The Mutant Human ND4 Subunit of Complex I Induces Optic Neuropathy in the Mouse

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PURPOSE. To produce a mouse model of Leber hereditary optic neuropathy.

METHODS. A mutant ND4 subunit made compatible with the universal genetic code and containing an arginine-to-histidine substitution at residue 340, or a synthetic normal human ND4 gene was delivered to the mouse visual system. The expression and effects of the mutant ND4 gene on the optic nerve and cultured retinal ganglion cells was assessed by magnetic resonance imaging, immunohistochemistry, and light and transmission electron microscopy.

RESULTS. The ATPc mitochondrial targeting sequence directed the allotopically expressed mutant human R340H and wild-type ND4FLAG polypeptides into mitochondria. Expression of normal human ND4 in murine mitochondria posed no ocular toxicity. In contrast, the mutant ND4 disrupted mitochondrial cytoarchitecture, elevated reactive oxygen species, induced swelling of the optic nerve head, and induced apoptosis, with a progressive demise of ganglion cells in the retina and their axons comprising the optic nerve.

CONCLUSIONS. Allotopic expression of the mutant human R340H ND4 subunit of complex I replicated the hallmarks of human mitochondrial disease in the mouse. In contrast, occult expression of the wild-type human ND4 subunit in lower mammals appears safe, suggesting that it may be useful for treatment of patients with Leber hereditary optic neuropathy.

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Leber Hereditary Optic Neuropathy (LHON) is a maternally inherited disorder that results in loss of vision during the second and third decades of life. The G-to-A transition at nucleotide 11778 in mitochondrial DNA (mtDNA) in the gene specifying the ND4 dehydrogenase subunit 4 (ND4) of complex I causes half the cases.1,2 It results in an arginine-to-histidine substitution at amino acid 340.3 Still, approximately 45 other pathogenic point mutations in human polypeptide-coding mtDNA genes have been linked to LHON.4 Of all diseases caused by mutated mitochondria LHON is the most common.5

To deter the effects of LHON, we have adapted the approach of "allotopic expression,"6 in which a nuclear-encoded version of a gene normally encoded by mitochondrial DNA (ND4 in this case) specifies a protein expressed in the cytoplasm that is then imported into the mitochondria. Using this approach we rescued LHON cybrid cells homoplasmic for the G11778A mutation in mitochondrial DNA.7,8 The ND4 protein is highly hydrophobic, and others have reported that it cannot be imported into the organelle from the cytoplasm.9 In this report, we describe expression of the wild-type gene in the mouse visual system and replication of the human disease LHON in the mouse by delivery of a nuclear-encoded version of the mutant human ND4 subunit gene.

MATERIALS AND METHODS

Construction of Mutant ND4 and AAV Vectors

To construct the fusion gene containing the mitochondrial targeting sequences and epitope tag, synthetic 80 mer oligonucleotide pairs were created in the nuclear genetic code and codons prevalent in highly expressed nuclear genes to conserve the amino acid sequence. The synthetic oligonucleotides were overlapped by approximately 20 complementary nucleotides serving as primers for PCR with the high fidelity of pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) until the entire 13777-nucleotide-ncRNA-encoded ND4 gene was constructed. Using this technique, we then fused the ND4 gene in-frame to the ATP1 and FLAG epitope tags (Fig. 1A). To complete generating the wild-type ND4, base deletions and substitutions in the reading frame were corrected using an in vitro mutagenesis kit (QuickChange; Stratagene). To generate the mutant R340H ND4, we exchanged the AGG encoding for arginine to the CAC codon for histidine at amino acid 340 by using site-directed mutagenesis. Flanking Xbal restriction sites were added for cloning the mutant and wild-type versions of P1ND4Flag into AAV vectors. The entire reading frame of the mutant and wild-type versions of the P1ND4Flag fusion gene was cloned in the Xbal sites of AAV plasmid vectors pTR-UF22 (regulated by the 381-bp CMV immediate early gene enhancer/1532-bp chicken β-actin promoter-exon 1-intron 1/woodchuck postregulatory element [WPRE]). To generate mitochondrial targeting expression of mutant or wild-type versions of P1ND4Flag and cytoplasm-targeted expression of green fluorescent protein (GFP) in the same cell, we used the pTR-UF22 vector that had P1ND4Flag linked to GFP via a 657-bp poliovirus internal ribosomal entry site (IRES). Visualization of cytoplasmic GFP enabled us to identify those cells that were also expressing mutant or wild-type versions of P1ND4Flag, which had been inserted upstream of the IRES. The plasmids were amplified and purified by cesium chloride gradient centrifugation and then packaged into AAV-2 capsids by transfection into human 293 cells using standard procedures. Genome titers of the recombinant AAV (rAAV) were determined using real-time PCR.10 The resultant rAAV-packaged ND4s and humanized GFP control viruses were assayed, and each virus preparation was found to contain 1011 to 1012 particles per milliliter.

Cell Culture and Viral Transfection

Human 143B osteosarcoma cells containing wild-type mtDNA,8 human embryonic kidney (293) cells, murine fibroblasts (3T3) and rat retinal ganglion cell-like cells (RGC-5) were cultured. For AAV infections, cells at approximately 50% to 70% confluence were transfected with 5.0 × 107 AAV viral particles in complete high-glucose medium. Selection in galactose was performed in 10 separate wells, with the cells treated with selective medium for three days.8 Cells were trypsinized...
and counted with an automated particle counter (Z-100; Beckman Coulter, Hialeah, FL).

Detection of Reactive Oxygen Species and Apoptosis

The probe dihydroethidium was used to detect intracellular superoxide (O$_2^-$). Superoxide oxidizes dihydroethidium to yield a red fluorescent signal. RGC-5 cells infected with the AAV-expressing mutant ND4 or with control virus were incubated for 15 minutes at 37°C with 1 μM of dihydroethidium (Invitrogen-Molecular Probes, Eugene, OR). We assessed apoptotic cell death with a terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) reaction kit, according to the manufacturer’s specifications (Roche, Indianapolis, IN). The cells were washed and then observed under a fluorescence microscope. The intensity of fluorescence was quantitated with fluorospectrometer (Eclipse; Varian, Palo Alto, CA) and normalized to protein content using a kit according to the manufacturer’s specifications (Bio-Rad, Hercules, CA).

Experimental Animals

For the intraocular injection of recombinant rAAV, 40 DBA/1J mice were sedated by inhalation with 1.5% to 2% isoflurane. A local anesthetic (proparacaine HCl) was applied topically to the cornea, and then a 32-gauge needle attached to a syringe (Hamilton, Reno, NV) was inserted through the pars plana. The needle tip was visualized in the vitreous with the use of an indirect ophthalmoscope, and it was positioned directly over the optic nerve head. Ten animals received intraocular injection in the right eye of 2 μL of AAV containing the wild-type ND4, and another group of 30 animals received 2 μL of AAV containing the mutant ND4. The left eyes were mock treated by
injection with 2 μL of a control AAV vector containing the gene for GFP. The animals were maintained in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Magnetic Resonance Imaging**

One and 6 months after the viral inoculations, high-resolution, three-dimensional magnetic resonance imaging (3D MRI) was performed. Animals, under isoflurane anesthesia, were placed in a prone position with their heads firmly fixed in a custom-made surface coil. T1 weighting was performed immediately after intraperitoneal administration of gadolinium-DTPA (Berlex Laboratory, Montville, NJ) at a dose of 0.2 mmol/kg of body weight. Three-dimensional images were acquired with a 4.7-T magnet (Oxford Instruments, Oxford, UK; with ParaVision 3.0.2 software; Bruker BioSpin MRI GmbH, Ettlingen, Germany). For measurement of the total volume of the optic nerve, 10 slices of optic nerve were evenly acquired longitudinally through the three-dimensional images and quantified (IDL Virtual Machine software; Research Systems, Inc., Boulder, CO). The volume of each optic nerve was expressed as the ratio of the mutant inoculated right eye to the mock-treated left eye.

**Light and Electron Microscopy**

Ten mice inoculated with the AAV-expressing mutant ND4 and 20 mice inoculated with the AAV expressing wild-type human ND4 were overdosed with pentobarbital sodium 1 month after the intracocular injections, and an additional 10 mice inoculated with the mutant ND4 were overdosed at 6 months. They were then perfused by cardiac puncture with fixative consisting of 4% paraformaldehyde in 0.1 M PBS buffer (pH 7.4) or, for detection of in vivo H2O2, with a mixture consisting of 2 mM cerium chloride, 10 mM 3-amino-1,2,4-triazole, 0.8 mM NADH, 0.1 M PBS buffer (pH 7.5), and 7% sucrose followed by perfusion with the fixative. The eyes with attached optic nerves were dissected and further processed by either of the following procedures: (1) For H2O2 localization, tissue specimens were immersion 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. For immunohistochemistry, tissue specimens were postfixed in 5.0% acrolein, 0.1 M sodium cacodylate-HCl buffer (pH 7.4) and 7% sucrose and then dehydrated through an ethanol series to propylene oxide, infiltrated, and embedded in epoxy resin that was polymerized at 60°C overnight. (2) For immunocytochemistry, tissue specimens were postfixed in 5.0% acrolein, 0.1 M sodium cacodylate-HCl buffer (pH 7.4) and 7% sucrose and then dehydrated through an ethanol series and embedded in resin (LR White; Ted Pella, Redding, CA) that was polymerized at 60°C overnight. Semithin longitudinal sections (0.5 μm) of the optic nerve head and retrobulbar nerve were stained with toluidine blue for light microscopic examination. Ultrathin sections (90 nm) were placed on nickel grids for immunocytochemistry. Examinations were made with a transmission electron microscope (H-7000 or H-7600; Hitachi), operating at 75 to 80 kV.

**Immunohistochemical Analysis**

For immunohistochemistry, the infected cells were incubated for 30 minutes with 250 nM of a mitochondria-specific fluorescent dye (Mitotracker Red; Invitrogen-Molecular Probes). Immunostaining with mouse monoclonal anti-FLAG M2 antibodies conjugated to Cy3 (Sigma-Aldrich, St. Louis, MO) or anti-GFP antibodies (BD-Clontech, Palo Alto, CA) was performed on the cells and ocular specimens. Secondary anti-mouse Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) or anti-mouse immunogold was used for immunodetection. Immunofluorescence was visualized in a fluorescence microscope (Leitz, Wetzlar, Germany) and immunogold labeling by transmission electron microscopy.

**Immunoblot Analysis and Immunoprecipitation**

Mitochondrial proteins were isolated from RGC-5 cells, as well as optic nerves and retinas of mice infected with AAVs expressing human ND4s and optic nerves of control animals. Briefly, this involved washing tissues in cold PBS, followed by resuspension in a buffer consisting of 50 mM Tris-HCl, 0.21 M Na-mannitol, 70 mM sucrose, 0.1 M phenyl-methylsulfonyl fluoride (PMSF), 3 mM CaCl2, and 20 mM EDTA (pH 7.5). Tissues were then manually homogenized. The homogenates were centrifuged at 1200g for 10 minutes at 4°C. The resultant supernatant containing the mitochondrial fraction was collected and then centrifuged at 12,000g for 20 minutes at 4°C. The pellet containing the mitochondria was washed and resuspended in buffer consisting of 50 mM Tris-HCl, 10 mM EDTA, and 20% sucrose (pH 7.5), then stored at −80°C for later analysis.

We used a kit (MS101 Complex I Immunocapture Kit) for the immunoprecipitation of complex I according to the manufacturer’s specifications (MitoSciences, Eugene, OR). Briefly, this involved resuspending the mitochondrial isolates in buffer that consisted of 50 mM Tris-HCl (pH 7.5), 1% protease inhibitor cocktail (Calbiochem EMD Biosciences, San Diego, CA), and 1 mM phenylmethylsulfonyl fluoride (PMSF, pH 7.5) and then adding 100 μL of 10% n-dodecylβ-D-maltoside. This mixture was incubated on ice for 30 minutes and then centrifuged for 50 minutes at 21,000g at 4°C. The beads saturated with antibody (Complex I Capture Matrix; MitoSciences) provided with the kit was then added. The suspension was incubated overnight at 4°C with gentle agitation. After a spin at 3200g for 3 minutes at 4°C, the pellet was washed in PBS plus 1% n-dodecylβ-D-maltoside. To elute proteins, beads with bound complex I were resuspended in 40 μL of 1% SDS, incubated for 10 minutes at room temperature. After centrifugation at 3200g for 5 minutes at 4°C, the supernatant was saved and stored at −80°C for later analysis.

For Western blot analysis, proteins extracted or immunoprecipitated from mitochondria isolated from the infected RGC-5 cells, optic nerves or retinas were separated by electrophoresis through a 10% polyacrylamide gel and electrotransferred to a polyvinylidene fluoride membrane (Bio-Rad). For immunodetection, the membrane was stained with murine anti-FLAG antibody and then with rabbit anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma-Aldrich). For normalization of sample loading we used a mitochondrial loading control VDAC1/Porin antibody (Abcam, Inc., Cambridge, MA). We detected complexes using the enhanced chemiluminescence (ECL) system (GE Healthcare, Piscataway, NJ).

**Morphometric Analysis**

Quantitative analysis was performed in masked fashion, as previously described. Briefly, images of toluidine blue-stained sections of the retina and optic nerve were captured with a video camera mounted on a light microscope, and then the data were entered into the computer memory. Using NIH Image software (available by ftp at zippy.nimh.nih.gov or at http://rsb.info.nih.gov/nih-image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD), myelinated fibers were enumerated by thresholding of the darker-staining myelin with the mean myelin area (in square micrometers) expressed per 107 μm2 for each optic nerve. Optic nerve head areas (in square micrometers) delimited by the peripapillary retina, vitreous body, and lamina sclera were manually traced. The number of ganglion cells in the retina was enumerated per 8.64 × 105 μm2. For statistical analysis, mean values for mutant ND4 inoculated right eyes were compared with the left eyes, which received a control virus expressing only GFP.

**Statistical Analysis**

Right eyes inoculated with the AAV-expressing the mutant ND4 were compared with the left eyes that received the control AAV expressing only GFP. Statistical analysis was performed by Student’s t-test for unpaired data. P ≤ 0.05 was considered significant.

**RESULTS**

**Detection of Allotopic Expression**

The ATPc mitochondrial targeting sequence (MTS) directed the allotopically expressed mutant R340H ND4FLAG polyep-
tide into mitochondria. Immunoblots of mitochondria isolated from cultured retinal ganglion cells (RGCs) transfected with AAV containing the mitotargeted ND4FLAG fusion gene and the gene for GFP, but without a MTS, showed that GFP expression was cytoplasmic and nuclear. (B) When reacted with anti-FLAG Cy3 antibodies, the red perinuclear punctate pattern and lack of a colocalization of GFP with the mitochondrial FLAG seen in the merged image (C) suggests that the recoded R340H mutant human gene was imported into the mitochondria. (D) Transmission electron micrographs of human embryonic kidney cells transfected with the bicistronic AAV show cytoplasmic localization of GFP immunogold (arrows) and (E) mitochondrially targeted wild-type ND4FLAG (Arg) immunogold labeling the interior of the mitochondria (arrows). (F) A month after inoculation of the mouse eye, mutant ND4FLAG (His) immunogold labeled the interior of mitochondria (arrows) in optic nerve axons. (G) In the retina, GFP immunogold (arrows) was distributed diffusely throughout the cytoplasm of the ganglion cells. (H) Wild-type ND4FLAG (Arg) immunogold labeled the interior of RGC mitochondria (arrows). (I) Mutant ND4FLAG (His) immunogold is seen in the mitochondria (arrows) of a degenerating axon (J). Mitochondria of RGCs transfected with the wild-type ND4FLAG (Arg) were further identified by detection of endogenous mitochondrial SOD by using 10 nm anti-MnSOD immunogold (larger arrows; K, L). These mitochondria also expressed the ND4FLAG fusion detected by the smaller 5-nm anti-FLAG immunogold (smaller arrows).
formed the complex I immunoprecipitation on pooled mitochondrial samples isolated from 20 optic nerves and 20 retinas of 10 mice that had received intravitreal injections of AAV expressing the mutant human FLAG tagged ND4 into both eyes 1 month earlier. Immunoblot analysis with the anti-FLAG antibody revealed a strong 52-kDa band in the retina (Fig. 1C, right), but not in the earlier. Immunoblot analysis with the anti-FLAG antibody revealed the mutant human FLAG tagged ND4 into both eyes 1 month after inoculation of the mouse eye, ND4FLAG were well labeled with a mitochondria-specific dye (MitoTracker Red; Invitrogen-Molecular Probes). (F) Mutant ND4 transfected RGCs were poorly labeled by the dye, indicating loss of mitochondrial membrane potential. (G) Relative to mock treatment, (H) superoxide production increased by one-third in RGCs transfected with mutant ND4. (I) Few mock-treated RGCs grown in high-glucose medium were TUNEL positive. (J) In this medium, slightly more mutant ND4 transfected RGCs were TUNEL positive. (K) Growth in galactose medium slightly increased TUNEL staining in mock-treated RGCs. (L) In contrast, after transfection with mutant ND4, growth in restrictive medium markedly increased TUNEL-positive RGCs. (M) Cell counts of cybrid cells harboring normal mitochondrial DNA that were transfected with mutant ND4 and grown in the glucose-deficient galactose medium for 3 days was reduced by one-third, relative to mock treatment with AAV-GFP (P < 0.05; mean ± SD, n = 10). (N) The intensity of fluorescence (per milligram protein content) from membrane potential dye-labeled cells decreased by half in mutant ND4 transfected RGCs relative to mock-treated cells (P < 0.01; mean ± SD, n = 10). (O) The intensity of fluorescence induced by superoxide production detected by dihydroethidium increased by one-third in RGCs transfected with mutant ND4 relative to mock treatment (P < 0.05; mean ± SD, n = 10). (P) The intensity of fluorescence from TUNEL-positive cells. Relative to the control, AAV-GFP mutant ND4 gene transfection increased TUNEL-positive RGCs by 40% in galactose-containing medium (P < 0.01; mean ± SD, n = 10).
to RGCs, mitochondria of axons of the optic nerve (Fig. 2J) were also labeled by wild-type ND4FLAG immunogold. When mutant ND4FLAG was delivered to the eye, it was also detectable in the mitochondria of axons (Fig. 2F). Many of these axons were degenerating (Fig. 2I), suggesting a correlation between uptake of mutant ND4 and axonal injury. That these structures were mitochondria of RGCs was verified by detection of endogenous mitochondrial superoxide dismutase (MnSOD), by using 10 nm anti-MnSOD immunogold. These organelles also expressed the ND4FLAG fusion seen in this study as the smaller 5-nm anti-FLAG immunogold (Figs. 2K–L). Therefore, allotopic gene delivery of a human mitochondria gene appears feasible in the mammalian eye.

**Effect of Mutant ND4 on In Vitro Cell Survival**

Next, we reasoned that introduction of the mutant R340H ND4 by allotopic gene transfer into cells with normal mitochondrial DNA may mimic some of the defects observed in LHON cells with 100% mutated G11778A mitochondrial DNA. To evaluate the functional effects of our mutant ND4 gene transfer, we transfected the 143B osteosarcoma cells harboring normal mitochondrial DNA with AAV containing the mutant P1ND4FLAG gene. Immediately after the transfection, the cells were grown in glucose-rich medium for 3 days. Like mock-treated cells (Figs. 3A, 3C) they appeared normal (Fig. 3B). When the medium was exchanged for glucose-free medium containing galactose as the main carbon source for glycolysis, they began to die (Fig. 3D). In fact, relative to mock treatment with AAV-GFP, survival of these cybrid cells harboring normal mitochondrial DNA and transfected with mutant ND4 was reduced in number by one third when grown in the glucose-deficient galactose medium for 3 days (P < 0.05; Fig. 3M). This result is unlike the improved survival that we observed in LHON cybrids rescued by allotopically expressed wild-type ND4.8 In the current study, The glucose-free galactose medium killed cybrids with normal mtDNA that were transfected with mutant ND4.

**Effect of Mutant ND4 on ROS and Apoptosis**

Growth in the restrictive medium led to a 55% decrease in mitochondrial membrane potential in RGCs transfected with mutant ND4 (Figs. 3E, 3N) relative to mock treatment with AAV-GFP (Fig. 3E; P < 0.01). Next, we tested for increased ROS activity, which has been linked to loss of mitochondrial membrane potential that can lead to apoptotic cell death.13 We found superoxide production increased by one-third in RGCs...
transfected with mutant ND4 (Figs. 3H, 3O) relative to mock treatment with AAV-GFP (Fig. 3G; $P < 0.05$). Last, whereas no significant differences in apoptosis, as measured by TUNEL staining, were seen with the cells grown in glucose medium (Figs. 3L, 3J, 3P), transfection of RGCs with mutant ND4 increased TUNEL positive RGCs by 40% (Fig. 3L, 3P) in the galactose media relative to mock treatment with AAV-GFP (Fig. 3K; $P < 0.01$). It seems likely that the R340H mutant ND4 gene transfer resulted in the demise of cultured RGCs by increases in oxidative stress that led to apoptotic cell death.

**Mutant ND4 Swells the Optic Nerve Head**

Mice exhibited histologic swelling of the optic nerve head (Fig. 4C), a month after AAV inoculations with the mutant ND4 gene. This observation has been made ophthalmoscopically in patients with LHON in the weeks or months preceding and following their acute loss of vision. In contrast, optic nerve heads of mice inoculated with AAV-GFP (Fig. 4B) or AAV expressing the allotopic version of wild-type human ND4 (Fig. 4A) appeared histologically normal. When we quantified the degree of swelling by measuring the area of the swollen optic nerve head delimited by the peripapillary retina, vitreous body and lamina sclera, we found an almost three-fold increase relative to mock treatment with AAV-GFP (Fig. 4M; $P < 0.005$).

As seen in patients with LHON, optic disc edema was pathologic in mutant ND4 inoculated eyes. Light microscopy showed dramatic and progressive loss of toluidine blue staining (Figs. 6M–O). Clearly, introduction of mutant R340H ND4 induced degeneration of the murine optic nerve that was progressive.

**Mutant ND4 Causes Optic Nerve Atrophy**

Resolution of optic nerve head swelling in our mice gave way to the typical optic atrophy characteristic of the later stages of LHON. Using serial 3D MRIs to quantitate the volume of optic nerve tissue from the eye to the brain in live mice, we found that shrinkage of the right optic nerve became apparent 3 months after inoculation of the AAV-expressing mutant ND4 relative to mock treatment with AAV-GFP (Fig. 5C). Previous MRIs of the same animal obtained before (Fig. 5A) and 1 month after inoculation with the mutant ND4 expressing AAV (Fig. 5B) showed the diameters of both optic nerves were equivalent. Quantitative analysis of optic nerve volumes revealed a 4% dip at 1 month (not statistically significant), a 10% decline at 3 months ($P < 0.02$) that increased to 17% 6 months ($P < 0.0005$) after mutant ND4 gene inoculation of the right eyes relative to mock therapy of the left eye (Fig. 5E). The loss of optic nerve tissue seen on MRI was confirmed after death, in the ND4 inoculated nerves excised at 6 months (Fig. 5D). This finding was also apparent in the 6-month histologic specimen shown in Figure 4D.

Microscopy confirmed that the retrobulbar optic nerve, was pathologic in mutant ND4 inoculated eyes. Light microscopy showed dramatic and progressive loss of toluidine blue staining (Figs. 4K, 4L) and cystic spaces suggesting axonal loss and/or demyelination. In contrast, eyes inoculated with AAV-expressing normal human ND4 (Fig. 4I) or GFP (Fig. 4J) did not. Mutant ND4 gene inoculation reduced myelin area by 23% relative to mock treatment with AAV-GFP at 1 month ($P < 0.005$; Fig. 4O). By 6 months, it reduced myelin area by 35% ($P < 0.0001$). These conclusions were borne out by examination in the electron microscope. Ultrastructural analysis confirmed that loss of toluidine blue staining was caused not by a multiple sclerosis-like demyelination, but primarily by progressive loss of optic nerve fibers composed of axons and their enveloping myelin sheaths (Figs. 6M–O).

**Effects of Mutant ND4 on the Retina**

Neuronal cell bodies whose axons comprise the optic nerve reside in the ganglion cell layer of the retina. In our experiments, the mutant human ND4 induced RGC degeneration. In

![Figure 5](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933236/ on 11/26/2018)
contrast, inoculation with AAV expressing normal human ND4 (Fig. 4E) or GFP (Fig. 4F) did not. One month after mutant ND4 gene inoculation, cells exhibiting hyperchromatic cytoplasm and nuclei were evident in the ganglion cell layer of the retina (Fig. 4G). Still, at this time point, their numbers were not significantly diminished (Fig. 4N). By 6 months, one third of the RGCs were lost (Figs. 4H, 4N; \( P < 0.0001 \)).

**Mutant ND4 Induces Apoptosis**

Ultrastructural examinations of the murine retina revealed condensation of nuclear chromatin and increased electron density of the cytoplasm (Figs. 6D, 6E) that gave way to the pyknotic nucleus with thin cytoplasmic rim seen in the latest stages of apoptosis (Fig. 6F). These typical ultrastructural features of apoptosis were not seen in control eyes (Fig. 6A). Clearly, apoptosis led to the progressive decline in the RGC population (Figs. 6B, 6C), with optic neuropathy (Figs. 6K, 6L, 6N, 6O) in these animals most likely attributable to degeneration and death of RGCs.

**Effects of Mutant ND4 on Mitochondria**

Relative to control cells (Fig. 6J) optic nerve head mitochondria of murine eyes inoculated with mutant ND4 were distended with dissolution of cristae (Figs. 6K, 6L). Mitochondrial proliferation and accumulation were evident in RGCs and the nerve fiber layer of the retina (Figs. 6G–D) 1 month after mutant gene inoculation. Such proliferation is characteristic of mitochondrial diseases. These findings were not evident in eyes mock treated with AAV-GFP (Fig. 6A) or in eyes inoculated with AAV expressing wild-type human ND4, which appeared normal. Clearly, the presence of the arginine-to-histidine sub-
Effects of Mutant ND4 on In Vivo ROS Activity

Perfusion of the mice with cerium chloride in the presence of endogenous hydrogen peroxide results in generation of cerium perhydroxide. These particles are small but electron dense, thus visible by transmission electron microscopy. The ROS induced reaction product was increased in the vascular, perivascular space (Figs. 7B, 7C) and in the mitochondria of mutant ND4 inoculated eyes (Figs. 7D–F). Relative to the control (mock treatment with AAV-GFP), mutant ND4 gene inoculation resulted in a threefold increase in hydrogen peroxide-derived reaction product 1 (P < 0.01) and 6 (P < 0.01) months later (mean ± SD, n = 10).

DISCUSSION

We have shown that a nuclear version of the mutant human ND4 subunit of complex I containing an arginine-to-histidine substitution at amino acid 340 is translocated into the mitochondria of rodent RGCs. Using a kit (MitoSciences) that contained agarose beads irreversibly cross-linked to highly specific MS101c monoclonal antibodies for immunocapture of the NADH ubiquinone oxidoreductase complex from small amounts of tissue such as the murine retina and optic nerve, we were able to pull down the 52-kDa FLAG tagged ND4 protein from the pooled retinal mitochondria. Although this finding seems to suggest incorporation of human mutant ND4FLAG into the murine RGC complex I, we acknowledge that the MS101c monoclonal antibody (though it is not directed against the ND4 subunit) may also have nonspecifically brought down mutant ND4FLAG that was imported but not integrated in the murine complex I.

Still, allotopic expression of mutant human ND4 in the mouse visual system induced an animal model of LHON. Resolution of optic disc swelling was followed by optic atrophy in our mice. Unlike human LHON that requires 100% mutated G11778A mtDNA for expression of the phenotype, our model system was initiated by expression of the mutant human complex I subunit in murine mitochondria with a normal genome. Thus, in the mouse it appeared to have a dominant impact. We do not believe that this effect was due to a mixing of the ND4 gene products of two different species that has been shown to have an adverse effect on mitochondrial bioenergetics. We found no histologic abnormality in the retina or optic nerve of mice expressing the normal human complex I subunit, but only in those inoculated with the mutant ND4. Thus, retinal and optic nerve degeneration in our model system appears to be due to expression of the human mutant R340H ND4 in murine mitochondria.

A threefold increase in reactive oxygen species appeared to mediate the accumulating damage to RGCs and the optic nerve in our model system, inducing a progressive disease not unlike that of LHON. This heightened level of ROS activity continued 6 months after mutant gene inoculation, the longest interval that we evaluated. It provided an environment conducive to further injury. Although this result had been anticipated by our in vitro introduction of the mutant complex I subunit into RGC-5 cells harboring normal mitochondrial DNA and those of cultured LHON hybrid cells with mutated mtDNA, the magnitude of ROS activation in our mouse model of LHON was 10-fold higher than that observed in cultured cells. We had shown in earlier work that loss of complex I activity induced by a ribozyme targeting the NDUFA1 complex I subunit also increases ROS activity that also resulted in RGC and optic nerve degeneration. The insult was similar to the ocular injury caused by genetically reducing mitochondrial protection against reactive oxygen species with a ribozyme targeting SOD2. Taken together, these studies support the pathogenicity of complex I mutations to be due to an increased generation of reactive oxygen species that results in death of RGCs directly or by triggering apoptosis.

Our findings in the mouse point to two avenues for intervention in the disease process. First, antioxidant gene therapy...
may be useful to treat patients with LHON. 26 Though it had no counterpart in human disease, rescue of our animal model of complex I deficiency induced by the NDUFA1 ribozyme with a gene that detoxifies ROS (mitochondria superoxide dismutase, SOD2) proved that, in that model system, suppression of ROS inhibited death of RGCs, a phenomenon that we showed in the current study also to be involved in the pathogenesis of disease caused by the mutated human ND4 complex I subunit gene. Second, complementation of the defective ND4 subunit gene with the normal gene is an alternate way to treat LHON. We showed that when injected into the mammalian visual system, an AAV containing the normal human ND4 subunit of complex I is expressed in ganglion cells of the retina and axons of the optic nerve, the very cells impacted by LHON. 27 Unlike expression of the human mutant R340H ND4 that induced optic neuropathy, the wild-type version of the human ND4 subunit gene appeared to have no adverse effect on the mouse visual system, suggesting that delivery of ND4 is feasible in mammals and perhaps even in humans.

That allotopic expression can rescue complex I deficiency was recently shown in a murine model of Parkinson’s disease. Rather than complementing the defective 8kDa complex I subunit 28 with a human gene, the investigators used the AAV vector to deliver the single-subunit NADH dehydrogenase (ND1) of yeast (Saccharomyces cerevisiae). 29 Despite the marked mismatch in the amino acid sequence and size of the yeast relative to the murine complex I, a 50% rescue of complex I activity was seen in their mice. They have also used the yeast ND1 to rescue complex I deficiency in a mutant ND4 cell line. 30 We and others have used allotopic complementation with wild-type human ND4, 31 human ATP6, 7 or even an algae counterpart subunit of ATP6, 31 to rescue cultured LHON or NARP cells. However, animal models of these disorders did not exist for further test application of this methodology. We believe that we have developed a murine model system that can be used to test the effectiveness of treatments for LHON.

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3. Wallace DC, Singh G, Lott MT, et al. Mitochondrial DNA mutation in LHON: evidence for further test application of this methodology. We believe that we have developed a murine model system that can be used to test the effectiveness of treatments for LHON.