Gene Therapy With Mitochondrial Heat Shock Protein 70 Suppresses Visual Loss and Optic Atrophy in Experimental Autoimmune Encephalomyelitis

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PURPOSE. To rescue visual loss and optic neuropathy in experimental autoimmune encephalomyelitis (EAE).

METHODS. Encephalomyelitis was induced in mice that received intravitreal injections of AAV2-*mtHSP70Flag* or AAV2-*Cox8-mCherry*. Additional mice were injected with AAV2-*Cox8-mCherry*, but not sensitized for EAE. Visual function was assessed by pattern electroretinograms (PERG) at 1, 3, and 6 months post injection (MPI). Optical coherence tomography (OCT) evaluated the thickness of the inner plexiform layer + nerve fiber layers at 1, 3, and 6 MPI. Retinas and optic nerves (ONs) of mice euthanized 6 MPI were processed for light and electron microscopy. Expression of *mtHSP70Flag* in the retina and ONs was evaluated by RT-PCR, immunofluorescence, and Western blotting. The activities of respiratory complexes I and III, as well as mitochondrial protein import were quantitated.

RESULTS. Expression: immunofluorescence revealed punctate and perinuclear expression of *mtHSP70Flag* that colocalized with mitochondrial porin in thy1.2 labeled retinal ganglion cells (RGCs). Immunoblotting and RT-PCR confirmed *mtHSP70Flag* expression in the retina and ON. Rescue: treatment with *mtHSP70Flag* resulted in a 44% increase in PERG amplitude and less delays in latency relative to the EAE-*mCherry* group that also showed progressive inner retinal thinning. At 6 MPI, the almost 50% loss of RGCs and optic nerve axons in EAE mice was suppressed by *mtHSP70Flag*. In addition, retinas of EAE-*mtHSP70Flag* mice showed nearly complete rescue of complex I and III activities that was reduced by one-third in the EAE-*mCherry* retinas. Lastly, reductions in import of *COX8-mCherry* into mitochondria of mice sensitized for EAE improved by 30% with *mtHSP70Flag* gene therapy.

CONCLUSIONS. Mitochondrial HSP70 ameliorates mitochondrial dysfunction that culminates in irreversible visual loss and atrophy of the optic nerve in EAE suggesting that it may be useful to prevent irreversible disability in patients with optic neuritis and multiple sclerosis (MS)

Keywords: *mtHSP70*, gene therapy, optic neuropathy

Experimental autoimmune encephalomyelitis (EAE) is an extensively used animal model for multiple sclerosis (MS).^{1–6} In both disorders, symptomatic lesions typically involve the optic nerve and spinal cord.^{7–13} Traditionally, MS is considered a primary disorder of demyelination that is mediated by inflammatory cells.¹⁴ More recently, MS is increasingly recognized as a neurodegenerative disorder where neuronal and axonal loss contribute to permanent disability.^{15,16} Currently available treatments are directed primarily against the inflammatory component of the disease, and are ineffective in preventing neurodegeneration that leads to irreversible loss of function.¹⁷ The molecular mechanisms associated with neurodegeneration in MS and EAE are poorly understood.

Mitochondrial dysfunction is increasingly recognized as playing a major role in the pathogenesis of neurodegeneration in MS. In particular, functional deficits involving mitochondrial

respiratory complex I and III,¹⁸ along with large kilobase deletions in mitochondrial DNA, contribute to impairment of oxidative phosphorylation in autopsied MS tissues.^{19,20} Increases in reactive oxygen species (ROS) mediated by mitochondria are also recognized as key mediators of central nervous system (CNS) injury in EAE animal models and in MS patients.^{21,22} Previously, we have shown ROS-mediated oxidative damage to key subunits of complex I and IV along with the mitochondrial heat shock protein 70 (*mtHSP70*) that primes the neuronal degeneration of EAE.²³ We know from the previous studies that the *mtHSP70* chaperone plays an essential role in import, folding, and assembly of nuclear encoded mitochondrial proteins and sequestration of damaged proteins, thereby resulting in cell survival or death.^{24–35} In this study, we explored the possibility of neuroprotection in the visual system by overexpressing *mtHSP70* in retinal ganglion cells (RGCs) of mice sensitized for EAE.

TABLE. Animal numbers in each experiment

Experiment	Animal	Number of Animals, N
Electrophysiology	Mice	30
SD-OCT	Mice	30
RT-PCR	Mice	6
Western blotting	Mice	20
Immunofluorescence	Mice	5
Histology and electron Microscopy	Mice	30
Complex I+III, III, and I activity	Mice	20
EAE-Cox8mCherry protein import 22 DPI	Mice	20

DPI, days post injection.

MATERIALS AND METHODS

Cloning and Packaging of Mthsp70 Into Adeno Associated Viral Serotype 2 (AAV2) Vector

The complementary (c) DNA of the mouse mthsp70 gene (Hspa9) was appended with a FLAG epitope to the C terminus then cloned into pTR-UF11 at *Xba*I and *Not*I restriction sites. Expression of the recombinant gene was driven by the 381-bp cytomegalovirus (CMV) immediate early gene enhancer/1260-bp chicken β -actin promoter-exon 1-intron 1. The reading frame of the insert was verified by Sanger sequencing. The ssAAV2-*mtHSP70Flag*, *sAAV2-GFP*, or *sAAV2-COX8mCherry* (23 amino acid COX8 mitochondrial targeting sequence appended to the N terminus of cherry) recombinant constructs were packaged into AAV2 virions with either single (444) or triple tyrosine (Y) to phenylalanine (F) mutations at positions 444, 500 and 730 (Y444F+Y500F+Y730F) in the VP3 capsid^{36,37} using the plasmid cotransfection method.³⁸ Briefly, plasmids were purified by cesium chloride gradient centrifugation and transfected into human embryonic kidney 293 cells. Recombinant viral particles extracted by freeze thaw cycles were purified using a fast protein liquid chromatography system (AKTA; Amersham Pharmacia, Piscataway, NJ, USA). Vector was eluted from the column using 215 mM sodium chloride (pH, 8.0), and the recombinant adeno associated virus (rAAV) peak was collected. Vector-containing fractions were 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). Vector was then titered for DNase resistant vector genomes by RT-PCR relative to a standard. 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 .

Intravitreal Injections

Female DBA/1J mice (The Jackson Laboratory, Bar Harbor, ME, USA) were sedated by inhalation with 1.5% to 2% isoflurane. A local anesthetic (proparacaine hydrochloride) was applied topically to the cornea and pupils were dilated with 1% tropicamide. A 32-G needle attached to a Hamilton syringe (Hamilton Company, Reno, NV, USA) was inserted through the pars plana under the dissecting microscope and one microliter of ssAAV2 *mtHSP70Flag* (3.45×10^{12} VG/mL) or *sAAV2-GFP* (1.03×10^{12} VG/mL) or *sAAV2-COX8mCherry* (8.66×10^{12} VG/mL) was injected into the vitreous. The use of animals was approved by the University of Miami IACUC and performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

EAE Sensitization and rAAV Injections

Female DBA/1J mice ($n = 20$) were sensitized for EAE by subdermal injection of 0.1 mL sonicated homologous spinal cord emulsion in complete Freund's adjuvant (Difco, Detroit, MI, USA) in the nuchal area and randomly divided into two groups. One group was rescued with intravitreal injection of 1 μL ssAAV2 *mtHSP70Flag* (3.45×10^{12} VG/mL) into both eyes, whereas a second group received 1 μL *sAAV2-COX8mCherry* (8.66×10^{12} VG/mL) into both eyes acted as disease control. Control animals sensitized with Freund's adjuvant alone also received 1 μL *sAAV2-COX8mCherry* (8.66×10^{12} VG/mL) into both eyes. Encephalomyelitis sensitization and intravitreal injections were done simultaneously at the same time for all the experiments except, mitochondrial import of COX8mCherry in EAE-AAV2GFP vs EAEAAV-mthsp70 where, the first injection (GFP or mthsp70) was done at 3 days after sensitization and COX8mCherry injections done 6 days after EAE sensitization. The number of animals used in each experiment is summarized in the Table.

Flat Mounts, Cryosections, and Immunofluorescence

One week after rAAV injections, mice were euthanized and the globes were dissected out and fixed in 4% paraformaldehyde (PFA) for 1 hour, then immersed in 0.4% PFA overnight. The cornea and lens were removed and the retina was carefully dissected from the eyecup and flattened by making four radial cuts from edge to the equator. Retinas were washed in PBS (pH, 7.4) three times, permeabilized in 0.5% Triton X-100 (Dow Chemical Corporation, Midland, MI, USA) in PBS for 1 hour and blocked in PBS containing 0.5% Triton X-100 and 10% goat serum for 1 hour. The flat-mounted retinas were rinsed in PBS and incubated with a mixture of mouse monoclonal anti-flag (1:100; Sigma, Cambridge, MA, USA), rabbit polyclonal porin (1:100; Abcam, Cambridge, MA, USA) and rat polyclonal Thy1.2 (1:100; Abcam) antibodies overnight at 4°C . Retinas were washed with PBS three times and were incubated with 1:500 dilution of goat anti-mouse Cy3 (flag), goat anti-rabbit Cy5 (porin), and goat anti-rat Cy2 (thy1.2) antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), along with 4',6-diamidino-2-phenylindole (DAPI; $2\mu\text{g/mL}$; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), at 4°C overnight. All the primary and secondary antibodies were diluted in PBS containing 0.2% Triton X-100 and 10% goat serum. The retinas were washed three times and were transferred to glass slides with the RGC layer facing upward, a coverslip was placed on the specimen and 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) and placed at -80°C overnight, serial sections of 8 μm in thickness were cut using a cryostat, mounted with fluorescent mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA) and examined with the confocal microscope.

RNA Isolation and Reverse Transcriptase PCR

Total RNA was isolated from the retina, optic nerve, brain, and spinal cord from the mice killed 50 days post injection (DPI) of ssAAV2 *mtHSP70Flag* using the RNeasy mini Kit (Qiagen Sciences, Germantown, MD, USA). Ribonucleic acid was quantified and 500-ng total RNA was used to reverse transcribe into cDNA (iScript; Bio-Rad, Hercules, CA, USA) and 2 μL of cDNA mix was used to further amplify using gene specific primers, 18S rRNA was used as a housekeeping

control. The primer sets used for PCR amplification include: mtHSP70F-5'-GCTCCTTGCTCGAAAGGACAGT-3', FlagR: 5'-TGTCATCGTCGTCCTTGAGTC-3', which amplifies cDNA of the recombinant mtHSP70Flag and not the endogenous mtHSP70, with an expected PCR product size of 200 bp. 18S rRNA F-5'-GAACTGAGGCCATGATTAAGAG-3', 18S rRNA R-5'-CATTCTTGGCAAATGCTTTC-3' primer set amplifies endogenous 18S rRNA with an expected PCR product size of 120 bp. Resultant PCR products were electrophoresed on a 1% agarose gel.

Mitochondria Isolation and Western Blotting

Whole-tissue lysates, mitochondrial, and cytoplasmic protein fractions were isolated from retina, optic nerve, brain, and spinal cord from the mice euthanized 15 DPI of ssAAV2-*mtHSP70-Flag* or *scAAV2-Cox8mCherry*. Briefly, tissues were washed twice in ice-cold PBS, resuspended in buffer containing 20 mM HEPES-KOH pH 7.5, 0.2 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, and 1 mM EDTA, and then homogenized using the Omni THQ-Digital tissue homogenizer (OMNI International, Kennesaw, GA, USA). A 100 μ L of the whole-tissue homogenate was separated and stored at -80°C until use. Differential centrifugation was performed on the rest of the tissue homogenates at 750g for 3 minutes, 3500g for 3 minutes, and 5000g for 3 minutes at 4°C . The supernatants were centrifuged at 10,500g for 15 minutes at 4°C . The cytoplasmic fraction was carefully separated from the mitochondrial pellet and further concentrated using the Amicon Ultra centrifugal filters with 3 kDa cut off (Millipore, Carrigtwohill, Co., Cork, Ireland) according to the manufacturer's instructions and stored at -80°C until use. The pellets containing the mitochondria were washed with the same buffer twice more and then were stored at -80°C until use. Proteins were quantified using the Bio-Rad protein assay kit. Equal amounts of protein were loaded on the 4% to 12% NuPage Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and were electrotransferred onto polyvinylidene difluoride membranes. Membranes were blocked in tris buffered saline tween (TBST) containing 5% nonfat dry milk and 0.5% Tween 20 for 1 hour and incubated with respective primary antibodies that included rat monoclonal to FLAG epitope (Sigma), rabbit monoclonal to mtHSP70 (Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit polyclonal to porin, and rabbit polyclonal to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Abcam, Inc.). The membranes were washed three times with TBST buffer and incubated with the respective secondary antibodies (goat anti-rat IgG and goat anti-rabbit IgG conjugated with horseradish peroxidase [HRP; Santa Cruz Biotechnology]). Membranes washed in TBS tween 20 buffer three times and immunodetected using the enhanced chemiluminescence (ECL) system (GE Healthcare, Piscataway, NJ, USA).

Pattern Electroretinography (PERG) Recordings

All mice were monitored for visual defects. PERGs were recorded for all three groups (control-mCherry, EAE-mCherry, and EAE-mtHSP70Flag at 1, 3, and 6 MPI as previously described.^{39,40} Briefly, mice were anesthetized with intraperitoneal injections of ketamine/xylazine hydrochloride solution (80 and 10 mg/kg body weight, respectively) and were restrained with the use of bite bar and nose holder. Body temperature of the mice was maintained constant at 37°C using a feedback controlled heating pad. The eyes of the mice were wide open and pupils pointing laterally and upward. The ERG silver electrode configured to 0.25-mm diameter, semicircular loop was positioned on the corneal surface encircling the pupil without limiting the field of view. The reference and ground

electrodes made of stainless steel needles were inserted under the skin of the scalp and tail, respectively. A drop of balanced saline solution was applied to the corneal surface to prevent drying. 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. The retinal signals obtained 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 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).

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

All mice were monitored for changes in retinal thickness due to optic neuritis using spectral-domain optical coherence tomography (SD-OCT; BiopTigen, Inc., Durham, NC, USA). Imaging was performed on live mice at 1, 3, and 6 MPI and high-resolution three-dimensional (3D) retinal images were obtained as described previously.^{39,41} Briefly, the mice were anesthetized by intraperitoneal injection of ketamine and xylazine (180 and 10 mg/kg body weight, respectively) and the pupils were dilated using 1% tropicamide. Systane Ultra (Alcon Laboratories, Inc.) lubricating eye drops were applied to the cornea to maintain hydration and clarity. Mice were placed on a custom stage which allowed free rotation to align the eye for imaging the optic nerve head (ONH) and retina. Rectangular volume scans measuring 512×128 (horizontal $\times$ vertical) and 1024×64 depth scan patterns, with the fast scan in the horizontal direction were acquired for each eye. The scan length was approximately 30° for mouse eye. The images were processed using the macro written in MATLAB software (Mathworks, Natick, MA, USA). For thickness measurements, 25 images around the ONH were used from each eye. The images were manually segmented by drawing the boundaries from the nerve fiber layer (NFL) to the inner boundary of inner nuclear layer and this information was used to construct 3D geometrical plots of the retina using algorithms in MATLAB software. Eyes with images showing shadowing due to media opacities were excluded from analysis.

Histologic Sectioning of the Optic Nerves and Retina

Six months after EAE sensitization and rAAV injections mice were euthanized and perfused with 4% PFA in 0.1 M PBS (pH 7.4). The globes were dissected out, the anterior segments, lens, and cornea of the eye was removed. Optic nerves and eye cups with retina were separated leaving 2 mm of optic nerve attached to the eye cup. The specimens were further processed for light and electron microscopic examination as described previously. Briefly, specimens were fixed 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. Semithin longitudinal sections (0.5 μ m) of the optic nerve and retina stained with toluidine blue were used for light microscopic examination. The number of cells in the RGC layer was counted from 10 random sections from each eye of each group. Ultrathin sections (90 nm) were placed on copper grids for transmission electron microscopic examination.

Transmission Electron Micrographs and Optic Nerve Demyelination

Ultrathin optic nerve and retinal sections were examined on a Hitachi H-7000 transmission electron microscope (Tokyo, Japan) operating at 80 kV. Photographs were obtained at a magnification of $\times 2500$. Ten micrographs of each optic nerve were used for quantitative analysis. The number of axons in each image were counted manually and averaged for all the optic nerves in each group.

Complex I, III, and I+III Activity Assay

Three months post injection unsensitized and EAE sensitized mice rescued with *mtHSP70Flag* or injected with *mCherry* were euthanized and the retinas were dissected. The tissue was homogenized in mitochondrial isolation buffer to disrupt the cells, but the mitochondria were intact. The nicotinamide dehydrogenase (NADH)-cytochrome oxidoreductase activity was assayed in 0.1 M potassium phosphate pH 7.5, 2 mM NADH, 10 mM KCN, and 1 mM oxidized cytochrome C at 30°C using equal amounts of tissue homogenate. The NADH oxidoreductase activity was measured spectrophotometrically at 550 nm for 2 minutes and the sensitivity to rotenone was tested 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.

Mitochondrial Import of *Cox8mCherry* in EAE-GFP Versus EAE-*mtHSP70* Rescued Mice

Three days after EAE sensitization, mice were rescued with intravitreal injection of ss-AAV2 *mtHSP70Flag* or scAAV2-*GFP*. To look at import of *Cherry* into mitochondria, all mice received an intravitreal injection of scAAV2-*COX8-mCherry* 6 days after EAE sensitization. The expression of GFP and *mCherry* was monitored in live mice by confocal laser scanning ophthalmoscopy (CSLO; Heidelberg Carlsbad, CA, USA) imaging 14 days after sensitization as previously described.³⁹ Mice were euthanized 22 days after sensitization and the mitochondria were isolated from the retinas then immunoblotted for *mCherry*. Band intensities in EAE-GFP and EAE-*mtHSP70Flag* injected group were measured using ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and normalized with the porin band intensity. The ratio of *mCherry* to porin was plotted.

Statistical Analysis

All the data obtained were analyzed using one-way ANOVA. If the overall *P* value was significant then comparisons between the groups were done by Newman-Keuls multiple comparison test and adjusted *P* less than 0.05 was considered as statistically significant.

RESULTS

AAV2-Mthsp70flag Efficiently Transduces Retinal Ganglion Cells

Confocal microscopy of retinal flat mounts of normal mice 1 week after intravitreal injection with single-stranded AAV serotype 2 (ssAAV2) containing the murine *mtHSP70Flag*, revealed DAPI-labeled nuclei (Fig. 1A), mitochondrial marker voltage dependent anion channel (VDAC)/porin (Fig. 1B),

RGCs labeled by *thy1.2* (Fig. 1C) expressing *mtHSP70Flag* (Fig. 1D), and a merged image (Fig. 1E). Longitudinal retinal sections show DAPI (Fig. 1F) and porin (Fig. 1G), *Thy1.2*-labeled RGCs (Fig. 1H), *mtHSP70Flag* expression in the RGC layer (Fig. 1I), and a merged image (Fig. 1J). Higher magnification shows DAPI-labeled RGC nuclei (Fig. 1K), porin (Fig. 1L), *thy1.2*-positive RGCs (Fig. 1M) exhibiting perinuclear *mtHSP70* expression (Fig. 1N) within RGCs (Fig. 1O). Thus, *mtHSP70Flag* appears to be translated in the cytoplasmic and mitochondrial compartments of the targeted RGCs.

We also detected transcription of *mtHSP70Flag* in the retina. Reverse transcriptase analysis using sequence specific primers on total RNA extracted and pooled independently from the optic nerve, retina, brain, and spinal cord samples 50 days after intravitreal injections with *mtHSP70Flag* revealed *mtHSP70Flag* transcripts exclusively in the infected retina and optic nerve samples (Fig. 1P). An internal control, 18S rRNA, was seen in all tissue samples tested. Immunoblotting with an anti-FLAG antibody on pooled whole-tissue lysates from the optic nerve, retina, brain, and spinal cord showed the approximate 75 kDa band of *mtHSP70Flag* exclusively in the optic nerve and retina samples of mice injected with AAV-*mtHSP70Flag* (Fig. 1Q, top row). Mice injected with *COX8-mCherry* did not show the corresponding approximate 75 kDa band in any of the tissues examined (Fig. 1R, top row). Immunoblotting for endogenous *mtHSP70* (GRP75) detected bands in all four tissues of *mtHSP70Flag* and *COX8-mCherry*-injected groups, but it was overexpressed only in the optic nerve and retina samples of *mtHSP70Flag*-injected mice (Fig. 1Q), but not in *COX8-mCherry*-injected mice (Fig. 1R). Porin and GAPDH loading controls showed the samples contain both cytoplasmic and mitochondrial components. Thus, infection with AAV-*mtHSP70Flag* overexpressed mitochondrial HSP70 in the visual system of mice.

To determine the sites of overexpression, we examined the distribution of *mtHSP70* in mitochondrial and cytoplasmic fractions of the optic nerve and retina of mice injected with *mtHSP70Flag* or *COX8-mCherry*. Immunoblotting using the anti-FLAG antibody detected the approximate 75 kDa band in the mitochondrial enriched fractions (Fig. 1S) and in the cytoplasmic (Fig. 1T) fractions of *mtHSP70Flag*-injected mice that was absent in the *COX8-mCherry* mice. Endogenous mitochondrial HSP70, detected using an antibody against *mtHSP70* showed bands in both mitochondrial and cytoplasmic fractions of *mtHSP70FLAG* and *COX8-mCherry*-injected mice (Fig. 1S, T). VDAC/Porin was detected in mitochondrial samples (Fig. 1S) GAPDH detected cytoplasmic fractions (Fig. 1T). Thus, mice injected with *mtHSP70Flag* showed overexpression of the *mtHSP70* in the cytoplasm and mitochondria of retinal ganglion cells and their axons in the optic nerve. Next, we evaluated the effects of *mtHSP70* overexpression on experimental optic neuritis.

Mitochondrial HSP70 Overexpression Suppresses Visual Loss in EAE

The PERG, a sensitive measure of RGC and visual function, was used to evaluate progression of optic neuritis in mice that were either unsensitized and vitreally injected with *Cox8-mCherry* or EAE sensitized and injected with *Cox8-mCherry* or *mtHSP70Flag* at 1, 3, and 6 months. At 1 month, there were no significant differences in the mean PERG amplitudes. Compared with the unsensitized *Cox8-mCherry* group at 3 months, the mean PERG amplitude decreased significantly in the EAE-*Cox8-mCherry* group by 42% ($P < 0.01$) and by 28% in EAE-*mtHSP70Flag* eyes ($P < 0.05$; Fig. 2A). While the EAE-*mtHSP70Flag*-injected group showed a 32% greater mean PERG amplitude compared with the EAE-*Cox8-mCherry* group,

FIGURE 1. Expression of mitochondrial HSP70Flag. At 1 week post injection of recombinant AAV2-mtHSP70Flag retinal flat mounts show DAPI (A), porin (B), thy1.2 (C), mtHSP70Flag (D), and all four channels merged (E). Longitudinal retinal sections of DAPI (F) porin (G), and thy1.2 (H), mtHSP70Flag (I), and merged (J). The RGC layer at higher magnification shows DAPI (K), porin (L), thy1.2 (M), mtHSP70Flag (N), and merged (O). At 50 days post injection, RT-PCR shows 18S rRNA transcripts in the optic nerves, retinas, brains, and spinal cords with mtHSP70Flag transcripts only in the retinas and optic nerves (P). Western blotting of mtHSP70Flag-injected mice revealed the transgene in whole-tissue lysates (Q, top row) that was absent in Cox8mCherry-injected mice (R, top row). Mitochondrial (S) and cytoplasmic fractions (T) are also shown. The square box shown in longitudinal sections were zoomed in RGC images (K–O). Scale bars: 100 μ m for whole mounts, 25 and 5 μ m for longitudinal sections; $n = 5$ for immunofluorescence, $n = 6$ for RT-PCR, $n = 20$ for Western blots, number of repetitions = 3 for each experiment. RE, retina; ON, optic nerve; INL, inner nuclear layer; ONL, outer nuclear layer.

this difference was not statistically significant. At 6 months, mean PERG amplitudes dropped further in the EAE-Cox8-mCherry group by 50% compared with the unsensitized Cox8-mCherry group, ($P < 0.001$). In contrast, PERG amplitudes remained stable in the EAE-mtHSP70Flag group. The EAE-mtHSP70Flag group showed a 44% rescue of PERG amplitude compared with the EAE-Cox8-mCherry group (Fig. 2A, Supplementary Table S1; $P < 0.05$).

While no differences in amplitudes were detected at 3 months, the EAE-mtHSP70Flag group showed less delay in latency relative to the EAE-Cox8-mCherry at 3 MPI ($P < 0.05$) that was maintained at 6 MPI ($P < 0.05$; Fig. 2B, Supplementary Table S2). Compared with the unsensitized Cox8-mCherry group, the EAE-mtHSP70Flag mice showed no significant delays in latency at 3 or 6 MPI. However, PERG latencies were delayed in the EAE-Cox8-mCherry group by 16% ($P < 0.05$) and 18% ($P < 0.05$) at 3 and

6 MPI, respectively. Averaged PERG waveforms at 6 MPI illustrate a decrease in amplitude and delay in latency associated with EAE was suppressed by *mtHSP70Flag* (Fig. 2C). Thus, *mtHSP70Flag* expression preserved RGC function in EAE mice. We next evaluated if *mtHSP70* would suppress loss of RGCs, the dreaded hallmark of human optic neuritis and MS.

Overexpression of Mitochondrial HSP70 Preserves the RGC and the Nerve Fiber Layer of EAE Mice (OCT)

Our results of serial high-resolution SD-OCT imaging and a subsequent analysis using MATLAB showed normal thickness of the RGC and inner plexiform layers in unsensitized mice that were injected with *Cox8-mCherry* at 3 (Fig. 3A) and 6 MPI (Fig. 3B). While the EAE mice injected with *Cox8-mCherry* showed thinning of these retinal layers (RGC and inner plexiform layers) at 3 (Fig. 3C) and 6 MPI (Fig. 3D), the *mtHSP70Flag*-injected mice showed normal thickness at 3 (Fig. 3E) and 6 MPI (Fig. 3F). Quantitative analysis of mean thickness revealed a 15% thinning of the inner retina at 6 MPI, in the EAE *Cox8-mCherry* group compared with the unsensitized *Cox8-mCherry* group ($P < 0.001$; Fig. 3G, Supplementary Table S3). Interestingly, *mtHSP70Flag*-injected mice showed a 92% rescue when compared with the EAE-*Cox8-mCherry* group ($P < 0.001$). Thus, as gauged by OCT, *mtHSP70Flag* ameliorated RGC loss that results in permanent disability in optic neuritis and MS.

Mitochondrial HSP70 Prevents EAE Mediated RGC Loss (Histopathology)

Light micrographs of retinal longitudinal sections of mice euthanized 6 months after EAE sensitization and rAAV injections confirmed that loss of RGCs was prevented by mitochondrial HSP70Flag infection. The EAE-*Cox8-mCherry* retina showed loss of cells in the RGC layer (Fig. 4A) relative to the EAE-*HSP70Flag* retina (Fig. 4B). Transmission electron micrographs of the RGC layer showed loss of RGCs in the EAE *Cox8-mCherry* retina (Fig. 4C) relative to the *mtHSP70Flag* rescued mice (Fig. 4D). Apoptotic RGCs with condensed cytoplasm and increased electron density of the nucleus were evident in the *Cox8-mCherry* retina (Fig. 4E). Quantitative analysis revealed a mean RGC count of 98 ± 2.6 cells/mm ($\pm$ SE) in the EAE-*Cox8-mCherry* group, that was 40% less than the unsensitized *Cox8-mCherry* control group (164 ± 2 cells/mm, $\pm$ SE). The *mtHSP70Flag*-rescued mice showed a mean RGC count of 148 ± 3 cells/mm ($\pm$ SE), (i.e., a 10% loss compared with the unsensitized *Cox8-mCherry* control group; $P < 0.01$; Fig. 4F, Supplementary Table S4). Relative to mock treatment, *mtHSP70Flag* rescued 76% of cells in the RGC layer ($P < 0.001$). As *mtHSP70* preserved RGC function and structure, we next studied the effects on the optic nerve.

FIGURE 2. Visual function. A bar plot shows mean PERG amplitudes (A), latencies (B), and averaged PERG waveforms (C) of unsensitized mice vitreally injected with AAV2-*Cox8-mCherry* (Con/mCh), EAE mice vitreally injected with AAV2-*Cox8-mCherry* (EAE-mCh) and EAE mice vitreally injected with AAV2-*mtHSP70Flag* (EAE-mtHSP70) at 1 MPI ($n = 18, 17$, and 18 , respectively), 3 MPI ($n = 15, 16$, and 10 , respectively) and 6 MPI, respectively ($n = 10, 17$, and 13). Number of repetitions = 3, for each group and time point, * $P = 0.05$ to 0.01 ; ** $P = 0.001$ to 0.009 ; *** $P = 0.0001$ to 0.0009 .

FIGURE 3. In vivo imaging of the retina. Cross-sectional OCT images of the retina (top of panels) and 3D thickness maps (bottom of panels) show the thickness of the RGC+IPL layers in unsensitized *Cox8-mCherry*-injected mice at 3 MPI ($n = 11$; [A]) and 6 MPI ($n = 10$; [B]).

Mitochondrial HSP70 Suppresses Axonal Loss in EAE Mice

Transmission electron micrographs of optic nerves obtained from mice euthanized 6 months after AAV injections showed loss of axons in the EAE-*Cox8-mCherry* group, whereas treatment with mtHSP70Flag suppressed axon loss. The EAE-*Cox8-mCherry* optic nerves showed few infiltrating mononuclear inflammatory cells, many cystic spaces where axons were presumably lost and remaining axons with thin or absent myelin (Fig. 5A) and electron dense aggregates indicative of ongoing axonal degeneration (Fig. 5B). In contrast, the EAE-mtHSP70Flag-treated group showed greater axon density (Fig. 5C) and axons with intact microtubules (Fig. 5D). Quantitative analysis revealed a 41% loss of axons in the optic nerves of EAE-*Cox8-mCherry* compared with the unsensitized control-*Cox8-mCherry* group ($P < 0.01$) (Fig. 5E, Supplementary Table S5). Mice injected with mtHSP70Flag showed 85% more axons than the EAE-*Cox8-mCherry* mice ($P < 0.01$). In fact, axon counts in mtHSP70Flag rescued mice were comparable with those of the unsensitized *Cox8-mCherry*-injected mice. Therefore, axonal loss associated with the neurodegeneration of EAE was ameliorated by mitochondrial HSP70. Next, we tested whether this rescue improved mitochondrial function.

Mitochondrial HSP70 Improves Complex I and III Function

We examined mitochondrial respiratory complex I and III activities in EAE mice that were mock-treated with *Cox8-mCherry*, mice rescued with mtHSP70Flag and unsensitized mice injected with *Cox8-mCherry* at 3 months post antigenic sensitization. We found that relative to unsensitized controls injected with *Cox8-mCherry*, complex I activity was reduced by 28% ($P < 0.001$) complex III by 40% ($P < 0.0001$), and the overall total activity of complexes I+III was reduced by 34% ($P < 0.0001$) in the EAE *Cox8-mCherry* group (Fig. 6, Supplementary Table S6). Treatment with mtHSP70 showed 100% rescue of complex I, III activities independently, and complex I+III total activity compared with mock-treated EAE mice ($P < 0.0001$). Next, we wondered if the effects of EAE reduced protein import into mitochondria and if mtHSP70Flag rescued this dysfunction.

Mitochondrial HSP70Flag Improves Protein Import Into Mitochondria

Three days after EAE sensitization, mice were vitreally injected with mtHSP70Flag or GFP. Three days later, all the mice received intravitreal injection of *Cox8-mCherry*, followed by confocal laser scanning ophthalmoscopy a week later (i.e., 2 weeks post-EAE sensitization). While the GFP-positive cells were observed in EAE mice injected with GFP (Fig. 7A), absent in the mtHSP70Flag group (Fig. 7B), *COX8-mCherry* was observed in all groups (Figs. 7A, 7C, 7D). Twenty-two days after sensitization, immunoblotting of retinal mitochondrial fractions using anti-Cherry antibody revealed the mCherry band had a greater intensity in mtHSP70Flag-injected eyes than GFP

Encephalomyelitis-mCherry mice show RGC+IPL thinning at 3 MPI ($n = 9$; [C]) and 6 MPI ($n = 8$; [D]). The EAE-mtHSP70 retinas show normal thickness of RGC+IPL layers at 3 MPI ($n = 7$; [E]) and 6 MPI ($n = 7$; [F]). A bar plot shows the mean thickness of the RGC+IPL layers at 3 and 6 MPI in con-mCherry, EAE-mCherry, and EAE-mtHSP70 groups (G). * $P = 0.05$ to 0.01; ** $P = 0.001$ to 0.009; *** $P = 0.0001$ to 0.0009.

FIGURE 4. Histology and ultrastructure of the retina. At 6 months post-rAAV injection and EAE sensitization, toluidine blue stained light micrographs of EAE-*Cox8-mCherry* mice show thinning of the inner retina (A) relative to the mthHSP70Flag rescued retina (B). Transmission electron micrographs of the EAE-*Cox8-mCherry* retina show loss of RGCs (C). The mthHSP70Flag rescued eyes show more RGCs (D). Apoptotic RGCs (arrowheads) are seen in the EAE-*Cox8-mCherry* retinas (E). A bar graph shows mean ($\pm$ SE) cell counts of the RGC layer (F). $n = 9$ for Control-mCherry, $n = 12$ for EAE-mCherry, and $n = 15$ for EAE-mthHSP70Flag. * $P = 0.05$ to 0.01 ; ** $P = 0.001$ to 0.009 ; *** $P = 0.0001$ to 0.0009 .

controls (Fig. 7E). Quantitative analysis of the band intensities of mCherry normalized to mitochondrial control VDAC/Porin showed 30% more mCherry in *mtHSP70Flag*-injected eyes compared with those injected with *GFP* (Fig. 7F; $P < 0.05$). Thus, mthHSP70 overexpression improved defective mitochondrial protein import associated with EAE.

DISCUSSION

In the present study, we demonstrated that overexpression of the mthHSP70 in retinal ganglion cells and their axons in EAE mice preserves vision, prevents neuronal apoptosis, and axonal demise, improves mitochondrial complex I and III activities,

FIGURE 5. Ultrastructure of the optic nerve. A representative electron micrograph of the EAE-Cox8-mCherry optic nerve shows axons with thin or absent myelin, few inflammatory cells (IC), and many cystic spaces (*; **A**). At higher magnification axons are undergoing wallerian degeneration (arrow; **B**). Micrographs of the mHSP70 rescued optic nerve show more axons (**C**). At higher magnification axonal microtubules are intact (**D**). A bar plot shows mean ($\pm$ SE) axon counts of unsensitized controls, EAE-mCherry-, and EAE-mHSP70-injected mice (**E**). $n = 12$ for each group, $**P = 0.001$ to 0.009 , Ax = axon.

and protein import into mitochondria. Although not directly involved in the mitochondrial electron transport chain (ETC), mtHSP70 is an import chaperone crucial for translocation of cytoplasmically synthesized proteins into the mitochondrial matrix. The mitochondrial DNA encodes only 13 of the approximately 100 proteins involved in oxidative phosphorylation. The rest are imported from the cytoplasm. As suggested by the defective import of mCherry here, inactivation or loss of mtHSP70 function can attenuate the import of proteins into the organelle contributing to respiratory dysfunction. Oxidative damage to mtHSP70 was reported in the CNS of EAE mice, which resulted in loss of mitochondrial membrane potential and reduced mitochondrial ATP synthesis.²³ Spontaneous upregulation of HSP70 is observed in autopsied MS brains suggesting that it may have a protective role in neurons and oligodendrocytes.^{42,43} On the other hand, higher titers of cerebrospinal fluid antibodies against HSP70 were detected in progressive MS cases, suggesting that loss of mtHSP70 activity occurs when relapsing remitting MS converts to the neurodegenerative phase of progressive MS.⁴⁴ In light of these reports,

our study here shows that overexpression of mtHSP70 in RGCs of EAE mice improves mitochondrial dysfunction, preserves RGCs and their axons, thereby contributing to preservation of visual function.

Our results here corroborate those of others (i.e., that heat shock preconditioning resulting in overexpression of HSP70 in the rat EAE model was neuroprotective).⁴⁵ Similarly, drugs that induce the expression of HSP70 ameliorated EAE in rodents.^{46,47} While the loss of mtHSP70 function resulted in altered mitochondrial morphology and biogenesis,⁴⁸ its overexpression in rat brains improved mitochondrial function and protected against focal ischemia.⁴⁹ Our study adds support to these existing reports that mtHSP70 is crucial for mitochondrial function and its overexpression prevents the neurodegeneration of EAE.

As the therapeutic potential of HSP70 chaperones in various neurodegenerative diseases is well studied,^{50–59} why did we use gene therapy? We did so because there are ambiguous reports on HSP70's role in EAE and MS. While intracellular HSP70 is neuroprotective,^{28–30,32,60,61} HSP70 released into the

FIGURE 6. Complex I and III function. A bar plot shows the activities of complex I + III, complex I, or complex III (mean $\pm$ SE) of the pooled retinas obtained from unsensitized control-mCherry ($n = 15$), EAE-mCherry ($n = 15$), or EAE-mtHSP70 ($n = 9$) mice at 3 MPI. Number of repetitions = 3; ** $P = 0.001$ to 0.009 ; *** $P = 0.0001$ to 0.0009 .

extracellular environment by necrotic cells exerts adaptive and innate immune responses either by acting as a danger signal, which promotes dendritic cell maturation^{62,63} or by acting as an antigen adjuvant.^{64,65} It is reported that extracellular HSP70 protein in the CNS exacerbates inflammation in EAE and MS by binding and presenting myelin-antigens to antigen presenting cells.^{44,66–68} In conjunction with this, HSP70 knockout mice developed a milder form of EAE upon sensitization.⁶⁷ In contrast, the drugs like geldamycin, or triptolide, which induce expression of HSP70, also suppress the glial and pro-inflammatory responses by avoiding nuclear factor- κ B (NF- κ B) activation and by promoting regulatory T cells, thereby ameliorating EAE in rodents.^{46,47} It is also observed that HSP70 can also act via an anti-inflammatory mechanisms by inducing the expression of anti-inflammatory cytokines like IL10⁶⁹ or by downregulating the production of IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1.⁷⁰ The results presented in this article demonstrate that overexpression of the mtHSP70 chaperone in RGCs prevents the RGC and axonal demise of EAE preserving vision.

Mitochondrial dysfunction is increasingly identified as a major contributing factor in neurodegeneration of MS patients and EAE animal models. Oxidative stress mediated damage to mitochondrial proteins, lipids, and mitochondrial (mt) DNA were reported in EAE and MS autopsied brain lesions. Large base pair deletions in mitochondrial DNA encoding complex I subunits (i.e., ND3, ND4L, ND4, ND5, and ND6)²⁰ and point mutations within ND1, ATP6/8, and transfer (t) RNA (leu/Lys) encoding genes were detected in autopsied MS brain neurons,³⁴ and in peripheral leukocytes of optic neuritis patients that had poor recovery of function.⁷¹ Peroxynitrite mediated damage to the mitochondrial complex I subunit ND6A6, complex IV subunit of COX VIII and mtHSP70 were detected in EAE²³ reducing ATP production below levels seen in maternally transmitted disorders associated with mutated mitochondrial DNA. Overexpression of mtHSP70 overcame mitochondrial dysfunction and ameliorated the neuronal and axonal degeneration in EAE suggesting it may be of benefit in addressing this dreaded consequence of human optic neuritis and MS.

We have focused on the optic nerve for several reasons. The optic nerves have a high density of mitochondria and are therefore more susceptible to perturbations of mitochondrial

FIGURE 7. Mitochondrial protein import. Confocal scanning laser ophthalmoscopy of the retina of live EAE mice injected with scAAV2-GFP (A, C) or scAAV2-mtHSP70Flag (B, D) followed by scAAV2-Cox8m-Cherry show expression of mCherry in both GFP- and mtHSP70-infected mice in the red channel (C, D) and expression of GFP in GFP-infected mice in the green channel (A). Immunoblotting of the mitochondrial fractions obtained 22 days post-EAE sensitization shows mCherry in both GFP- and mtHSP70FLAG-injected mice, with the band intensity greater in mtHSP70Flag rescued mice (E). A bar graph compares the band intensities of mCherry normalized with porin in both groups (F). $n = 10$ in each group; * $P = 0.05$ to 0.01 .

dysfunction.⁷² Lesions in the optic nerve of EAE mice directly correlate to the loss of function and can be easily monitored by noninvasive techniques like PERG and OCT, as also done for MS and optic neuritis patients.^{73–75} In addition, the RGCs and their axons comprising the optic nerve are readily accessible for therapeutic intervention. Previous studies have shown effective and safe AAV2 gene delivery system that can target RGCs and the optic nerve.^{76,77} Tyrosine to phenylalanine mutations in the capsid proteins used in our study increase the speed and efficiency of transgene expression, crucial to intervention in optic neuritis with its characteristic rapid onset of permanent visual loss.^{78–82} Due to the large size of mitochondrial HSP70 we were unable to fit it into a self-complementary AAV cassette. In summary, we used a gene therapy approach to specifically target neurons and to increase

intracellular mitochondrial HSP70. We did this because it is well known that extracellular HSP70 exacerbates inflammation in EAE and MS.^{44,66–68} Taken together these findings suggest that gene therapy with mitochondrial HSP70 may be useful in patients with optic neuritis and MS.

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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, Taylor J, Chan-Ling T. 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, Mancuso A, Quisling RG. 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, Mancuso A. Gadolinium-DTPA-enhanced magnetic resonance imaging in experimental optic neuritis. *Ophthalmology.* 1990;97:601–607.
- Guy J, Mancuso A, Quisling RG, Beck R, Moster M. 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.
- Frohman EM, Racke MK, Raine CS. Multiple sclerosis—the plaque and its pathogenesis. *N Engl J Med.* 2006;354:942–955.
- Bjartmar C, Trapp BD. Axonal and neuronal degeneration in multiple sclerosis: mechanisms and functional consequences. *Curr Opin Neurol.* 2001;14:271–278.
- Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mörk S, Bö L. Axonal transection in the lesions of multiple sclerosis. *N Engl J Med.* 1998;338:278–285.
- 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.
- 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.
- 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.
- Cowden WB, Cullen FA, Staykova MA, Willenborg DO. Nitric oxide is a potential down-regulating molecule in autoimmune disease: inhibition of nitric oxide production renders PVG rats highly susceptible to EAE. *J Neuroimmunol.* 1998;88:1–8.
- Ruuls SR, Van Der Linden S, Sontrop K, Huitinga I, Dijkstra CD. Aggravation of experimental allergic encephalomyelitis (EAE) by administration of nitric oxide (NO) synthase inhibitors. *Clin Exp Immunol.* 1996;103:467–474.
- Qi X, Lewin AS, Sun L, Hauswirth WW, Guy J. Mitochondrial protein nitration primes neurodegeneration in experimental autoimmune encephalomyelitis. *J Biol Chem.* 2006;281:31950–31962.
- Kang PJ, Ostermann J, Shilling J, Neupert W, Craig EA, Pfanner N. Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature.* 1990; 348:137–143.
- Endo T, Yamano K. Multiple pathways for mitochondrial protein traffic. *Biol Chem.* 2009;390:723–730.
- Mokranjac D, Neupert W. The many faces of the mitochondrial TIM23 complex. *Biochim Biophys Acta.* 2010;1797:1045–1054.
- van der Laan M, Hutu DP, Rehling P. On the mechanism of preprotein import by the mitochondrial presequence translocase. *Biochim Biophys Acta.* 2010;1803:732–739.
- Flynn GC, Chappell TG, Rothman JE. Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science.* 1989;245:385–390.
- Beckmann RP, Mizzen LE, Welch WJ. Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. *Science.* 1990;248:850–854.
- Murakami H, Pain D, Blobel G. 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. *J Cell Biol.* 1988;107:2051–2057.
- Buka B, Deuerling E, Pfund C, Craig EA. Getting newly synthesized proteins into shape. *Cell.* 2000;101:119–122.
- Hartl FU, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science.* 2002;295:1852–1858.
- Young JC, Barral JM, Ulrich HE. More than folding: localized functions of cytosolic chaperones. *Trends Biochem Sci.* 2003; 28:541–547.
- Burbulla LF, Schelling C, Kato H, et al. Dissecting the role of the mitochondrial chaperone mortalin in Parkinson's disease:

- functional impact of disease-related variants on mitochondrial homeostasis. *Hum Mol Genet.* 2010;19:4437–4452.
35. Blamowska M, Neupert W, Hell K. Biogenesis of the mitochondrial Hsp70 chaperone. *J Cell Biol.* 2012;199:125–135.
 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. Petrs-Silva H, Dinculescu A, Li Q, et al. High-efficiency transduction of the mouse retina by tyrosine-mutant AAV serotype vectors. *Mol Ther.* 2009;17:463–471.
 38. Hauswirth WW, Lewin AS, Zolotukhin S, Muzyczka N. Production and purification of recombinant adeno-associated virus. *Methods Enzymol.* 2000;316:743–761.
 39. 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.
 40. Talla V, Yang C, Shaw G, Porciatti V, Koilkonda RD, Guy J. Noninvasive assessments of optic nerve neurodegeneration in transgenic mice with isolated optic neuritis. *Invest Ophthalmol Vis Sci.* 2013;54:4440–4450.
 41. 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.
 42. Aquino DA, Capello E, Weisstein J, et al. Multiple sclerosis: altered expression of 70- and 27-kDa heat shock proteins in lesions and myelin. *J Neuropathol Exp Neurol.* 1997;56:664–672.
 43. Cwiklinska H, Mycko MP, Luvsannorov O, et al. Heat shock protein 70 associations with myelin basic protein and proteolipid protein in multiple sclerosis brains. *Int Immunol.* 2003;15:241–249.
 44. Chiba S, Yokota S, Yonekura K, et al. Autoantibodies against HSP70 family proteins were detected in the cerebrospinal fluid from patients with multiple sclerosis. *J Neurol Sci.* 2006;241:39–43.
 45. Zhang JF, Huang R, Xu J, Jin SJ, Yang YJ. Protective effects of heat shock preconditioning on the experimental autoimmune encephalomyelitis rats [in Chinese]. *Zhongguo Dang Dai Er Ke Za Zhi.* 2007;9:563–566.
 46. Kizelsztejn P, Komarnytsky S, Raskin I. Oral administration of triptolide ameliorates the clinical signs of experimental autoimmune encephalomyelitis (EAE) by induction of HSP70 and stabilization of NF-kappaB/IkappaBalpha transcriptional complex. *J Neuroimmunol.* 2009;217:28–37.
 47. van Eden W. Protective and therapeutic effect of triptolide in EAE explained by induction of major stress protein HSP70. *J Neuroimmunol.* 2009;217:10–11.
 48. Sanjuan Szklarz LK, Guiard B, Rissler M, et al. Inactivation of the mitochondrial heat shock protein zim17 leads to aggregation of matrix hsp70s followed by pleiotropic effects on morphology and protein biogenesis. *J Mol Biol.* 2005;351:206–218.
 49. Xu L, Voloboueva LA, Ouyang Y, Emery JF, Giffard RG. Overexpression of mitochondrial Hsp70/Hsp75 in rat brain protects mitochondria, reduces oxidative stress, and protects from focal ischemia. *J Cereb Blood Flow Metab.* 2009;29:365–374.
 50. Aridon P, Geraci F, Turturici G, D'Amelio M, Savettieri G, Sconzo G. Protective role of heat shock proteins in Parkinson's disease. *Neurodegener Dis.* 2011;8:155–168.
 51. Kalia SK, Kalia LV, McLean PJ. Molecular chaperones as rational drug targets for Parkinson's disease therapeutics. *CNS Neurol Disord Drug Targets.* 2010;9:741–753.
 52. Evans CG, Chang L, Gestwicki JE. Heat shock protein 70 (hsp70) as an emerging drug target. *J Med Chem.* 2010;53:4585–4602.
 53. Nagai Y, Fujikake N, Popiel HA, Wada K. Induction of molecular chaperones as a therapeutic strategy for the polyglutamine diseases. *Curr Pharm Biotechnol.* 2010;11:188–197.
 54. Witt SN. Hsp70 molecular chaperones and Parkinson's disease. *Biopolymers.* 2010;93:218–228.
 55. Broadley SA, Hartl FU. The role of molecular chaperones in human misfolding diseases. *FEBS Lett.* 2009;583:2647–2653.
 56. Brown IR. Heat shock proteins and protection of the nervous system. *Ann N Y Acad Sci.* 2007;1113:147–158.
 57. Zheng Z, Yenari MA. The application of HSP70 as a target for gene therapy. *Front Biosci.* 2006;11:699–707.
 58. Franklin TB, Krueger-Naug AM, Clarke DB, Arrigo AP, Currie RW. The role of heat shock proteins Hsp70 and Hsp27 in cellular protection of the central nervous system. *Int J Hyperthermia.* 2005;21:379–392.
 59. Chung KK, Dawson TM. Parkin and Hsp70 sacked by BAG5. *Neuron.* 2004;44:899–901.
 60. Benn SC, Woolf CJ. Adult neuron survival strategies—slamming on the brakes. *Nat Rev Neurosci.* 2004;5:686–700.
 61. Beere HM. “The stress of dying”: the role of heat shock proteins in the regulation of apoptosis. *J Cell Sci.* 2004;117:2641–2651.
 62. Asea A, Rehli M, Kabingu E, et al. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem.* 2002;277:15028–15034.
 63. Kuppner MC, Gastpar R, Gelwer S, et al. The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. *Eur J Immunol.* 2001;31:1602–1609.
 64. Becker T, Hartl FU, Wieland F. CD40, an extracellular receptor for binding and uptake of Hsp70-peptide complexes. *J Cell Biol.* 2002;158:1277–1285.
 65. Suto R, Srivastava PK. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science.* 1995;269:1585–1588.
 66. Mansilla MJ, Montalban X, Espejo C. Heat shock protein 70: roles in multiple sclerosis. *Mol Med.* 2012;18:1018–1028.
 67. Mycko MP, Cwiklinska H, Walczak A, Libert C, Raine CS, Selmaj KW. A heat shock protein gene (Hsp70.1) is critically involved in the generation of the immune response to myelin antigen. *Eur J Immunol.* 2008;38:1999–2013.
 68. Galazka G, Stasiolek M, Walczak A, et al. Brain-derived heat shock protein 70-peptide complexes induce NK cell-dependent tolerance to experimental autoimmune encephalomyelitis. *J Immunol.* 2006;176:1588–1599.
 69. Detanico T, Rodrigues L, Sabritto AC, et al. Mycobacterial heat shock protein 70 induces interleukin-10 production: immunomodulation of synovial cell cytokine profile and dendritic cell maturation. *Clin Exp Immunol.* 2004;135:336–342.
 70. Luo X, Zuo X, Zhou Y, et al. Extracellular heat shock protein 70 inhibits tumour necrosis factor-alpha induced proinflammatory mediator production in fibroblast-like synoviocytes. *Arthritis Res Ther.* 2008;10:R41.
 71. Bosley TM, Constantinescu CS, Tench CR, Abu-Amro KK. Mitochondrial changes in leukocytes of patients with optic neuritis. *Mol Vis.* 2007;13:1516–1528.
 72. Barron MJ, Griffiths P, Turnbull DM, Bates D, Nichols P. The distributions of mitochondria and sodium channels reflect the specific energy requirements and conduction properties of

- the human optic nerve head. *Br J Ophthalmol*. 2004;88:286-290.
73. Sergott RC. Optical coherence tomography: measuring in-vivo axonal survival and neuroprotection in multiple sclerosis and optic neuritis. *Curr Opin Ophthalmol*. 2005;16:346-350.
 74. 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.
 75. Pasol J, Feuer W, Yang C, Shaw G, Kardon R, Guy J. Phosphorylated neurofilament heavy chain correlations to visual function, optical coherence tomography, and treatment. *Mult Scler Int*. 2010;2010:542691.
 76. Zaiss AK, Muruve DA. Immunity to adeno-associated virus vectors in animals and humans: a continued challenge. *Gene Ther*. 2008;15:808-816.
 77. Peden CS, Burger C, Muzyczka N, Mandel RJ. Circulating anti-wild-type adeno-associated virus type 2 (AAV2) antibodies inhibit recombinant AAV2 (rAAV2)-mediated, but not rAAV5-mediated, gene transfer in the brain. *J Virol*. 2004;78:6344-6359.
 78. Zhong L, Li B, Mah CS, et al. Next generation of adeno-associated virus 2 vectors: point mutations in tyrosines lead to high-efficiency transduction at lower doses. *Proc Natl Acad Sci U S A*. 2008;105:7827-7832.
 79. Mingozzi F, Maus MV, Hui DJ, et al. CD8(+) T-cell responses to adeno-associated virus capsid in humans. *Nat Med*. 2007;13:419-422.
 80. Zhong L, Li B, Jayandharan G, et al. Tyrosine-phosphorylation of AAV2 vectors and its consequences on viral intracellular trafficking and transgene expression. *Virology*. 2008;381:194-202.
 81. Pien GC, Basner-Tschakarjan E, Hui DJ, et al. Capsid antigen presentation flags human hepatocytes for destruction after transduction by adeno-associated viral vectors. *J Clin Invest*. 2009;119:1688-1695.
 82. Markusic DM, Herzog RW, Aslanidi GV, et al. High-efficiency transduction and correction of murine hemophilia B using AAV2 vectors devoid of multiple surface-exposed tyrosines. *Mol Ther*. 2010;18:2048-2056.