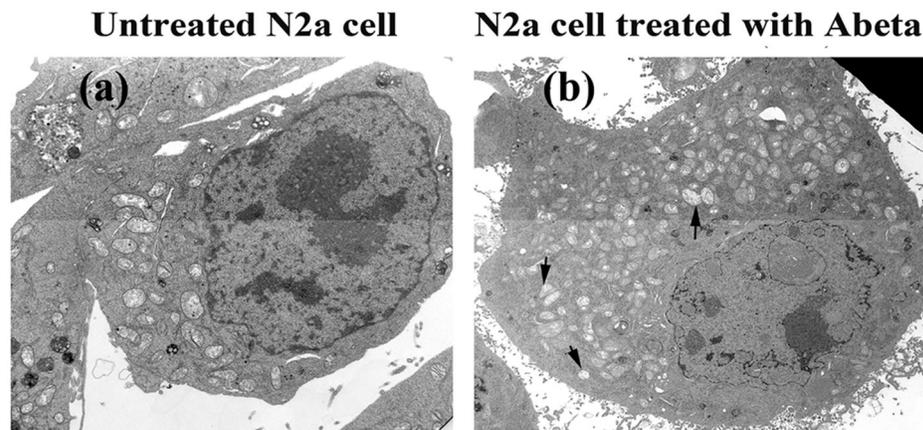
Figure 2.

Quantitative immunoblotting analyses of mitochondrial structural proteins, peroxiredoxins, neuroprotective proteins (n=4). Figure 2A shows the quantitative protein data on mitochondrial structural proteins; 2B, represents quantitative protein data on peroxiredoxins; and 2C, quantitative protein data on neuroprotective proteins. \* denotes statistical significance.

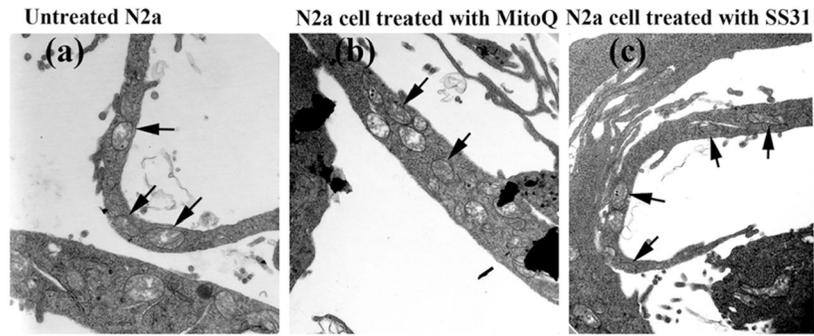


**Figure 3.**

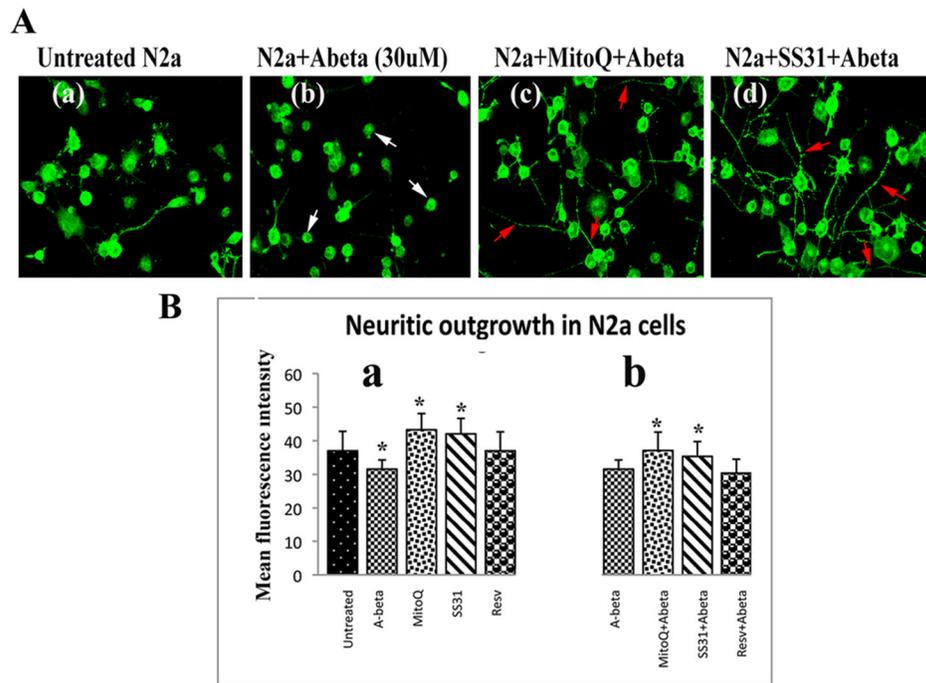
Electron microscopy in control N2a neurons, A $\beta$ -incubated N2a cells, and N2a cells treated with MitoQ and SS31, and then incubated with A $\beta$  (n=4). **Image A** shows (a) a control N2a cell compared to (b) a representative A $\beta$ -incubated N2a cell. Mitochondria increased in the A $\beta$ -incubated N2a cell, and they exhibited morphological changes, including swollen and damaged inner mitochondrial membrane and cristae. **Image B** shows high magnification of mitochondrial fragmentation of an A $\beta$ -incubated N2a cell. **Image C** shows results from quantitative analysis of mitochondria: (a) in control N2a cell compared to N2a cells treated with MitoQ, SS31, and resveratrol, and then incubated with A $\beta$ . Significantly increased mitochondria were found in A $\beta$ -incubated N2a neurons ( $P < 0.05$ ) compared to the control N2a cells, and (b) in A $\beta$ -incubated N2a cells compared to N2a cells treated with MitoQ, SS31, and resveratrol, and then incubated with A $\beta$ . Significantly decreased mitochondria were found in the N2a cells treated with MitoQ and then incubated with A $\beta$  ( $P < 0.05$ ), and in N2a neurons treated with SS31 and then incubated with A $\beta$  ( $P < 0.05$ ), compared to A $\beta$ -treated N2a cells.



**Figure 4.** Mitochondrial fragmentation in A $\beta$ -treated N2a cell. Increased mitochondria were found in A $\beta$ -treated N2a cell (image b).

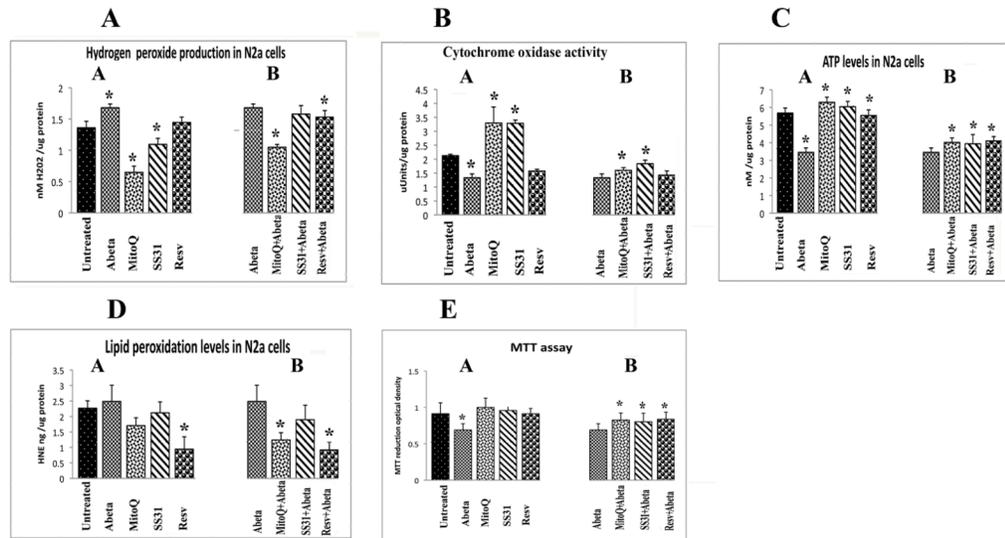


**Figure 5.** Electron microscopy of N2a cells, with a focus on neuronal processes. **(a)** Elongated mitochondria were found in neuronal processes and even in terminals of neurons. Black arrows indicate intact mitochondria in neuronal processes of **(b)** MitoQ- and **(c)** SS31-treated N2a cells.

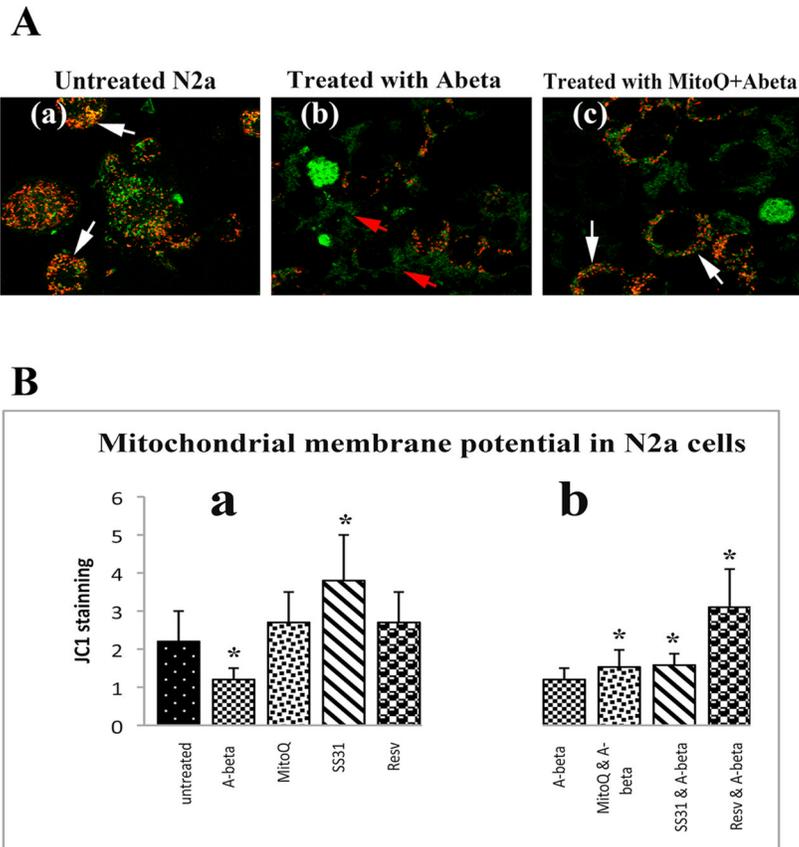


**Figure 6.**

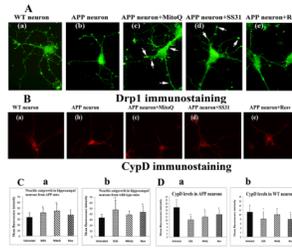
Neurite outgrowth in control N2a cells, A $\beta$ -incubated N2a cells, N2a cells treated with MitoQ and SS31 and then incubated with A $\beta$  (n=4). **Image A** presents representative images of (a) Drp1 immunostaining of control N2a cells, (b) A $\beta$ -incubated N2a cells, (c) N2a cells treated with MitoQ and then incubated with A $\beta$ , and (d) N2a cells treated with SS31 and then incubated with A $\beta$ . White arrows indicate the loss of neuronal processes in the A $\beta$ -incubated N2a cells, and red arrows indicate an increase in neuronal processes in N2a cells treated with MitoQ and SS31. **Image B** shows quantitative analysis of Drp1 immunostaining in N2a cells. (a) Significantly decreased Drp1 immunostaining in neuronal processes was found in A $\beta$ -incubated N2a cells ( $P<0.01$ ) compared to control N2a cells, and significantly increased Drp1 immunostaining (neurite outgrowth) was found in neurons treated with MitoQ ( $P<0.01$ ) and SS31 ( $P<0.03$ ) compared to control N2a cells. (b) Significantly increased Drp1 immunostaining (neurite outgrowth) was found in N2a cells treated with MitoQ and SS31 and then incubated with A $\beta$  ( $P<0.01$  and  $P<0.03$  respectively), compared to A $\beta$ -incubated N2a cells.



**Figure 7.** Mitochondrial functional parameters in control N2a cells, in A $\beta$ -incubated N2a cells, in N2a neurons treated with MitoQ and SS31, and in N2a cells treated with MitoQ and SS31 and then incubated with A $\beta$  (n=4). We analyzed mitochondrial functional data in 2 ways: (1) the control N2a cells were compared to the N2a cells treated MitoQ, SS31 and resveratrol, and incubated with A $\beta$  (image a); and (2) A $\beta$ -incubated N2a cells were compared to (i) N2a cells treated with MitoQ and then incubated with A $\beta$ , (ii) N2a cells treated with SS31 and then incubated with A $\beta$ , and (iii) N2a cells treated with resveratrol and then incubated with A $\beta$  (image b). We performed statistical analysis using ANOVA following the Dunnett correction, for: **(A)** cytochrome oxidase activity, **(B)** H<sub>2</sub>O<sub>2</sub> production. **(C)** ATP levels, **(D)** lipid peroxidation, and **(E)** cell viability.

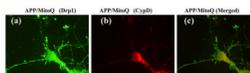


**Figure 8.** Mitochondrial membrane potential using live-cell imaging and JC1 staining. **Image A** is a representative image of N2a cells with an accumulation of JC1 in the mitochondria (in red): **(a)** in control N2a cells, **(b)** in A $\beta$ -incubated N2a cells, where JC1 aggregates remain in the cytosol (in green), and **(c)** in N2a cells treated with MitoQ and then incubated with A $\beta$ , where JC1 aggregates are in the mitochondria (in red). **Image B**, from quantitative analysis of mitochondrial membrane potential in N2a cells (n=4): **(a)** in A $\beta$ -incubated N2a cells, in MitoQ-treated N2a cells, in SS31-treated N2a cells, and in resveratrol-treated N2a cells compared to control N2a cells, and **(b)** in N2a cells treated with Mito, SS31, and resveratrol, and then incubated with A $\beta$ , compared to A $\beta$ -incubated N2a cells. Significantly decreased mitochondrial membrane potential was found in A $\beta$ -incubated N2a cells (P<0.001). Increased levels of the mitochondrial membrane potential were found in the SS31-treated N2a cells (P<0.002). In N2a cells treated with MitoQ, SS31, and resveratrol, and then incubated with A $\beta$ , the membrane potential significantly increased in all 3 treatments (MitoQ+A $\beta$ , P<0.04; SS31+A $\beta$ -P<0.02; and resveratrol+A $\beta$ , P<0.001) compared to A $\beta$ -incubated N2a neurons, indicating that MitoQ, SS31, and resveratrol prevented the membrane potential from A $\beta$  toxicity.



**Figure 9.**

Neurite outgrowth in primary hippocampal neurons from A $\beta$ PP transgenic mice and wild-type mice with its hippocampal neurons treated with MitoQ, SS31, and resveratrol. **Image A** shows representative Drp1-stained hippocampal neurons: (a) wild-type, (b) A $\beta$ PP transgenic, (c) MitoQ-treated APP, (d) SS31-treated APP, and (e) resveratrol-treated A $\beta$ PP transgenic. Immunostaining was conducted using a Drp1 antibody (a marker for synaptic branching). White arrows indicate increased synaptic branching in hippocampal neurons treated with MitoQ and SS31. Fig. 9B. Cyclophilin D levels in hippocampal neurons from A $\beta$ PP transgenic mice and wild-type mice. **Image B** shows representative CypD-stained hippocampal neurons: (a) wild-type, (b) A $\beta$ PP transgenic, (c) MitoQ-treated A $\beta$ PP transgenic, (d) SS31-treated A $\beta$ PP transgenic, and (e) resveratrol-treated A $\beta$ PP transgenic. Immunostaining was performed using the CypD antibody. Fig. 9C shows quantitative analysis of Drp1 immunostaining in hippocampal neurons from APP and wild-type mice. (a) Significantly increased Drp1 was found in hippocampal neurons from APP mice treated with MitoQ ( $P < 0.024$ ) and with SS31 ( $P < 0.044$ ), compared to control hippocampal neurons from A $\beta$ PP transgenic mice. (b) Significantly increased Drp1 was found in hippocampal neurons from wild-type mice treated with SS31 ( $P < 0.03$ ) and resveratrol ( $P < 0.038$ ), compared to control hippocampal neurons from wild-type mice. Figure 9D presents quantitative analysis of CypD in hippocampal neurons from A $\beta$ PP transgenic mice and wild-type mice. (a) Significantly decreased CypD was found in hippocampal neurons from A $\beta$ PP transgenic mice treated with SS31 ( $P < 0.0004$ ), MitoQ ( $P < 0.006$ ), and resveratrol ( $P < 0.013$ ), compared to control hippocampal neurons from A $\beta$ PP transgenic mice, and (b) Hippocampal neurons from wild-type mice treated with SS31 and resveratrol showed significantly decreased levels of CypD, compared to control hippocampal neurons.



**Figure 10.** Double-labeling analysis of (a) Drp1 (synaptic branching protein), (b) CypD (mitochondrial matrix protein), and (c) the proteins Drp1 and CypD merged in MitoQ-treated A $\beta$ PP transgenic mice hippocampal neurons.

**Table 1**

Summary of real-time RT-PCR oligonucleotide primers used in measuring mRNA expression in mitochondrial structural genes, ETC-encoded genes, peroxiredoxins and neuroprotective genes of N2a cells treated with A $\beta$  and MTAs, and N2a cells treated with MTA followed by incubation with A $\beta$ .

Gene	DNA Sequence (5'-3')	PCR Product Size
<b>Mitochondrial Structural Genes</b>		
Drp1	Forward Primer GCGCTGATCCCGCGTCAT	54
	Reverse Primer CCGCACCCACTGTGTGA	
Fis1	Forward Primer GCCCTGCTACTGGACCAT	62
	Reverse Primer CCCTGAAAGCCTCACACTAAGG	
MFN1	Forward Primer TCTCCAAGCCCAACATCTTCA	62
	Reverse Primer ACTCCGGCTCCGAAGCA	
MFN2	Forward Primer ACAGCCTCAGCCGACAGCAT	56
	Reverse Primer TGCCGAAGGAGCAGACCTT	
Opa1	Forward Primer TGGGCTGCAGAGGATGGT	59
	Reverse Primer CCTGATGTCACGGTGTGATG	
TOMM40	Forward Primer CCTGCCCTCTGACCTTCC	60
	Reverse Primer GAGAGCTGGCAGCCAACAC	
Cyclophilin D	Forward Primer CAGCCAAGCCCTCCAATC	58
	Reverse Primer GCCGATGTCCACGTCAAAG	
BCL2	Forward Primer TGAGACTCCAGCATGAAGGA	60
	Reverse Primer GCCTTCCACGCACTCCAT	
HSP60	Forward Primer GAAATGCTTCGACTACCCACAGT	57
	Reverse Primer CCAGTGCCCGGGACACT	
<b>Mitochondrial-encoded Electron Transport Chain Genes</b>		
NADH (sub1)	Forward Primer CGGGCCCCCTTCGAC	72
	Reverse Primer GGCCGGCTGCGTATTCT	
NADH (sub3)	Forward Primer TGTACTCAGAAAAGCAAATCCATATG	75
	Reverse Primer AATAATAGAAATGTAATTGCTACCAAGAAAAA	
NADH (sub5)	Forward Primer CGGACGAACAGAACGCAAATA	70
	Reverse Primer TAAAATGAATCCGATGTCTCCGA	
CYT B	Forward Primer TTATCGCGGCCCTAGCAA	75
	Reverse Primer TAATCCTGTTGGGTTGTTGATCC	
COX 1	Forward Primer GAAGAGACAGTGTTCATGTGGTGT	73
	Reverse Primer TCCTGGCCCTTCAGGAATA	
COX3	Forward Primer CAGGATCTTCTGAGCGTTCTATCA	75
	Reverse Primer AATCCTGTTGGAGGTCAGCA	
ATP6	Forward Primer TGTGGAAGGAAGTGGCAA	72
	Reverse Primer CCACTATGAGCTGGAGCCGT	
<b>Cytoprotective Antioxidant Enzyme Genes</b>		

Gene	DNA Sequence (5'-3')	PCR Product Size
Prx1	Forward Primer TGGCTCGACCCTGCTGATAG	61
	Reverse Primer GGAGCAGGATACCCAATTTTG	
Prx2	Forward Primer CCCCTGAATATCCCTCTGCTT	57
	Reverse Primer CGCCGTAATTCTGGGACAA	
Prx3	Forward Primer GGCCCCATTTCTGGAT	60
	Reverse Primer CAGGCAGGCTAAGGGAAAG	
Prx4	Forward Primer CCTGTTGCGGACCGAATCT	55
	Reverse Primer GGGTCCGGAACCGTTCAT	
Prx5	Forward Primer CCCGATCAAGGTGGGAGAT	56
	Reverse Primer CCCGGTCCCTTCAAATA	
Prx6	Forward Primer TCTGGCAAAAAATACCTCCGTTA	58
	Reverse Primer GCCCAATTTCCGCAAAG	
<b>Neuroprotective Genes</b>		
FOXO1	Forward Primer CCCGTCCTAGGCACGAACT	56
	Reverse Primer ACGCGCCAGAACTTAACTTC	
PGC1 $\alpha$	Forward Primer GGACAGTCTCCCGTGGAT	57
	Reverse Primer TCCATCTGTCAGTGCATCAAATG	
NMDA receptor	Forward Primer GGTCAGTTCTGCTGCACATC	62
	Reverse Primer TGA CTCTCCCGGAAAC	
<b>Housekeeping Genes</b>		
Beta Actin	Forward Primer ACGGCCAGGTCATCACTATTC	65
	Reverse Primer AGGAAGGCTGGAAAAGAGCC	
GAPDH	Forward Primer TTCCCGTTCAGCTCTGGG	59
	Reverse Primer CCCTGCATCCACTGGTGC	

**Table 2**

Fold changes of mRNA expression in mitochondrial structural genes, electron transport chain genes, and neuroprotective genes in Aβ-treated N2a cells, treated with mitochondrial targeted antioxidants, and treated with mitochondrial targeted antioxidants+ Aβ peptide.

Gene	Untreated N2a cells Vs Aβ-Treated N2a Cells, and MTAs-Treated N2a Cells. Comparison A				AβTreated N2a Cells Vs MTAs+Aβ-Treated N2a Cells. Comparison B			
	Aβ	MitoQ	SS31	Resv	MitoQ+ Aβ	SS31+ Aβ	Resv+ Aβ	Resv+ Aβ
<b>Mitochondrial structural genes</b>								
DRP1	1.7*	1.1	1.5*	1.1	-1.2*	-1.2*	-1.4*	-1.4*
FIS1	3.4*	1.9*	1.6*	1.0	-1.4*	-2.7*	-2.9*	-2.9*
MFN1	-1.4*	1.8*	1.4	1.3	1.0	1.1	1.2	1.2
MFN2	-1.8*	1.5*	1.3	1.0	1.0	1.0	1.7*	1.7*
OPA1	-2.5*	1.9*	2.0*	1.0	2.0*	2.1*	2.8*	2.8*
TOMM40	-1.7*	1.2	2.3	1.1	1.4	1.5	1.5	1.5
Cyclophilin D	2.2**	-1.6*	-1.3	1.0	1.6	1.3	1.1	1.1
BCL-2	5.1**	3.5**	5.0**	2.2*	-3.0*	-2.6*	-1.6*	-1.6*
HSP60	5.7*	2.8*	5.5**	1.3	4.9**	3.4*	1.5	1.5
<b>Electron transport chain genes</b>								
NADH (sub1)	2.2*	1.6	7.4**	1.6	1.5	1.0	-1.6*	-1.6*
NADH (sub3)	2.5	1.5	8.2**	2.1*	1.2	6.6**	1.7*	1.7*
NADH (sub5)	2.5*	1.4	6.6**	2.0*	1.2	-1.6*	-1.3*	-1.3*
Cyt B	2.1*	1.8*	9.5**	1.9*	1.6	1.3	-1.2*	-1.2*
COX1	2.7*	1.5	9.4**	2.1*	1.2	1.1	-1.4*	-1.4*
COX3	2.1*	2.5*	8.9**	1.5	2.0*	7.3**	1.2	1.2
ATP-6	2.2*	1.8*	8.2**	1.8*	1.0	1.1	-1.3*	-1.3*
<b>Antioxidant enzymes - Peroxiredoxins</b>								
PRX1	-5**	1.6	1.5	1.0	5.5**	4.4**	3.5**	3.5**
PRX2	-2.3**	1.3	2.8*	2.3*	4.3**	3.3**	3**	3**
PRX3	-1.3*	1.3	4.5**	5.3**	1.5	4.2**	2.2*	2.2*

Gene	Untreated N2a cells Vs A $\beta$ -Treated N2a Cells, and MTAs-Treated N2a Cells, Comparison A				A $\beta$ Treated N2a Cells Vs MTAs+A $\beta$ -Treated N2a Cells, Comparison B			
	A $\beta$	MitoQ	SS31	Resv	MitoQ+ A $\beta$	SS31+ A $\beta$	Resv+ A $\beta$	
PRX4	-1.7*	1.9*	3.4**	1.8*	2.3*	4.1**	3.7**	
PRX5	-2.8**	1.5	2.6*	1.7*	4.2**	3.7**	5.0**	
PRX6	-4.5**	4.4**	3**	6**	2.9**	3.0**	3.1**	
<b>Neuroprotective genes</b>								
FOXO1	7.5**	3.1*	14.9**	3.7*	1.6*	1.6*	1.2	
NMDA receptor	3.1*	8.2**	8.8**	9.4**	4.3*	3.7**	1.3	
PGC-1 $\alpha$	2.4*	4.8**	2.5*	1.8*	2.5*	1.4	-1.4*	

\* indicates statistical significance level P<0.05

\*\* indicates statistical significance level P<0.005