Complex I Subunit Gene Therapy With NDUFA6 Ameliorates Neurodegeneration in EAE

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PURPOSE. To address the permanent disability induced by mitochondrial dysfunction in experimental autoimmune encephalomyelitis (EAE).

METHODS. Mice sensitized for EAE were rescued by intravitreal injection of adeno-associated viral vector serotype 2 with the complex I subunit gene *scAAV-NDUFA6Flag*. Controls were injected with a mitochondrially targeted red fluorescent protein (*scAAV-COX8-cherry*). Another group received *scAAV-COX8-cherry*, but was not sensitized for EAE. Serial pattern electroretinograms (PERGs) and optical coherent tomography (OCT) evaluated visual function and structure of the retina at 1, 3, and 6 months post injection (MPI). Treated mice were killed 6 MPI for histopathology. Immunodetection of cleaved caspase 3 gauged apoptosis. Complex I activity was assessed spectrophotometrically. Expression of NDUFA6Flag in the retina and optic nerve were evaluated between 1 week to 1 month post injection by RT-PCR, immunofluorescence and immunoblotting.

RESULTS. Reverse transcription-PCR and immunoblotting confirmed NDUFA6Flag overexpression with immunoprecipitation and blue native PAGE showing integration into murine complex I. Overexpression of NDUFA6Flag in the visual system of EAE mice rescued retinal complex I activity completely, axonal loss by 73%, and retinal ganglion cell (RGC) loss by 88%, RGC apoptosis by 66%, and restored the 33% loss of complex I activity in EAE to normal levels; thereby, preventing loss of vision indicated by the 43% reduction in the PERG amplitudes of EAE mice.

CONCLUSIONS. *NDUFA6* gene therapy provided long-term suppression of neurodegeneration in the EAE animal model suggesting that it may also ameliorate the mitochondrial dysfunction associated with permanent disability in optic neuritis and MS patients.

Keywords: mitochondria, optic neuritis, multiple sclerosis

Experimental autoimmune encephalomyelitis (EAE) is an inflammatory autoimmune disease of the central nervous system. It is frequently used as an animal model to understand the disease pathogenesis and to test treatment options for multiple sclerosis (MS).^{1–6} The optic nerve, brain, and spinal cord are the sites affected in EAE and MS.^{7–13} Traditionally, MS has been viewed as an immune mediated demyelinating disease. Decades of research in this area resulted in the development of various immunomodulatory drugs, which lately are found to be ineffective in preventing permanent disability.¹⁴ More recently, MS has been increasingly considered a neurodegenerative disease, where neuronal and axonal demise result in irreversible loss of function.^{15–17} The underlying molecular mechanisms associated with neuronal and axonal loss in MS are poorly understood,^{18,19} but mitochondrial dysfunction is recognized as one of the major contributing factors.^{15,19–21}

Mitochondrial involvement in other neurologic diseases is well documented for almost 30 years.^{22–24} However, mitochondrial dysfunction in MS was first reported by Dutta and coworkers²¹ approximately 10 years ago. They showed mitochondrial complex I and III activity loss in autopsied MS brains. During the same year, Qi et al.,²⁵ reported oxidative stress mediated damage to the central nervous system (CNS)

mitochondrial proteins including the complex I subunit NDUFA6, complex IV subunit *cox VIII*, and mitochondrial HSP70 that occurred prior to the infiltration of inflammatory cells and primed the neurodegeneration of EAE. Furthermore, we reported decreased complex I activity in EAE mice optic nerves that increased mitochondrial oxidative stress, which contributed to the neurodegeneration associated with permanent visual loss.²⁶ We had found that administration of the single subunit complex I of yeast (NDI) ameliorated mitochondrial oxidative stress, thus preventing neuronal and axonal demise. Genetic knock-down of a different complex I subunit, NDUFA1-mediated neurodegeneration of the optic nerve in mice,²⁷ suggesting the neurodegeneration associated with permanent visual disability in optic neuritis and multiple sclerosis may have a mitochondrial component. In addition, recent reports have documented impaired activity of several respiratory complexes, large kilobase deletions or point mutations within the mitochondrial DNA of autopsied MS brains.^{28–30} It is known that damage to these mitochondrial proteins alters the mitochondrial electron transport chain, resulting in oxidative stress, axonal energy deficits, loss of cellular homeostasis, and ultimately culminating in neuronal degeneration.^{23,31,32} We hypothesize that dysfunction of

mitochondrial respiratory complex I results in increased reactive oxygen species (ROS) generation that may damage various proteins, lipids, and mtDNA, thus priming axonal degeneration in MS overexpressing complex I subunits, which are otherwise susceptible to oxidative damage is expected to be beneficial in mitigating complex I dysfunction, thereby rescuing neuronal degeneration. Here, we explore the possibility of neuroprotection by overexpressing the mouse *NDUFA6* subunit of complex I in retinal ganglion cells (RGC) and their axons comprising the optic nerves of EAE sensitized mice.

METHODS

NDUFA6 Cloning and Viral Packaging

The cDNA of the mouse *NDUFA6* gene appended with a flag epitope tag at the C terminus was cloned into single-stranded AAV (ssAAV) plasmid vector pTR-UF22, regulated by the 381-bp cytomegalovirus (CMV) immediate early gene enhancer/1352-bp chicken β -actin promoter-exon 1-intron 1 that was digested with the restriction enzyme *EcoRI*. The 2170-bp fragment carrying the promoter, regulatory elements and the *NDUFA6Flag* cDNA were gel purified. Similarly, the parent sc-CMV-COX8-*Cherry* vector was digested with *EcoRI* to remove the 1170-bp fragment containing the CMV promoter followed by the *COX8-Cherry* gene with the 23 amino acid COX8 mitochondrial targeting sequence appended to the N terminus of the cherry gene. The vector backbone was ligated with the 2170 fragment released from the pTR-UF22 vector carrying the *NDUFA6Flag* cDNA along with the regulatory elements. The reading frame and orientation of the inserts were confirmed by Sanger sequencing, then the recombinant plasmids were packaged into AAV2 particles by the plasmid cotransfection method.³³ Briefly, plasmids were amplified and purified by cesium chloride gradient centrifugation and then packaged into AAV2 capsids by transfection into human embryonic kidney cells using standard procedures.³³ The crude iodixanol fractions were purified using a fast protein liquid chromatography system (AKTA; Amersham Pharmacia, Piscataway, NJ, USA). The vector was eluted from the column using 215 mM sodium chloride (pH, 8.0), and the recombinant AAV (rAAV) peak was collected. Vector-containing fractions were then concentrated and buffer exchanged in balanced salt solution (Alcon Laboratories, Fort Worth, TX, USA) with 0.014% polysorbate 20 (Tween20; Jiangsu Haian Petrochemical Plant, Jiangsu, China), using a centrifugation concentrator (Biomax 100 K; Millipore, Billerica, MA, USA). The vector was then tittered for DNase resistant vector genomes by real-time PCR relative to a standard.³³ Finally, the purity of the vector was validated by silver-stained SDS-PAGE, assayed for sterility and lack of endotoxin, then divided into aliquots, and stored at -80°C .

Animals

Adult female DBA/1J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animal experiments were performed in accordance with the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Miami.

Induction of EAE and Intravitreal Injections

Female DBA/1J mice ($n = 20$) 6 months of age were sensitized for EAE by injecting 0.1 mL of sonicated homologous spinal

cord emulsion in complete Freund's adjuvant (Difco, Detroit, MI, USA) subdermally into the nuchal area. Control animals ($n = 10$) received subdermal inoculation with Freund's adjuvant.

For intraocular injection of recombinant AAV, DBA/1J mice were sedated by inhalation with 1.5% to 2% isoflurane. A local anesthetic (proparacaine hydrochloride) was applied topically to the cornea and the pupils were dilated by a drop of 1% tropicamide. A 32-G needle attached to a Hamilton syringe (Sigma-Aldrich Corp., St. Louis, MO, USA) was inserted through the pars plana under the dissecting microscope. One microliter of sc-*NDUFA6Flag* AAV2 (1.0×10^{11} VG/mL) or sc-CMV-COX8-*Cherry* AAV2 (8.66×10^{10} VG/mL) was injected into both eyes of each animal.

Ten of 20 EAE sensitized mice received bilateral intravitreal injections of scAAV2-*NDUFA6Flag*. Ten EAE sensitized mice received scAAV2-CMV-COX8-*Cherry* as disease controls. Ten unsensitized animals received sc-CMV-COX8-*Cherry* as injection controls. The number of mice used for each experiment is summarized in the Table. Experimental autoimmune encephalomyelitis sensitization and intravitreal injections were done at the same sitting.

Pattern Electroretinography (PERG)

Serial PERGs were obtained for Control-*cherry* ($n = 10$), EAE-*cherry* ($n = 10$), and EAE-*NDUFA6Flag* ($n = 10$) mice at 1, 3, and 6 months post injection. The procedure used was similar to those previously published.^{26,34} Briefly, mice were weighed and anesthetized by intraperitoneal injection of ketamine/xylazine hydrochloride solution (ketamine 80 mg/kg body weight and xylazine 10 mg/kg body weight). Mice were gently restrained with the use of bite bar and a nose holder in order to allow unobstructed vision. Body temperature was maintained at 37°C using a feedback controlled heating pad. The eyes of anesthetized mice were wide open and steady, with undilated pupils pointing laterally and upward. The ERG electrode (0.25-mm diameter silver wire configured to a semicircular loop of 2-mm radius) was placed on the corneal surface and it was positioned in such a way as to encircle the pupil without limiting the field of view. Reference and ground electrodes made of stainless steel needles were inserted under the skin of the scalp and tail, respectively. A small drop of balanced saline was topically applied on the cornea to prevent drying during recording. A visual stimulus of contrast-reversing bars (field area, $50^{\circ} \times 58^{\circ}$; mean luminance, 50 cd/m²; spatial frequency, 0.05 cyc/deg; contrast, 100%; temporal frequency, 1 Hz) was aligned with the projection of the pupil at a distance of 20 cm. Retinal signals were amplified by 10,000-fold and band-pass filtered by 1 to 30 Hz. Three consecutive responses to each of the 600 contrast reversals were recorded and checked for their consistency by superimposition and then were averaged. Averaged PERG values were analyzed to evaluate the major positive and negative waves using commercially available software (Sigma Plot; Systat Software, Inc., San Jose, CA, USA).

TABLE. Animal Numbers in Each Experiment

Experiment	Animal	Animal No. (N)
Electrophysiology	Mice	30
SD-OCT	Mice	30
RT-PCR	Mice	4
Western blot	Mice	30
Immunofluorescence	Mice	5
Histology and electron microscopy	Mice	30
Complex I+III, III, and I activity	Mice	23
Apoptosis	Mice	4

Spectral-Domain Optical Coherence Tomography (SD-OCT) Imaging

All three groups (Control-*cherry* [$n = 10$], EAE-*cherry* [$n = 10$], and EAE-*NDUFA6Flag* [$n = 10$] mice) were followed up for 1, 3, and 6 months post injection by spectral-domain optical coherence tomography (SD-OCT; BiopTigen, Inc., Durham, NC, USA) for retinal thickness measurements. High-resolution three-dimensional (3D) retinal images were obtained from live mice as previously described.³⁵ Briefly, mice were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). Pupils were dilated using a drop of tropicamide (1%). Corneal hydration and clarity was maintained by applying Systane Ultra (Alcon Laboratories, Inc., Fort Worth, TX, USA) lubricating eye drops. The mice were secured on a custom stage, which allowed free rotation, to align the eye for imaging of the optic nerve head (ONH). Rectangular volume scans centered on the ONH, were acquired from both eyes. For analysis of SD-OCT images, 25 images around the ONH were used from each eye. The images were manually segmented by drawing the boundaries and measuring the distance from the NFL to the inner boundary of the inner nuclear layer using algorithms in MATLAB software (MathWorks, Natick, MA, USA). The segmented image information was used to construct 3D geometric plots of the retina using algorithms for 3D thickness maps in MATLAB.

Expression Studies

Immunofluorescence. We tested for expression of the transgene 1 week after viral injections. Mice ($n = 4$) were humanely euthanized. The globes and optic nerves were dissected out and fixed in 4% paraformaldehyde in PBS for 1 hour and then 0.4% paraformaldehyde overnight. To make flat mounts, the cornea and lens were removed, and the retina was carefully dissected from the eyecup. Four radial cuts were made to flatten the retina. After 3 washes in PBS, retinas were permeabilized with 0.5% Triton X-100 (Dow Chemical Corporation, Midland, MI, USA) in PBS for 1 hour, then blocked with 10% goat serum containing 0.5% Triton X-100 for 1 hour. The flat-mounted retinas were rinsed in PBS and incubated with a mixture of rat monoclonal anti-Flag (1:100) antibody (Sigma, Cambridge, MA, USA) along with rabbit monoclonal anti-TUJ1 (1:100; Covance, Inc., FL, USA) and mouse monoclonal GRIM19 (1:200; Abcam, Cambridge, MA, USA) antibodies overnight at 4°C. Flat-mounted retinas were washed with PBS three times (5 minutes each) and were incubated in 1:500 dilution of goat anti-mouse Cy3 (GRIM19), goat anti-rabbit Cy5 (Tuj1), and goat anti-rat Cy2 (FLAG; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) antibodies, along with 4',6-diamidino-2-phenylindole (2 µg/mL) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), at 4°C overnight. The working concentrations of the primary and secondary antibodies were prepared in 10% goat serum in PBS (pH, 7.4) containing 0.2% Triton X-100. The retinas were washed 3 times (5 minutes each time) in PBS and then transferred to glass slides with the RGC layer facing up. Retinas were observed under a confocal microscope (Leica TCS SP5; Leica, Wetzlar, Germany). After imaging, the retinas were embedded in optimal cutting temperature embedding compound (Sakura Finetek, Torrance, CA, USA) at -80°C overnight. Serial sections, 8-µm thick, were cut using a cryostat, mounted with fluorescent mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA) and examined for fluorescence using the confocal microscope.

Transcription. Mice injected with *sCAAV2-NDUFA6Flag* were killed 1 month after viral injections. The retinas, optic nerves, brains, and spinal cords were dissected out ($n = 3$) and

total RNA was isolated using the RNeasy mini Kit (Qiagen Sciences, Valencia, CA, USA). Ribonucleic acid was quantified, 500 ng total RNA was used to reverse transcribe into cDNA (iScript; Bio-Rad, Hercules, CA, USA); 2 µL of cDNA was used for further amplification using gene specific primers. The 18S rRNA was used as a house keeping control. The following primer sets were used for PCR amplification; *NDUFA6Flag*-5'-AAGAATGCCCCATGTCACAGACC-3', *FlagR*: 5'-TGTCATC GTCGTCCTTGTAGTC-3', which amplifies the cDNA of the recombinant construct, but not endogenous *NDUFA6*, with an expected PCR product size of 200 bp. 18S rRNA F-5'-GAAC TGAGGCCATGATTAAGAG-3', 18S rRNA R-5'-CATTCTTGGCA AATGCTTTC-3' primer sets amplified endogenous 18S rRNA with an expected PCR product size of 120 bp. Resultant PCR products were electrophoresed on a 1% agarose gel.

Mitochondria Isolation

Mice were killed 1 month after *sCAAV2-NDUFA6Flag* ocular injections ($n = 15$) or uninjected controls ($n = 15$). The retinas, optic nerves, brains, and spinal cord tissues were collected. Mitochondria were isolated from excised tissues using a standard protocol.³² Briefly, the tissues were washed in ice-cold PBS twice, resuspended in a buffer consisting of 20 mM HEPES-KOH pH 7.5, 0.25 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, and 1 mM EDTA, and homogenized using the Omni THQ-Digital Tissue Homogenizer (OMNI International, Kennewick, WA, USA). Differential centrifugation was performed at 750g for 3 minutes, 3500g for 3 minutes, and 5000g for 3 minutes. The supernatant was collected in a fresh 1.5-mL tube and centrifuged at 10,500g for 15 minutes. The supernatant containing the cytoplasmic protein fraction was carefully removed and was further concentrated using Amicon Ultra centrifugal filters with a 3 kDa cut off (Millipore, Carrigtwohill County Cork, Ireland). The pellets containing the mitochondria were washed with the same buffer for two more times, then stored at -80°C until use. All centrifugations were performed at 4°C. The protein fractions were quantified using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

Complex I Immunoprecipitation

Complex I was immunoprecipitated from mitochondrial pellets using a kit (MS101 complex I immunocapture kit) following the manufacturer's specifications (MitoSciences, Eugene, OR, USA). Briefly, mitochondrial pellets obtained from the *NDUFA6Flag* or control retinas (500 µg each) were thawed on ice and suspended in solubilization buffer (50 mM Tris-HCl pH 7.5, 1:100 protease inhibitor cocktail [Sigma] and 1% n-dodecyl-β-D-maltoside; MitoSciences, Eugene, OR, USA). The mitochondrial suspension was incubated on ice for 30 minutes and then centrifuged for 30 minutes at 21,000g at 4°C. The complex I immunocapture beads were added to the solubilized mitochondrial supernatant and incubated overnight at 4°C with gentle mixing in a tube rotator. After spinning at 3200g at 4°C, the beads containing the complex I captured proteins were washed three times in 1% n-dodecyl-B-maltoside in PBS. Beads were then dissolved in 2× SDS sample loading buffer and proteins were separated on a Novex Bis-Tris 4-12% gradient polyacrylamide gel (Invitrogen, Grand Island, NY, USA) and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen).

Blue Native Gel Electrophoresis

Blue native gel electrophoresis was performed on the mitochondrial samples using the Native PAGE Novex 4-16%

Bis-Tris gels (Invitrogen) according to the manufacturer's instructions. Briefly, 250 μ g of mitochondrial pellets obtained from *NDUFA6*Flag or uninjected control retinas were thawed on ice, suspended in 1 $\times$ native PAGE sample buffer (Invitrogen) containing 2.5% digitonin and incubated on ice for 15 minutes. The lysates were centrifuged at 20,000g for 30 minutes at 4°C. The supernatant was collected and stored at -80°C until use. The samples were mixed with G-250 sample additive with one-fourth the detergent concentration and were loaded on Native PAGE Novex 4-16% Bis-Tris gels. Native PAGE running buffer and the dark blue cathode buffers were prepared and electrophoresis was performed at 150 V for 110 minutes. For Western blotting we replaced the dark blue cathode buffer with light blue cathode buffer once the dye front migration was one-third down the gel. The protein complexes from the native PAGE gels were transferred onto PVDF membranes at 25 V constant for 1 hour using NuPAGE transfer buffer (Invitrogen). Once the transfer was completed, membranes were fixed in 8% acetic acid for 15 minutes, then rinsed with deionized water.

Immunoblotting

Membranes were blocked with 5% non-fat dry milk in TBST containing 0.5% Tween 20 for 1 hour and then incubated overnight with primary antibodies, rat monoclonal anti-Flag (Sigma), mouse monoclonal anti-*NDUFA9* (Abcam), and rabbit polyclonal anti-*NDUFA6* (Abcam). Membranes were washed with TBST tween 20 buffer and incubated for 1 hour with respective secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc.). Complexes were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pittsburgh, PA, USA). Band intensities were quantified using Image J software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Histologic Sectioning of the Optic Nerves and Retina

Six months after AAV injections and EAE sensitization, mice were euthanized by an overdose of sodium pentobarbital (0.3 mg/g of body weight), then perfused with 4% paraformaldehyde in 0.1 M PBS buffer (pH 7.4) by cardiac puncture. The retinas and optic nerves were dissected out and further processed by immersion fixation in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, 0.1 M sodium cacodylate-HCl buffer (pH 7.4), 7% sucrose in the cold, and then dehydrated through an ethanol series to propylene oxide, infiltrated, and embedded in epoxy resin that was polymerized at 60°C overnight. Semi-thin longitudinal sections (0.5 μ m) of the optic nerve and retina were obtained and counterstained with toluidine blue for light microscopic examination. The number of cells in the RGC layer were counted on 10 to 15 random sections from each eye and the mean cell number ($\pm$ SE) per millimeter retina of each group were plotted.

Transmission Electron Micrographs

Ultrathin sections (90 nm) were also obtained from all mice. They were placed on copper grids for transmission electron microscopic examination. Optic nerve and retinal specimens were examined without poststaining using a Hitachi H-7000 transmission electron microscope (Tokyo, Japan) operating at 80 kV. Digital photographs were made at a magnification of $\times 2500$. The number of axons in each image was counted manually and averaged for all the optic nerves of each group and plotted on a bar graph.

Apoptosis

Apoptosis of Thy 1.2 labeled RGCs were studied by immunofluorescence staining for cleaved caspase 3. These mice were sensitized for EAE 6 months earlier and injected with *mCherry* ($n = 4$) or rescued with *NDUFA6* ($n = 3$). Retinal sections 8- μ m thick were cut, transferred onto Superfrost Plus glass slides (VWR International, Bridgeport, NJ, USA) and stored at -80°C until use. The sections were permeabilized with 0.5% Triton X-100 in PBS for 1 hour, blocked with 10% goat serum in 0.5% Triton X-100 in PBS for 1 hour, then incubated with rabbit monoclonal cleaved caspase3 (1:100; Cell Signaling Technology, Inc., Danvers, MA, USA) and rat monoclonal thy1.2 (1:100; Abcam) antibodies. The sections were washed three times, 5 minutes each with PBS and incubated with goat anti rabbit-Cy5 (1:500) and goat anti-rat-Cy2 (1:500; Jackson ImmunoResearch Laboratories, Inc.). The sections were counterstained with DAPI for (2 μ g/mL; Santa Cruz Biotechnology, Inc.) to 10 minutes, washed with PBS three times, mounted with fluorescence media (Vectashield; Vector Laboratories) and observed under a confocal microscope (Leica TCS SP5; Leica, Wetzlar, Germany). For quantitative analysis 10 random sections covering the entire retina from each eye were counted for cleaved caspase 3 and DAPI-positive cells in the RGC layer. The percentage of cleaved caspase 3 cells relative to DAPI-positive cells were calculated, averaged, and compared using an unpaired Student's *t*-test. A *P* less than 0.05 was considered significant.

Complex I Activity Assay

Control ($n = 10$) and EAE mice ($n = 13$) were injected with *scAAV2-NDUFA6Flag* AAV into the right eyes. AAV-containing *scAAV2-CMV-COX8-Cherry* was injected into the left eyes. Mice were euthanized 3 months post injection and EAE sensitization. Retinas were immediately dissected out, homogenized in mitochondrial isolation buffer, and freeze thawed in liquid nitrogen for three cycles. NADH-cytochrome oxidoreductase activity was assayed in 0.1 M potassium phosphate (pH 7.5), 2 mM NADH, 10 mM KCN, 1 mM oxidized cytochrome C at 30°C, using 1.5 μ g of protein. NADH oxidoreductase activity was measured spectrophotometrically at 550 nm for 2 minutes and again 2 minutes after adding 10 μ L of 1 mM rotenone. The slope of the run was noted and the activity was expressed in nanomoles of NADH-Cytochrome C reduced per min per 1 μ g of protein.

Statistical Analysis

All data analysis was performed using a one way-ANOVA. The Brown Forsythe test was used to evaluate the homogeneity of the population. If the overall *P* value was significant ($P < 0.05$), then the Tukey's multiple comparisons test was performed to compare the groups and *P* less than 0.05 was considered statistically significant.

RESULTS

scAAV2-NDUFA6Flag Efficiently Transduces Mouse RGCs and Integrates Into a Functional Complex I

Confocal microscopic examination of the flat-mounted retinas obtained from mice ($n = 5$) 1 week after intravitreal injection with *NDUFA6Flag* revealed DAPI-labeled nuclei (Fig. 1A), in Tuj1-positive RGCs (Fig. 1B) expressing *NDUFA6Flag* (Fig. 1C) in mitochondria labeled by GRIM-19 (Fig. 1D). A merged image showed the colocalization of *NDUFA6Flag* with GRIM-19 markers (Fig. 1E). Longitudinal sections of these retinas

FIGURE 1. NDUF6 expression. Representative confocal microscopic images of retinal whole mounts at 1 week postinjection of scAAV2-CBA-NDUF6Flag show DAPI-labeled nuclei (A), Tuj1-labeled RGCs (B), NDUF6Flag (C), GRIM19-positive mitochondria (D), and merged (E). Longitudinal sections of the injected retina show DAPI-labeled nuclei (F), Tuj1-labeled RGCs (G), NDUF6Flag (H) in GRIM19-labeled mitochondria (I), and NDUF6Flag plus GRIM19 channels merged (J). At higher magnification the RGC layer shows DAPI (K), Tuj1 (L), NDUF6Flag (M), GRIM19 (N), NDUF6Flag, and GRIM19 merged (O). At 1 month post injection, RT-PCR shows NDUF6Flag transcripts exclusively in the optic nerve and retina with 18S rRNA transcripts in the optic nerve, retina, brain, and spinal cord (P). Western blotting of immunocaptured mitochondrial complex I shows the FLAG band in injected retinas that was absent in control retinas (Q). The NDUF9 band is seen in both tissues with similar intensity (Q). Blue native gel electrophoresis followed by immunoblotting with anti-Flag antibody detected a specific approximately 980-kDa band in injected retinas that was absent in control retinas (R). The NDUF9 band is seen in both injected and control retina (R). Scale bars: 5 μ m for retinal whole mounts and RGC cross sections and 20 μ m for longitudinal sections. $N = 5$ for IF, $n = 4$ for RT-PCR, $n = 30$ for immunoblotting. DAPI, 4',6'-Diamidino-2-Phenylindole; INL, inner nuclear layer.

showed DAPI labeled cell nuclei in the outer, inner nuclear, and RGC layers (Fig. 1F). Retinal ganglion cells stained positive for Tuj1 (Fig. 1G) were surrounded by punctate and perinuclear expression of NDUF6Flag (Fig. 1H) that were also stained positive for mitochondrial GRIM-19 (Fig. 1I) that colocalized with NDUF6Flag (Fig. 1J). The RGC layer at higher magnification shows DAPI-labeled nuclei (Fig. 1K), Tuj1-positive RGCs (Fig. 1L) exhibiting perinuclear NDUF6Flag

expression (Fig. 1M), mitochondrial GRIM-19 (Fig. 1N) colocalization with NDUF6Flag (Fig. 1O). Immunofluorescence analysis indicated expression of NDUF6Flag not only in RGCs but also in other retinal layers, thus consistent with the previous observations made with very high titer triple tyrosine to phenylalanine mutant AAV2.³⁶

One month after AAV injections, semiquantitative RT-PCR analysis ($n = 8$ eyes in each group) revealed NDUF6Flag

transcripts exclusively in the retina and optic nerves of *NDUFA6Flag* intravitreally-injected mice, with absence of expression in the brains and spinal cords. We used 18S rRNA as an internal control (Fig. 1P).

Immunoblotting with the anti-Flag antibody on complex I immunocaptured mitochondrial proteins revealed integration of *NDUFA6Flag* (~14-kDa band) in mitochondrial complex I. No band was seen in uninjected control retinas (Fig. 1Q). Immunoblotting for endogenous *NDUFA6* on complex I immunocaptured membranes revealed an approximately 14-kDa band in both injected and control retinas. Blotting for *NDUFA9* used here as an internal control was detected in both *NDUFA6Flag*-injected and uninjected samples (Fig. 1Q). Quantitative analysis of the band intensities of *NDUFA6* normalized with the band intensities of *NDUFA9* revealed a 3.2-fold higher expression of *NDUFA6* in the infected retinas compared with uninjected controls (Fig. 1Q). Immunoblotting with the anti-Flag antibody on retinal mitochondrial fractions separated on native blue gels revealed a 980-kDa band in *NDUFA6Flag*-injected mice that was absent in the uninjected control samples (Fig. 2R). Endogenous *NDUFA9* was seen in injected and control retinal samples (Fig. 2R). Thus, overexpressed *NDUFA6Flag* was transported into mitochondria of RGCs where it assembled into a functional complex I.

Overexpression of *NDUFA6Flag* Rescues the EAE-Mediated Visual Dysfunction

PERG analysis, a sensitive measure of visual function in the mice was used to monitor optic neuritis in unsensitized control mice intravitreally injected with *sCAAV-mCherry*, EAE-sensitized mice injected with *sCAAV-mCherry*, and EAE-sensitized mice injected with *sCAAV-NDUFA6Flag*. At 1 month post sensitization we found no significant differences in mean PERG amplitudes (Fig. 2A) and latencies (Fig. 2B) among the groups. By 3 months the EAE-*mCherry* mice showed a significant reduction in the PERG amplitude (43%, $P = 0.0099$) and a delay in latency (19%, $P = 0.0305$) compared with unsensitized control-*mCherry* mice. In contrast, *NDUFA6Flag*-protected mice showed a complete suppression of the amplitude loss ($P < 0.0001$) and the delay in latency ($P = 0.0011$). At 6 months post sensitization, EAE-*mCherry* mice showed a significant reduction in amplitude (44%, $P = 0.0035$) and delay in latency (21%, $P = 0.0378$) compared with unsensitized control *mCherry* mice. However, PERG amplitudes and latencies of mice that were injected with *NDUFA6Flag* were comparable to unsensitized control *mCherry* mice ($P > 0.05$) who were not sensitized for EAE. Compared with the EAE-*mCherry* group, *NDUFA6* treatment significantly suppressed the amplitude loss (76.5%, $P = 0.0039$) and the latency delay (76%, $P = 0.043$). Mean PERG waveforms showed the loss of amplitude and delay in latency associated with EAE were rescued with *NDUFA6Flag* injection (Fig. 2C). The mean PERG amplitudes $\pm$ SE and latencies $\pm$ SE at 1, 3, and 6 months post injection and the corresponding P values are listed in Supplementary Tables S1 and S2, respectively. Overall, there was a progressive loss of visual function in EAE-sensitized mice and *NDUFA6Flag* overexpression preserved visual function. None of the DBA/1J mice sensitized for EAE developed paralysis.

NDUFA6Flag Rescues EAE-Mediated RGC Loss and RNFL Thinning

High-resolution SD-OCT imaging revealed progressive thinning of the inner retina associated with the effects of EAE that was rescued by *NDUFA6Flag* overexpression. While the unsensitized *mCherry*-injected control mice did not show any changes in the inner retina at 3 and 6 months post injection (Figs. 3A–

D), the EAE sensitized mice showed inner retinal thinning at 3 and 6 months post sensitization (Figs. 3E–H). Interestingly, *NDUFA6Flag* expression preserved these retinal structures to near normal levels that were comparable to unsensitized controls (Figs. 3I–L). Quantitative analysis of the OCT data revealed a 9% ($P = 0.1048$) thinning of the inner retinal layers in EAE sensitized *mCherry* mice of 0.0567 ± 0.003 mm (mean $\pm$ SE) at 3 months and a 15% thinning ($P = 0.0002$) at 6 months (0.0566 ± 0.002) compared with the unsensitized control-*mCherry* retinas (0.062 ± 0.001 mm) at 3 months and 0.067 ± 0.001 mm at 6 months. The mean inner retinal thickness of the *NDUFA6Flag*-treated group at 3 months was 0.067 ± 0.001 mm and at 6 months it was 0.06745 ± 0.00133 mm. Thus, *NDUFA6Flag* treatment significantly prevented the retinal neurodegeneration effects of EAE at the timepoints evaluated ($P < 0.0005$; Fig. 3M).

NDUFA6Flag Overexpression Rescues EAE Axonal Degeneration

Transmission electron microscopic analysis ($n = 10$ eyes in each group) of EAE-*mCherry* optic nerves revealed marked reduction in axonal density (Fig. 4A), presence of few mononuclear inflammatory cells, thin or absent myelin lamellae surrounding axons some with electron dense aggregations characteristic of neurodegeneration (Fig. 4B). Optic nerve axons showed degenerating myelin and abnormal neurotubules (Fig. 4C). In contrast, *NDUFA6Flag* rescued EAE optic nerves showed higher axonal density (Fig. 4D) and axons appeared to be relatively normal and myelinated (Fig. 4E). Quantitatively, the mean number of axons in the unsensitized control-*mCherry* group was 0.1055 ± 0.0046 ($\pm$ SE) per μm^2 , whereas in the EAE-*mCherry* mice the axon count was reduced to 0.0580 ± 0.0112 per μm^2 . This axonal loss attributed to EAE was statistically significant (45%, $P = 0.0003$). However, mice treated with *NDUFA6Flag* showed a mean axonal count of 0.0924 ± 0.0049 per μm^2 , which corresponds to 73% axonal rescue compared with the EAE sensitized *mCherry* group ($P < 0.0082$) and it was comparable to the unsensitized *mCherry* group ($P > 0.05$; Fig. 4F). Thus, *NDUFA6Flag* overexpression suppressed axonal loss in the EAE optic nerve.

NDUFA6Flag Rescues RGC Apoptosis

Immunostaining for cleaved caspase-3 ($n = 7$ eyes) 6 months after EAE sensitization and mock-treatment with *sCAAV-mCherry* revealed DAPI-labeled nuclei in all three retinal layers (Fig. 5A), cleaved caspase-3 staining in RGCs (Fig. 5B) immunolabeled by Thy1.2 (Fig. 5C). A merged image shows colocalization of cleaved caspase-3 with the RGC marker (Fig. 5D). Retinal sections of *NDUFA6Flag*-treated mice showed DAPI staining in all three retinal layers (Fig. 5E), but minimal or no staining for cleaved caspase-3 (Fig. 5F) in Thy1.2-labeled RGCs (Fig. 5G) shown along with a merged image (Fig. 5H). Quantitative analysis of cleaved caspase 3-stained cells in the RGC layer indicated apoptosis in 15% of RGCs in EAE-*mCherry* retinas with 14.86 ± 47 (mean $\pm$ SE) cleaved caspase 3-stained cells per 100 DAPI-positive cells compared with 5% in *NDUFA6*-rescued retinas ($5.39 \pm 0.37/100$ DAPI positive cells), $P = 0.023$ (Fig. 5I). Thus, *NDUFA6* ameliorated EAE induced RGC apoptosis.

Toluidine blue stained retinas ($n = 27$ eyes) of the unsensitized control-*mCherry* group at 6 months after injection show a normal RGC layer (Fig. 6A), whereas the EAE sensitized-*mCherry* retinas showed relatively less cells, some with condensed nuclei (Fig. 6B). *NDUFA6Flag* overexpression preserved the cell number in EAE mice to near normal

FIGURE 2. Visual function. Bar plots of PERG amplitude (A), latency (B), and averaged PERG waveforms at 6 MPI (C) of the mCherry-control, EAE-mCherry, and EAE-NDUFA6 rescued mice at 1 ($n = 18$, 17,

Fig. 6C). Quantitative analysis revealed a mean count of 98.00 ± 2.6 (mean $\pm$ SE) cells in the RGC layer per millimeter for the EAE-mCherry group, which is 40% less compared with a mean of 164 ± 2.36 (mean $\pm$ SE) per millimeter in the control-mCherry ($P < 0.001$). In contrast, NDUFA6Flag-treated retinas showed a mean of 156 ± 6.1 (mean $\pm$ SE) cells per millimeter. Thus, NDUFA6-FLAG reduced the loss of cells in the RGC compared with the EAE-mCherry group ($P < 0.001$; Fig. 6D).

Transmission electron micrographs of the retina from control-mCherry mice revealed a normal nerve fiber layer beneath, which RGCs with large elliptical and electron lucent nuclei and cytoplasm were evident (Fig. 6E). In contrast, EAE-mCherry mice showed loss of the nerve fiber layer and RGCs (Fig. 6F) some with condensed nuclei (Fig. 6G). NDUFA6Flag-treated mice showed a relatively healthy RGC layer with electron lucent nuclei and cytoplasm similar to unsensitized controls (Fig. 6H).

NDUFA6Flag Overexpression Rescues EAE-Mediated Complex I Dysfunction

We measured respiratory function in unsensitized mice injected with mCherry and EAE sensitized mice injected with either mCherry or NDUFA6Flag at 3 months. We found that complex I+III activities were significantly lower (34%, $P < 0.0001$) in mCherry-injected EAE retinas (0.66 ± 0.02 nmol of NADH-cytochrome C reduced per minute per milligram protein, mean $\pm$ SE) compared with unsensitized control mice injected with mCherry (0.997 ± 0.037 nmol of NADH-cytochrome C reduced per minute per milligram protein). In contrast, EAE mice treated with NDUFA6Flag had a mean activity of 0.894 ± 0.054 nmol of NADH-cytochrome C reduced per minute per milligram protein; which correspond to a 69% rescue compared with the EAE-mCherry group, $P = 0.009$. Respiratory activity of the NDUFA6 group was statistically indistinguishable from the unsensitized control mCherry group, $P > 0.05$ (Fig. 7).

When we tested complex III activity, EAE sensitized mCherry mice showed a mean activity of 0.33 ± 0.015 nmol of NADH-cytochrome C reduced per minute per milligram protein, which is 36% ($P = 0.0001$) less than a mean 0.517 ± 0.035 for the unsensitized mCherry group. NDUFA6Flag-treated mice had a mean activity of 0.397 ± 0.037 , which is 23% less than the unsensitized mCherry-injected group ($P = 0.044$; Fig. 7) and not significantly different than the EAE-mCherry ($P > 0.05$) group.

Furthermore, when we measured complex I activity, we observed that EAE-mCherry mice had a mean activity of 0.333 ± 0.022 , which is 31% less ($P = 0.0063$) compared with a mean of 0.480 ± 0.038 for unsensitized controls injected with mCherry. In contrast, NDUFA6Flag-treated retinas had a mean activity of 0.497 ± 0.036 , which corresponds to 100% rescue ($P < 0.05$). Complex I activities in the EAE-NDUFA6Flag group was similar to that of the unsensitized control mCherry-injected mice, $P < 0.05$ (Fig. 7). Thus, deficient complex I activity in EAE mice was ameliorated by NDUFA6 gene therapy.

DISCUSSION

The results presented here demonstrate that neurodegeneration in the EAE animal model of MS can be ameliorated by

and 19), 3 ($n = 14$, 16, and 18), and 6 ($n = 7$, 17, and 16) MPI. Number of repetitions = 3, for each group and time point; mean $\pm$ SE; one-way ANOVA followed by Tukey's multiple comparison test; * $P = 0.01$ –0.05; ** $P = 0.001$ –0.009; *** $P = 0.0001$ –0.0009.

FIGURE 3. In vivo retinal imaging. Spectral-domain OCT images taken across the fundus and modified thickness maps derived using MATLAB software at 3 and 6 months post sensitization. *Top panel* shows representative cross sectional images of the retina at 3 and 6 MPI and the corresponding 3D thickness maps of the *mCherry*-controls ([A–D]; $n = 11$ and 10). *Middle panel* shows representative SD-OCT images and corresponding 3D thickness maps of EAE-*mCherry* mice ([E–H]; $n = 9$ and 8). *Bottom panel* shows representative SD-OCT images and corresponding 3D thickness maps of EAE-*NDUFA6Flag* mice ([I–L]; $n = 14$ and 11). A *bar plot* of the inner retinal thickness at 3 and 6 MPI of all three groups (M). Marked region in OCT images with *red lines*: RNFL/GCL/IPL, retinal nerve fiber layer/ganglion cell layer/inner plexiform layer. One-way ANOVA followed by Tukey's multiple comparisons test; $P < 0.05$ considered significant. *** $P = 0.001$ – 0.009 ; **** $P = 0.0001$ – 0.0009 .

overexpression of the respiratory complex I subunit NDUFA6, a crucial subunit involved in assembly of complex I. It is also a major target for damage induced by oxidative stress. By exposing isolated bovine mitochondria to peroxynitrite in vitro, Murray et

al.³⁷ showed for the first time that the NDUFA6 subunit is majorly damaged and this in turn lead to the inhibition of complex I activity. Interestingly, Qi et al.,²⁵ reported similar peroxynitrite-mediated damage to the NDUFA6 subunit in EAE

FIGURE 4. Optic nerve ultrastructure. Six months after intravitreal AAV injections transmission electron micrographs show axonal loss in the EAE-mCherry optic nerve (A), with axons exhibiting electron dense aggregations, mononuclear inflammatory cell (IC) infiltration (B), and axons with swelling and disruption of neurotubules and thin myelin (arrows; [C]). An NDUFA6Flag optic nerve shows higher axon (ax) density (D). At higher magnification mitochondria are swollen, but axonal neurotubules appear intact (E). Bar plot of mean optic nerve axon counts (F). $n = 10$ nerves in each group; one-way ANOVA, $**P = 0.001-0.009$; $***P = 0.0001-0.0009$.

mice. Ladha and coworkers³⁸ showed that downregulation of NDUFA6 in cultured T cells impaired complex I activity and introduced cellular apoptosis, whereas its overexpression reduced apoptosis. Similarly, thiazolidinedione, an antidiabetic agent caused complex I disassembly by specifically binding to NDUFA6, thereby resulting in a decrease in complex I

function.³⁹ Further, Dutta and coworkers⁴⁰ showed complex I and III activities decreased by one-half in autopsied MS brains. The impairment of complex I activity in MS lesions was also supported by other groups.⁴¹⁻⁴³ Overall, it is believed that mitochondrial NDUFA6 is a crucial member in normal functioning of mitochondrial complex I and any alterations to

FIGURE 5. Apoptosis. Immunostaining for cleaved caspase 3 and Thy1.2 performed on retinal sections obtained 6 months after EAE sensitization and mock treatment with *Cox8-Cherry* or rescue with *NDUFA6Flag*. Top panel shows DAPI-labeled nuclei (A), cleaved caspase 3 in the ganglion cell layer (B), Thy1.2 labeled RGCs (C), and all channels merged (D) in EAE-*Cox8-Cherry* mice. Bottom panel shows DAPI labeled nuclei (E) but no cleaved caspase 3 (F), Thy1.2 labeled RGCs (G), and merged (H) in NDUFA6Flag rescued retinas. A bar plot shows the percentage of cleaved caspase 3-labeled cells to DAPI labeled cells in the RGC layer of *Cox8-Cherry*-injected eyes and NDUFA6 rescued eyes (D). Error bars represent standard error. $n = 4$ for EAE and 3 for NDUFA6 rescue; unpaired Student's *t*-test; $*P = 0.023$; scale bars: 25 μm . *mCherry*, *Cox8-Cherry*.

FIGURE 6. Histology of retina and RGC ultrastructure. Six months after intravitreal AAV injections and sensitization; light micrographs of toluidine blue stained retinal sections of unsensitized control-*mCherry* mice (A) EAE-*COX8-Cherry* mice retina (B) and *NDUFA6Flag* rescued retinas (C). A bar plot shows the mean ($\pm$ SE) number of cells in the RGC layer of Control-*mCherry* ($n = 9$), EAE-*mCherry* ($n = 12$), and EAE-*NDUFA6Flag* ($n = 6$) on light micrographs (D). Representative transmission electron micrographs of unsensitized *mCherry* retinas show an elliptical and lighter RGC nucleus with overlying retinal nerve fiber layer (E). Experimental autoimmune encephalomyelitis-*mCherry* mice show chromatin condensation (arrow) and loss of cells in the RGC layer (F, G). Experimental autoimmune encephalomyelitis animals rescued with *NDUFA6Flag* show RGCs with the characteristic elliptical and lighter nucleus and overlying retinal nerve fiber layer (H). Scale bar for light micrographs = 50 μ m, for electron micrographs; 10 and 2 μ m; One-way ANOVA followed by Tukey's multiple comparisons test; $P < 0.05$ considered significant. $**P = 0.001-0.009$. V, vitreous.

this subunit adversely impacts respiratory chain function and ultimately cell viability. In our study, overexpression of the complex I subunit *NDUFA6* in RGCs and optic nerve axons successfully rescued complex I dysfunction thereby ameliorating neurodegeneration and visual dysfunction.

There is substantial evidence from various EAE animal models and MS tissue obtained at autopsy to show ROS-mediated mitochondrial dysfunction contributes to neuronal and axonal degeneration.^{25,44-47} Previously, we reported that RGC and optic nerve axonal injury is attributed to increased ROS generation in mitochondria^{25,26} and this is consistent with reports from other groups.^{48,49} Generation of ROS in mitochondria is predominantly mediated by complex I and III of the electron transport

chain.^{49,50} Complex I deficiency in longstanding MS has been attributed to oxidative stress.^{41,51} The effect of ROS on respiratory complex I is 2-fold, that is it can affect the rate of ATP synthesis, which directly contributes to neuronal degeneration and axonal loss, or it can increase production of ROS in mitochondria, which may further damage various proteins, lipids, and mtDNA. Studies showed, the loss of oxidative phosphorylation activity,^{41,52} altered expression of mitochondrial ATP synthase and cytochrome b in MS brains.⁵³ Furthermore, studies showed large kilobase deletions, point mutations in mitochondrial encoded complex I genes, and oxidative damage to the various mitochondrial proteins in the CNS of EAE animals and MS patients.^{25,29,54} We believe that, correcting complex I

FIGURE 7. Respiratory activity. A bar plot shows the activities of complex I + III, complex III, and complex I activities (mean $\pm$ SE) of the pooled retinas obtained from unsensitized control-*mCherry* ($n = 19$ eyes), EAE-*mCherry* ($n = 15$ eyes), and EAE-*NDUFA6Flag* ($n = 9$ eyes). Number of repetitions = 3; One-way ANOVA followed by Tukey's multiple comparison test; $*P = 0.01-0.05$; $**P = 0.001-0.009$; $***P < 0.001$, $****P < 0.0001$, *mCherry*, *COX8-Cherry*.

deficits as shown here or by alternative proteins like a single subunit yeast NDI²⁶ or boosting mitochondrial antioxidant enzyme expression^{48,55–62} are beneficial in overcoming oxidative stress induced RGC and axonal demise, thereby preventing neurodegeneration in EAE and perhaps the permanent disability of human optic neuritis and MS.

Lastly, despite the NDUFA6-induced improvements in the PERG signal and RGC counts, our apoptosis study demonstrated 6% of cells in the RGC layer were still undergoing active apoptosis 6 months post EAE sensitization even with treatment. This finding suggests that longer duration studies than performed here may be needed to determine if the neuroprotective effect of NDUFA6 is sustained 6 months to 1 year or longer after EAE sensitization when the inflammatory phase is subsided as evidenced by the few inflammatory cells seen at 6 months here and in our previous work.⁶¹ At the time points studied here, EAE becomes a neurodegenerative disorder as does relapsing and remitting MS that converts to secondary progressive MS no longer responsive to anti-inflammatory disease modifying drugs. Gene therapy targeting the neurodegeneration may be a hope for the future.

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References

- Baker D, Hankey DJ. Gene therapy in autoimmune, demyelinating disease of the central nervous system. *Gene Ther.* 2003; 10:844–853.
- Owens T. The enigma of multiple sclerosis: inflammation and neurodegeneration cause heterogeneous dysfunction and damage. *Curr Opin Neurol.* 2003;16:259–265.
- Giuliani F, Yong VW. Immune-mediated neurodegeneration and neuroprotection in MS. *Int MS J.* 2003;10:122–130.
- Petzold A, Rejdak K, Plant GT. Axonal degeneration and inflammation in acute optic neuritis. *J Neurol Neurosurg Psychiatry.* 2004;75:1178–1180.
- Kornek B, Storch MK, Weissert R, et al. Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. *Am J Pathol.* 2000;157:267–276.
- Bjartmar C, Wujek JR, Trapp BD. Axonal loss in the pathology of MS: consequences for understanding the progressive phase of the disease. *J Neurol Sci.* 2003;206:165–171.
- Hu P, Pollard J, Hunt N, et al. Microvascular and cellular responses in the optic nerve of rats with acute experimental allergic encephalomyelitis (EAE). *Brain Pathol.* 1998;8:475–486.
- Guy J, Fitzsimmons J, Ellis EA, et al. Intraorbital optic nerve and experimental optic neuritis. Correlation of fat suppression magnetic resonance imaging and electron microscopy. *Ophthalmology.* 1992;99:720–725.
- Guy J, Mao J, Bidgood WD Jr, et al. Enhancement and demyelination of the intraorbital optic nerve. Fat suppression magnetic resonance imaging. *Ophthalmology.* 1992;99:713–719.
- Guy J, Ellis EA, Rao NA. Hydrogen peroxide localization in experimental optic neuritis. *Arch Ophthalmol.* 1990;108: 1614–1621.
- Guy J, Fitzsimmons J, Ellis EA, et al. Gadolinium-DTPA-enhanced magnetic resonance imaging in experimental optic neuritis. *Ophthalmology.* 1990;97:601–607.
- Guy J, Mancuso A, Quisling RG, et al. Gadolinium-DTPA-enhanced magnetic resonance imaging in optic neuropathies. *Ophthalmology.* 1990;97:592–599.
- Guy J, Qi X, Hauswirth WW. Adeno-associated viral-mediated catalase expression suppresses optic neuritis in experimental allergic encephalomyelitis. *Proc Natl Acad Sci U S A.* 1998;95: 13847–13852.
- Shirani A, Zhao Y, Karim ME, et al. Association between use of interferon beta and progression of disability in patients with relapsing-remitting multiple sclerosis. *JAMA.* 2012;308:247–256.
- Bjartmar C, Trapp BD. Axonal and neuronal degeneration in multiple sclerosis: mechanisms and functional consequences. *Curr Opin Neurol.* 2001;14:271–278.
- Silber E, Sharief MK. Axonal degeneration in the pathogenesis of multiple sclerosis. *J Neurol Sci.* 1999;170:11–18.
- Trapp BD, Peterson J, Ransohoff RM, et al. Axonal transection in the lesions of multiple sclerosis [see comments]. *N Engl J Med.* 1998;338:278–285.
- Schattling B, Steinbach K, Thies E, et al. TRPM4 cation channel mediates axonal and neuronal degeneration in experimental autoimmune encephalomyelitis and multiple sclerosis. *Nat Med.* 2012;18:1805–1811.
- van HJ Witte ME, Ciccarelli O. The role of mitochondria in axonal degeneration and tissue repair in MS. *Mult Scler.* 2012; 18:1058–1067.
- Stys PK. General mechanisms of axonal damage and its prevention. *J Neurol Sci.* 2005;233:3–13.
- Dutta R, McDonough J, Yin X, et al. Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients. *Ann Neurol.* 2006;59:478–489.
- Kalman B, Leist TP. A mitochondrial component of neurodegeneration in multiple sclerosis. *Neuromolecular Med.* 2003; 3:147–158.
- Smith KJ, Lassmann H. The role of nitric oxide in multiple sclerosis. *Lancet Neurol.* 2002;1:232–241.
- Kalman B. Role of mitochondria in multiple sclerosis. *Curr Neurol Neurosci Rep.* 2006;6:244–252.
- Qi X, Lewin AS, Sun L, et al. Mitochondrial protein nitration primes neurodegeneration in experimental autoimmune encephalomyelitis. *J Biol Chem.* 2006;281:31950–31962.
- Talla V, Yu H, Chou TH, et al. NADH-dehydrogenase Type-2 suppresses irreversible visual loss and neurodegeneration in the EAE animal model of MS. *Mol Ther.* 2013;21:1876–1888.
- Qi X, Lewin AS, Hauswirth WW, et al. Suppression of complex I gene expression induces optic neuropathy. *Ann Neurol.* 2003;53:198–205.
- Nikic I, Merkler D, Sorbara C, et al. A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. *Nat Med.* 2011;17:495–499.
- Campbell GR, Ziabreva I, Reeve AK, et al. Mitochondrial DNA deletions and neurodegeneration in multiple sclerosis. *Ann Neurol.* 2011;69:481–492.
- Bosley TM, Constantinescu CS, Tench CR, et al. Mitochondrial changes in leukocytes of patients with optic neuritis. *Mol Vis.* 2007;13:1516–1528.
- Gilgun-Sherki Y, Melamed E, Offen D. The role of oxidative stress in the pathogenesis of multiple sclerosis: the need for effective antioxidant therapy. *J Neurol.* 2004;251:261–268.
- Talla V, Porciatti V, Chiodo V, et al. Gene therapy with mitochondrial heat shock protein 70 suppresses visual loss and optic atrophy in experimental autoimmune encephalomyelitis. *Invest Ophthalmol Vis Sci.* 2014;55:5214–5226.

33. Hauswirth WW, Lewin AS, Zolotukhin S, et al. Production and purification of recombinant adeno-associated virus. *Methods Enzymol.* 2000;316:743–761.
34. Talla V, Yang C, Shaw G, et al. Noninvasive assessments of optic nerve neurodegeneration in transgenic mice with isolated optic neuritis. *Invest Ophthalmol Vis Sci.* 2013;54:4440–4450.
35. Ruggeri M, Wehbe H, Jiao S, et al. In vivo three-dimensional high-resolution imaging of rodent retina with spectral-domain optical coherence tomography. *Invest Ophthalmol Vis Sci.* 2007;48:1808–1814.
36. Petrs-Silva H, Dinculescu A, Li Q, et al. Novel properties of tyrosine-mutant AAV2 vectors in the mouse retina. *Mol Ther.* 2011;19:293–301.
37. Murray J, Taylor SW, Zhang B, et al. Oxidative damage to mitochondrial complex I due to peroxynitrite: identification of reactive tyrosines by mass spectrometry. *J Biol Chem.* 2003;278:37223–37230.
38. Ladha JS, Tripathy MK, Mitra D. Mitochondrial complex I activity is impaired during HIV-1-induced T-cell apoptosis. *Cell Death Differ.* 2005;12:1417–1428.
39. Garcia-Ruiz I, Solis-Munoz P, Fernandez-Moreira D, et al. Pioglitazone leads to an inactivation and disassembly of complex I of the mitochondrial respiratory chain. *BMC Biol.* 2013;11:88.
40. Dutta R, McDonough J, Yin X, et al. Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients. *Ann Neurol.* 2006;59:478–489.
41. Lu F, Selak M, O'Connor J, et al. Oxidative damage to mitochondrial DNA and activity of mitochondrial enzymes in chronic active lesions of multiple sclerosis. *J Neurol Sci.* 2000;177:95–103.
42. Witte ME, Bo L, Rodenburg RJ, et al. Enhanced number and activity of mitochondria in multiple sclerosis lesions. *J Pathol.* 2009;219:193–204.
43. Kumleh HH, Riazi GH, Houshmand M, et al. Complex I deficiency in Persian multiple sclerosis patients. *J Neurol Sci.* 2006;243:65–69.
44. Nikic I, Merkler D, Sorbara C, et al. A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. *Nat Med.* 2011;17:495–499.
45. Hobom M, Storch MK, Weissert R, et al. Mechanisms and time course of neuronal degeneration in experimental autoimmune encephalomyelitis. *Brain Pathol.* 2004;14:148–157.
46. Trip SA, Schlottmann PG, Jones SJ, et al. Retinal nerve fiber layer axonal loss and visual dysfunction in optic neuritis. *Ann Neurol.* 2005;58:383–391.
47. Dutta R, McDonough J, Yin X, et al. Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients. *Ann Neurol.* 2006;59:478–489.
48. Qi X, Lewin AS, Sun L, et al. Suppression of mitochondrial oxidative stress provides long-term neuroprotection in experimental optic neuritis. *Invest Ophthalmol Vis Sci.* 2007;48:681–691.
49. Lieven CJ, Hoegger MJ, Schlieve CR, et al. Retinal ganglion cell axotomy induces an increase in intracellular superoxide anion. *Invest Ophthalmol Vis Sci.* 2006;47:1477–1485.
50. Pitkanen S, Robinson BH. Mitochondrial complex I deficiency leads to increased production of superoxide radicals and induction of superoxide dismutase. *J Clin Invest.* 1996;98:345–351.
51. Vyshkina T, Banisor I, Shugart YY, et al. Genetic variants of Complex I in multiple sclerosis. *J Neurol Sci.* 2005;228:55–64.
52. Kalman B, Leist TP. A mitochondrial component of neurodegeneration in multiple sclerosis. *Neuromolecular Med.* 2003;3:147–158.
53. Tajouri L, Mellick AS, Ashton KJ, et al. Quantitative and qualitative changes in gene expression patterns characterize the activity of plaques in multiple sclerosis. *Brain Res Mol Brain Res.* 2003;119:170–183.
54. Bosley TM, Constantinescu CS, Tench CR, et al. Mitochondrial changes in leukocytes of patients with optic neuritis. *Mol Vis.* 2007;13:1516–1528.
55. Guy J, Qi X, Hauswirth WW. Adeno-associated viral-mediated catalase expression suppresses optic neuritis in experimental allergic encephalomyelitis. *Proc Natl Acad Sci U S A.* 1998;95:13847–13852.
56. Guy J, Ellis EA, Hope GM, et al. Antioxidant enzyme suppression of demyelination in experimental optic neuritis. *Curr Eye Res.* 1989;8:467–477.
57. Guy J, Qi X, Wang H, et al. Adenoviral gene therapy with catalase suppresses experimental optic neuritis. *Arch Ophthalmol.* 1999;117:1533–1539.
58. Guy J, Qi X, Hauswirth WW. Adeno-associated viral-mediated catalase expression suppresses optic neuritis in experimental allergic encephalomyelitis. *Proc Natl Acad Sci U S A.* 1998;95:13847–13852.
59. Guy J, Ellis EA, Hope GM, et al. Antioxidant enzyme suppression of demyelination in experimental optic neuritis. *Curr Eye Res.* 1989;8:467–477.
60. Qi X, Hauswirth WW, Guy J. Dual gene therapy with extracellular superoxide dismutase and catalase attenuates experimental optic neuritis. *Mol Vis.* 2007;13:1–11.
61. Qi X, Lewin AS, Sun L, et al. SOD2 gene transfer protects against optic neuropathy induced by deficiency of complex I. *Ann Neurol.* 2004;56:182–191.
62. Qi X, Sun L, Lewin AS, et al. Long-term suppression of neurodegeneration in chronic experimental optic neuritis: antioxidant gene therapy. *Invest Ophthalmol Vis Sci.* 2007;48:5360–5370.