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Age-related accumulation of phosphorylated mitofusin 2 protein in retinal ganglion cells correlates with glaucoma progression

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Abstract

Dysregulation of axonal bioenergetics is likely a key mechanism in the initiation and progression of age-related neurodegenerative diseases. Glaucoma is a quintessential neurodegenerative disorder characterized by progressive deterioration of the optic nerve (ON) and eventual death of retinal ganglion cells (RGCs). Age and elevation of intraocular pressure are key risk factors in glaucoma, but the common early hallmarks of decreased axonal transport and increased bioenergetic vulnerability likely underlie disease initiation. We examined the correlation between bioenergetics and axonal transport with mitochondrial mutation frequency and post-translational modifications of mitofusin 2 (Mfn2) in RGCs during glaucoma progression. No increase in the frequency of mtDNA mutations was detected, but we observed significant shifts in mitochondrial protein species. Mfn2 is a fusion protein that functions in mitochondrial biogenesis, maintenance, and mitochondrial transport. We demonstrate that Mfn2 accumulates selectively in RGCs during glaucomatous degeneration, that two novel states of Mfn2 exist in retina and ON, and identify a phosphorylated form that selectively accumulates in RGCs, but is absent in ON. Phosphorylation of Mfn2 is correlated with higher ubiquitination, and failure of the protein to reach the ON. Together, these data suggest that post-translational modification of Mfn2 is associated with its dysregulation during a window of metabolic vulnerability that precedes glaucomatous degeneration. Future work to either manipulate expression of Mfn2 or to prevent its degradation could have therapeutic value in the treatment of neurodegenerative diseases where long-tract axons are vulnerable.

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Conflict of interest

The authors have no conflicts of interest to report.

Keywords

Mitofusin; Mitochondria; Phosphorylation; Axon transport; Neurodegeneration; Mutation frequency

1. Introduction

Decreased axonal transport, disruption of energy delivery, and organelle movement are common and early harbingers of many neurodegenerative diseases (Calkins, 2012; Millicamps and Julien, 2013; Rangaraju et al., 2014) such as amyotrophic lateral sclerosis, Huntington's, Parkinson's, Alzheimer's disease, and glaucoma. Deficits in axonal transport machinery are sufficient for neurodegenerative disease initiation (Perlson et al., 2010). Here, we focus on the experimentally tractable and common neurodegenerative disorder, glaucoma (Bosco et al., 2008; Morrison et al., 1997; Nguyen et al., 2011). Glaucoma is a neurodegenerative disease of the eye and optic nerve (ON), characterized by retinal ganglion cell (RGC) axonal degeneration and eventual RGC soma loss in the retina (Buckingham et al., 2008; Calkins and Horner, 2012; Della Santina et al., 2013). At the earliest stages of glaucoma, axonal transport mechanisms are disrupted, interfering with organelle transport, including mitochondria (Bosco et al., 2008; Cooper et al., 2016; Misko et al., 2010). We have previously shown disruptions in mitochondrial morphology (Coughlin et al., 2015) and established that the axons in a murine glaucoma model are more vulnerable to metabolic challenge (Baltan et al., 2010; Uo et al., 2009). A key therapeutic question is whether early changes in the ON transport mechanism and metabolic capacity can be targeted to slow or prevent neurodegeneration.

A number of neurodegenerative diseases are associated with abnormalities in mitochondrial dysfunction (Park et al., 2015). Defects in optic atrophy 1 (*Opa1*), an inner membrane fusion protein, cause increased mitochondrial fragmentation and severe cristae malformations (Cipolat et al., 2006; Frezza et al., 2006; Song et al., 2009). Genetic mutations in *Opa1* cause autosomal dominant optic atrophy (DOA) (Davies et al., 2007), one of the most common inherited optic neuropathies. Impaired mitochondrial dynamics are involved in the pathogenesis of Parkinson's and AD, with expression of levels of dynamin-related protein (Drp1), mitofusin 1 (Mfn1), and mitofusin 2 (Mfn2) significantly decreased in hippocampal neurons of human Alzheimer's disease brains (Cho et al., 2010). Mfn1 and Mfn2 are both outer mitochondrial membrane fusion proteins that are necessary for mitochondrial transport (Misko et al., 2010). Mutations of Mfn2 result in Charcot-Marie-Tooth (CMT) disease, the most common inherited neuromuscular disorder, and affect motor and sensory neurons. More than 40 mutations of Mfn2 have been identified in CMT neuropathy type 2A patients, with the majority of these being missense mutations (Cho et al., 2010). Misko and colleagues have demonstrated that Mfn2 is directly associated with the proteins Milton and Miro in dorsal root ganglion neurons, thus supporting the role of Mfn2 in mitochondrial transport (Misko et al., 2010).

Given that both Mfn1 and Mfn2 are tied to mitochondrial transport, we sought to examine their potential relevance in glaucoma. One barrier to the study of these proteins has been the

challenge of analyzing mitochondrial proteins from the small anatomical compartments of the visual pathway. We took advantage of a new nanoimmunoassay probe and optimized a novel method to measure low-abundance proteins as well as to begin to determine animal-to-animal variance of these proteins. The data indicate that a distinct phosphorylation state of Mfn2 is upregulated early in glaucoma and may thus be a unique biomarker and harbinger of early metabolic dysfunction that leads to blindness.

2. Materials and methods

2.1. Animals

Experiments were conducted in accordance with regulations set forth in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and were conducted under protocols approved by the Institutional Animal Care and Use Committee at the University of Washington. DBA/2J (DBA/diseased) and DBA/2J^{Gpnm^b+} (D2G/control) mice were initially obtained from Jackson Laboratories (Bar Harbor, ME), bred and maintained at a UW pathogen-free vivarium (South Lake Union), with new breeding pairs incorporated into the colony every year (Inman et al., 2006). All animals were kept on a 12-hour light/dark schedule with ad libitum access to food and water.

The DBA mouse is a well-established model of glaucoma (Howell et al., 2007; Libby et al., 2005). These mice spontaneously develop mutations in *Gpnm^b* and *Tyrl* (Anderson et al., 2002; Chang et al., 1999) that result in elevated intraocular pressure (IOP) and glaucoma with age, in a similar fashion as humans (see Supplementary Fig. 1). Animals of the following ages were used: 3 mo (young), 5–7 mo (6 mo), and 9–13 mo (aged). In early stages, the young DBA mice do not show outward manifestations of disease. By 6 months, the ON begins to retract, sight is compromised, and there is marked metabolic deficit in 42% of the mice. In the late, chronic stage, many RGCs have died, the aged mice are blind, and ATP production is severely curtailed in over 90% of the mice. Previously, we have demonstrated that the majority of DBA mice retinas have undergone significant neurodegeneration (Baltan et al., 2010; Buckingham et al., 2008; Inman et al., 2013; Lambert et al., 2008). In the current study, we confirmed this degeneration in the population of aged mice used in these studies by qualitative analysis of axon integrity by mitotracker staining in whole mount retinas (Supplementary Fig. 2).

The D2G mice serve as an ideal control for both disease and aging studies as they are genetically identical to the DBA mice, but have a functional *Gpnm^b* allele and do not develop elevated pressure or glaucoma. Age-matched animals were used in all experiments.

2.2. Tissue preparation

Animals were anesthetized with Beuthanasia (Merck Animal Health) and eyes were removed with the ON still connected. The retina was removed from the orb, and the ON was snipped free at the back of the eye globe to include the ON head, resulting in an approximate length of 5 mm. Tissue collected for protein analysis was placed into tubes on dry ice. Ice cold lysis buffer was added in a weight-to-volume ratio of 5–10 μ l lysis buffer per mg of

tissue. Tissue collected for RNA analysis was placed in cold TRIzol® (ThermoFisher) and processed using the manufacturer's protocol.

2.3. mtDNA random mutation capture assay

Quantification of random mtDNA mutations was performed as described (Vermulst et al., 2008), with alterations to enhance throughput. Specifically, DNA was diluted and digested with 100 units TaqαI restriction enzyme (New England Biolabs), 1× BSA and a TaqαI-specific digestion buffer (10 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl, pH 8.4) for 5 h, with 100 units TaqαI added at 1-h intervals. Mutation frequencies at sites in the 12S rRNA and ND5 genes were determined via real-time PCR with primers flanking a TaqαI restriction site to quantify the number of mutant molecules, i.e., those molecules resistant to digestion. Real-time PCR was also performed with a set of control primers in a region without a TaqαI restriction site to quantify the total number of molecules. Standard curves were included to determine the relationship between copy number and cycle threshold for each primer set, and the resulting formulae were used to quantify target DNA in each well. Real-time PCR was performed in duplicate in 25 µl reaction volumes, with 12.5 µl 2× GoTaq Hot Start Colorless Master Mix (Promega) supplemented with SYBR Green I Nucleic Acid Stain (Lonza) at 0.2× final concentration, 500 nM each of forward and reverse primers, and diluted TaqαI-digested DNA. Reactions were thermally cycled on a CFX96 instrument (BioRad) with the following conditions: 37 °C for 10 min, 95 °C for 10 min, and 45 cycles of 95 °C for 30 s, 65 °C for 1 min, and 72 °C for 90 s, followed by 72 °C for 5 min and then held at 4 °C. Mutant molecules were verified by a post-PCR TaqαI digest and agarose gel electrophoresis.

2.4. Quantitative reverse transcription-PCR

Whole retinas and ONs were removed from the DBA (disease model) and D2G (control) mice. RNA was isolated by placing the tissue into cold TRIzol® and processed using the manufacture's protocol, and amplified. For RNA analysis, it was necessary to use pooled tissue from retina and ON from age-matched DBA and D2G animal cohorts to acquire enough tissue for analysis. Each pooled sample contained tissue from three to seven animals (see Supplementary Table 1). cDNA was then prepared from the extracted RNA. The cDNAs from pooled tissue samples were probed with FAM-labeled primers for Mfn1, Mfn2, and GAPDH, a housekeeping gene used for normalization. All quantitative RT-PCR was performed in triplicate using three separate pools of each tissue type under standard cycling conditions for FAM-labeled primers. The accompanying software for the Rotor-Gene Q (Qiagen) was used to visualize the data. Final analysis of the data was completed using Excel (Microsoft).

2.5. DNA isolation

To obtain DNA, tissue samples were immersed in 1 ml of a homogenization solution (0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.8) and disrupted in a glass Dounce-type homogenizer. The homogenate was transferred to a 1.5 ml tube and centrifuged. The pellet was re-suspended in 600 µl lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 20 mM EDTA, 1% SDS, and 0.2 mg/ml Proteinase K) and incubated at 55 °C for 3 h. DNA was isolated by phenol-chloroform extraction followed by isopropanol precipitation.

2.6. Immunohistochemistry

Retinal cross-sections were prepared by placing unfixed eyecups containing the retina, but not the lens, in Tissue-Tek® OCT compound (Sakura Finetek, Torrance, CA) and snap freezing with liquid nitrogen. Frozen tissue was cryosectioned at 10 µm thickness. Slides were rinsed, blocked, and incubated with primary, then secondary antibodies. DAPI was applied prior to coverslipping.

Primary antibodies were applied in the following concentrations: anti-Mfn1 (chicken, 1:250; Novus), anti-Mfn2 (rabbit, 1:1000; AbCam), anti-NeuN (mouse, 1:1000; Chemicon), anti-ChAT (goat, 1:500; Chemicon), anti-GFAP (guinea pig, 1:250; Advanced Immuno-Chemical) all diluted in blocking solution (0.4% Triton X, 5% donkey serum in Tris Buffer). To visualize primary antibodies, a secondary antibody conjugated to RedX, cy2 or cy5 was applied at a dilution of 1:250 in blocking solution as above. Nuclei were labeled with DAPI (1:1000, Sigma) before coverslipping.

To validate Mfn2 antibody localization on mitochondria, retina from a mitochondrial reporter mouse was stained with Mfn2 and DAPI (see Supplementary Fig. 3).

2.7. Protein sample preparations

Retinas and ONs were removed from mice that had been lethally anesthetized. Tissue samples were placed in a tube on dry ice and weight was determined. Ice-cold Bicine/CHAPS lysis buffer (ProteinSimple), protease inhibitors, and DMSO were mixed and 5–10 µl/mg of tissue was added to the tube. Tissue was broken down using pipette tips to homogenize the mixture and spun to remove cellular debris. The supernatant was placed in chilled microcentrifuge tube and lysate concentration was determined using a BCA assay (Pierce). Lysates were frozen and stored at –20 °C.

2.8. Development and validation of a quantitative nanoimmunoassay for small amounts of protein

The need to pool tissue from multiple animals to conduct the protein and RNA assays limited the ability to adequately evaluate changes occurring with Mfn1 and Mfn2 protein expression on an individual animal level (see Section 2.4 above). Pooled samples made it particularly difficult to dissect out changes to the phosphorylated state or other variants of the protein. We sought to further characterize changes to Mfn2 and specifically look for post-translational or active kinase activity of Mfn2 during disease progression.

We used the NanoPro (NP) 1000 (ProteinSimple, formerly NanoPro) to develop a new nanoimmunoassay (Nivison and Horner, 2012a, 2012b; O'Neill et al., 2006) to detect both Mfn1 and Mfn2 in individual diseased and control animals. The NP uses capillary isoelectric focusing electrophoresis to detect signals from between 0.05 and 0.1 mg/ml of total protein. The assay we developed, nanoimmuno capillary isoelectric focusing (NICIF), is a combination western blot and ELISA, with proteins being separated by an isoelectric gradient in a capillary. Cell or tissue lysates are mixed with standards in a sample buffer that creates the gradient and an electrical field separates the proteins by their isoelectric points (pI). The proteins are then immobilized at different points along the length of the capillary

and can then be probed by primary and secondary antibodies. Ultimately applying a chemiluminescent signal allows the results to be measured and quantified.

Specifically, in developing the NICIF assays, the consistent position within the capillary of the antibody-tagged protein from each sample in relation to the standards is used to identify Mfn2 and Mfn1 protein. The NP proprietary software, Compass, then displays the signal intensity as peaks that can then be quantified. In the NICIF assay for Mfn2, these peaks occur at the approximate pIs of 6.2 and 6.5, the latter of which corresponds to the theoretical pI of 6.52 for Mfn2, further confirmation that these peaks represent the Mfn2 protein (Supplementary Fig. 4A). Each sample was run in triplicate to further clarify the actual protein signal. Other spikes tend to arise irregularly and inconsistently, and are attributable to background noise due to non-specific antibody binding. To validate the NICIF assay, several tissue types, control cells, and control antibodies were tested and cross-authenticated to further confirm the peaks for both Mfn2 and Mfn1. The antibodies with the most reproducible result and lowest background were ultimately used in the assays. See development assay protocols for more specific details (Nivison and Horner, 2012a, 2012b). The small, lower tracings in Supplementary Fig. 4B show ERK levels in HeLa cells (used for assay authentication), with the upper tracings showing ERK in retinal tissue, validating the presence of detectable protein in very small sample sizes. In analyzing the data, run-to-run variability was taken into account. Therefore, most comparisons were done within individual runs, usually with groups of three samples, with each sample run in triplicate. When comparing between runs, results were best analyzed by comparing total area percentages. Baselines and areas under the curves (peaks) were calculated using the Compass software.

2.9. NICIF assay preparation

For the Mfn1 and Mfn2 assays, lysates were diluted to a final concentration of either 0.05 mg/ml (retina) or 0.1 mg/ml (ON) and loaded in equal protein amounts using SERVLYT™ 4–7 carrier ampholyte (Universal Biologicals) and resolved by capillary isoelectric focusing electrophoresis using the NP 1000 according to our developed protocols (Nivison and Horner, 2012a, 2012b). Resolved proteins were immobilized using UV crosslinking, then probed with primary antibodies in the following concentrations: α -Mfn1 (rabbit, 1:300; Santa Cruz), α -Mfn2 (rabbit, 1:25; Sigma), and α -Ubiquitin (rabbit, 1:50; Cell Signaling). Secondary antibodies (ProteinSimple) used include: goat α -rabbit biotin conjugate (1:100) and streptavidin-HRP (1:100). Luminol/Peroxide XDR was used for signal detection.

2.10. Protein analyses

Phosphorylation-specific variants of Mfn1 and Mfn2 were identified using the Lambda Protein Phosphatase assay (Millipore). Lambda phosphatase was added to protein samples and incubated for 30 min at either room temperature or 37 °C prior to performing the NICIF assay. Four controls were used: untreated (no lambda phosphatase) protein samples 1) under standard NICIF assay conditions (on ice); incubated 2) at room temperature; and 3) at 37 °C; and finally, 4) lambda phosphatase alone (no protein).

For the immunoprecipitation assay, protein prepared for NICIF analysis (see Section 2.7, Protein sample preparations) was treated with urea to denature the protein. Treated protein was tagged using Mfn2 antibody (see Section 2.9, NICIF assay preparation) and precipitated out. Western blot of the protein was then probed with ubiquitin antibody.

In Western blots, protein detection was performed using the same primary antibodies (Section 2.9, NICIF assay preparation), as well as β -actin (rabbit, 1:1000; Cell Signaling). Secondary antibodies used include donkey α -rabbit HRP (Jackson, Amersham).

2.11. Imaging

Immunohistological images were visualized with a Zeiss Axioskop2 plus microscope with an Optronics camera. Confocal images were captured with a Nikon A1R microscope and 3D image stacks were further analyzed using Imaris 7.5 software (BitPlane) (Shen et al., 2008). Quantitative analysis of the confocal images was performed using Imaris MeasurementPro and ImarisCell to identify RGC nuclei and mitochondrial surface protein Mfn2, and to measure both Mfn2 proximity to the nuclei and its abundance in the cell soma. ImarisCell quantifies complex morphological relationships between cells, between cell components, and between components within a cell. Components are color coded to visually display these multiple complex relationships. Imaris MeasurementPro uses object detection features to perform complex quantifications, measurements, and statistical analyses of data extracted from microscopy images and classifies the results based on specified statistical parameters. Analysis is performed on a per cell basis on the combination of objects that meet pre-selected criteria. In this study, we used a distance of 4 μ m between the nucleus and Mfn2-tagged mitochondria to define the perinuclear space, with Mfn2 tags falling outside this parameter not being considered or quantified. To eliminate background staining, only Mfn2 fluorescent signal 4 pixels in size was included in the analysis.

2.12. Statistical analysis

In the NICIF assay, all samples were run in triplicate, with the average of the three used in any further statistical analysis. Most analyses were done within an individual run, to allow all background, scale, and probes to remain the same. Analyses across runs, involving all of one sample type, were done based on percentages to allow for run-to-run variability, with the closest-to-average run used for depictions or graphic quantification. Baselines for the curves (peaks) and areas under the curves were calculated using the NP-1000 Compass software. See Table 1 for total number of animals used for statistical analyses. Each animal provided two tissue samples of both retina and ON which were considered as separate samples due to eye-to-eye and nerve-to-nerve variability.

All data in this study, including those derived from the NICIF assay, are expressed as the mean $\pm$ SEM, except where otherwise indicated. Correlation coefficients, corresponding p values, and outliers were calculated using GraphPad Prism 3.0 (GraphPad Software) and Prism 6.0. Groups were compared using ANOVA with Welch's correction assuming a nonparametric comparison, ANOVA, two-way ANOVA, and unpaired t -tests. For all analyses, statistical significance was set at $p < 0.05$.

3. Results

3.1. Mitochondrial DNA mutation frequency

To determine the relationship of mtDNA mutations to the glaucoma phenotype, we measured the random mutation frequency in tissues from 9-month-old control D2G and diseased DBA mice using the random mutation capture (RMC) assay (Bielas and Loeb, 2005) adapted for mtDNA (Vermulst et al., 2007, 2008). When evaluating tissue types affected by glaucomatous degeneration (ON, Retina, superior colliculus; SC) and thus expected to sustain an increased mutation burden, we found no significant differences in mtDNA mutation load at two separate mutation sites between control and diseased tissues (see Supplementary Fig. 5A, B).

3.2. Protein and RNA levels change with disease

We found that RNA expression levels of Mfn1 were not significantly different in the retinas of control or diseased mice in the three age groups studied: 3, 6, and 9–12 months (data not shown). Mfn2 RNA expression levels were statistically similar in the retinal tissues of the three D2G/control cohorts and the three DBA cohorts. However, compared to age-matched controls, Mfn2 RNA levels were significantly lower in the DBA cohorts at 3 months ($p < 0.02$) and 9 months ($p < 0.02$). Overall, Mfn2 RNA expression was lower in the DBA mice when compared with the D2G controls ($p = 0.005$; Fig. 1A). Further analysis by 2-way ANOVA confirmed that disease status was statistically significant ($p < 0.0001$) and the interaction between age and disease was significant ($p < 0.04$).

Mfn1 RNA expression in the ON trended upward with age in the D2G control mice, but differences between groups were not significant. However, in the DBA mice, Mfn1 RNA expression trended downward with age with significant differences found between the three groups with one-way ANOVA ($p < 0.04$; Fig. 1B). Furthermore, Mfn1 RNA expression was significantly lower in the 6-month DBA mice compared to age-matched controls ($p < 0.02$; Fig. 1B). As with Mfn1, Mfn2 RNA expression in the ON of the DBA mice trended downward with age, although significance between the age groups was not quite reached (data not shown). There were no differences in Mfn2 RNA expression between groups in the control mice (data not shown).

We found by Western blot analysis that Mfn2 protein levels in the retina were higher overall in the DBA mice when compared to D2G controls (Fig. 1C). These data are similar to our previously published findings using B6 mice as controls (Uo et al., 2009). The Mfn2 protein levels in the retina of the diseased DBA mice were lowest in the youngest group and highest in the oldest group. Conversely, in the control D2G mice, we found the opposite trend with Mfn2 protein levels highest in the retinas of the youngest control mice, and lowest in the oldest mice.

Similar to our previous study (Uo et al., 2009), when evaluated by Western blot, Mfn1 protein levels in the ON were found to be lower in diseased DBA mice than in the D2G controls (Fig. 1D). In contrast to these earlier findings, we found Mfn1 protein levels were highest in the youngest groups and lowest in the oldest groups of both the glaucomatous and

control mice (Fig. 1D). Mfn2 protein levels in the ON were not significant (see Supplementary Fig. 6).

3.3. Immunohistochemistry showed Mfn2 accumulation in RGCs

Using immunohistological staining of retinal cross-sections to determine how Mfn2 protein is distributed during disease progression, we found Mfn2 was concentrated in RGCs and accumulated more rapidly in the surviving RGCs during disease progression (Fig. 2). Confocal imaging of 3-month-old DBA retina (prior to disease onset) shows an even distribution of Mfn2 immunoreactivity throughout the retina while being particularly concentrated in NeuN⁺ RGCs (Fig. 2A and B). This even distribution was the same in the age-matched control retina (data not shown). More limited expression of Mfn2 is visible in other cells and layers of the retina (Fig. 2A). The Fig. 2A inset shows the proximity of the Mfn2 to the nuclei. However, 10-month-old DBA retina had lower Mfn2 levels, as well as fewer RGCs overall, compared to those of the 3-month-old mice (Fig. 2C and D), while the Fig. 2C inset shows a higher accumulation of Mfn2 in close proximity to the nuclei.

We used Imaris imaging software to better visualize Mfn2 protein on intracellular structures and quantify total immunoreactivity in individual cells. We found Mfn2-tagged mitochondria were particularly concentrated in the cytoplasm of the perinuclear region of the RGCs in the young DBA mice (Fig. 2B). While we found fewer RGCs and less Mfn2 labeling in the older DBA mice, there was a greater accumulation of Mfn2 in the perinuclear space on a per-cell basis (Fig. 2D and Table 2). While not directly measured, Mfn2 levels in the remaining retinal layers appeared similar in both the young and older mice regardless of genotype (Fig. 2A and C; and data not shown).

We also quantified intracellular distribution of Mfn2 protein with Imaris imaging software. We found the number of NeuN⁺ cells (i.e., RGCs) in the retina of the aged DBA mice was significantly lower compared to the young DBAs ($p < 0.004$), and the aged controls ($p < 0.05$; Table 2). Data for the young D2G controls are not shown, as values were virtually identical to the young DBAs. The amount of Mfn2 protein in the aged DBAs was significantly lower when compared to young DBAs ($p < 0.03$), although not significantly different from the aged controls. We also found the amount of Mfn2 protein detected within the perinuclear space (defined as signal within 4 μm of the RGC nucleus) of the aged DBAs was two-fold higher than in the young DBAs ($p < 0.02$) and almost three-fold higher than in the aged controls ($p < 0.02$).

3.4. NICIF assay shows differences in protein expression based on age and disease

Using the NICIF assay, we determined that the Mfn2 peaks reflected protein level changes attributable to age or disease. Tracings of Mfn2 protein in retinal tissue reflect protein levels in young control (D2G) (Fig. 3A), aged D2G (Fig. 3B), and a comparison between the young and aged D2Gs (Fig. 3C). The corresponding panels, D–F, show the same thing in diseased retina of the DBA mice. The percentages given for each peak reflect the average amount of the total protein per peak for each age/condition.

3.5. NICIF assay shows phosphorylation-specific peaks in Mfn2

Protein analysis performed with the NICIF assay revealed two distinct Mfn2 peaks in both retina (see Fig. 3) and ON, with slightly different pIs. To characterize the two peaks, a phosphatase-stripping assay (Lambda Protein Phosphatase assay) was performed on the protein lysates. This created a near-complete disappearance of the 6.2 peak in both tissues (Fig. 4A and B) with a concurrent increase in the second 6.5 peak. This indicates that the 6.2 peak corresponds to the normally active phosphorylated form of Mfn2, while the 6.5 peak reflects the non-phosphorylated form of the protein. In the retina, we found more phosphorylated Mfn2 in the aged D2G control mice (Fig. 4C) and more non-phosphorylated Mfn2 in the aged DBA mice (Fig. 4D).

The shift in phosphorylation status of Mfn2 protein in control and diseased animals revealed significant reductions in overall peak size, as well as the loss of the 6.2 pI peak in diseased tissues (Fig. 5A–D). In young DBA diseased mice, phosphorylated Mfn2 was found to be already elevated compared to age-matched D2G controls (Fig. 5A and B). In aged DBA mice, there was a significant increase in non-phosphorylated Mfn2 in both the ON and retina (Fig. 5C and D). In contrast, in both young and aged control animals the ratio of phosphorylated Mfn2 to non-phosphorylated Mfn2 is close to one in both tissues.

3.6. Mfn2 protein levels increase significantly in glaucomatous retina, but decrease in affected ON

Examining total Mfn2 protein (both phosphorylated and non-phosphorylated forms), we found that Mfn2 is expressed at significantly higher levels in both the ON and retina of young DBA mice compared to controls (Fig. 5A, B, E, F). In the aged ON, Mfn2 levels are approximately 30-fold lower in the diseased DBA mice compared to controls (Fig. 5C, E). However, in the aged retina, Mfn2 levels are approximately 5.6-fold higher in the diseased DBAs compared to controls (Fig. 5D, F). In the ON, quantification of total Mfn2 protein shows that protein levels are ~6-fold higher in aged vs. young controls, however, strikingly, protein levels are ~20-fold lower in aged vs. young DBA animals (Fig. 5E). In the retina, Mfn2 quantification shows no significant differences between young and aged control mice, however protein levels are 4-fold higher in aged vs. young DBA mice (Fig. 5F).

3.7. Mfn1 protein levels are not compensatory in retina or ON

In contrast to Mfn2, we found very similar expression patterns of Mfn1 in the ON in both young control and DBA, as well as in aged control and DBA (diseased) animals (Fig. 6A). Quantification of total Mfn1 protein shows a significantly lower level in the aged mice ($p < 0.012$), although there is no significant difference between the control D2G and the DBA mice (Fig. 6B). However, when comparing the phosphorylated peak (P1) to the non-phosphorylated peak (P2) there is a significant shift to the non-phosphorylated peak ($p < 0.02$) that occurs with age (Fig. 6C).

In the retina, Mfn1 expression patterns were completely different in the control (D2G) and diseased (DBA) mice (Fig. 6D–F). Aged control mice had dramatically lower levels of Mfn1 than the young controls (Fig. 6D, G, $p < 0.004$) while, conversely, aged DBA animals expressed significantly higher levels of Mfn1 than the young DBAs (Fig. 6E, G, $p < 0.003$).

Mfn1 expression levels in aged D2G retina were significantly lower in comparison to aged DBA retina (Fig. 6F, G, $p < 0.004$). In the aged mice, the DBAs express significantly higher levels of Mfn1 compared to controls ($p < 0.004$). Thus, in the retina, Mfn1 protein levels reflect an inverse expression pattern with age between control and DBA animals ($p < 0.002$): the aged DBA mice had higher Mfn1 levels than the young DBAs, while the aged control mice had lower Mfn1 levels than the young controls (Fig. 6G).

3.8. Ubiquitin levels decrease in retina with disease progression

Ubiquitination is a post-translational modification that tags dysfunctional mitochondria for degradation (Whitworth and Pallanck, 2009). The level of ubiquitin expression in retina was similar between young and aged D2G (control) mice (Fig. 7A, C), but, levels were significantly lower in the aged DBA mice compared to the young mice (Fig. 7B, C; $p = 0.001$). This was confirmed via Western blot (Fig. 7D, D'), which showed that ubiquitin levels were lower in the retinas of the aged DBA mice compared to the young DBAs, while levels were similar in the control mice at both ages. Expression patterns for ubiquitin in the retina were similar to those of Mfn2 (see Fig. 5B, D). Using immunoprecipitation, we determined that ubiquitin is associated with Mfn2 in retinal tissue (Fig. 7E).

Ubiquitin was detectable by Western blot in the ON using pooled samples from 6 to 8 animals. Levels were lower in both aged control and aged DBA mice compared to the young counterparts. We were unable to detect ubiquitin in the ON using the NP 1000 (ON data not shown).

4. Discussion

Glaucoma, like many common neurodegenerative diseases, is a condition marked by disrupted energy balance (Baltan et al., 2010), transport deficits (Buckingham et al., 2008), and changes in mitochondrial form and function (Nguyen et al., 2011). Together, this evidence for mitochondrial dysfunction could be a result of direct dysregulation of mitochondrial dynamics and transport due to glaucomatous disease. Alternatively, mitochondrial dysfunction could result from DNA or protein damage due to oxidative stress or inflammation. One hallmark of an oxidative effect would be damage to mtDNA, which has been observed in patients with congenital glaucoma (Tanwar et al., 2010). It has been suggested that oxidative stress, induced by defective mitochondria, leads to an increase in mtDNA mutagenesis, and ultimately plays a causal role in the onset of glaucoma (Chrysostomou et al., 2013; Jarrett et al., 2013).

The role mitochondria play as either an initiator or a secondary contributor in neurodegenerative disease remains unknown. Here we sought to determine if increased mtDNA mutation frequency underlies the ATP crisis and metabolic vulnerability that we have previously described in DBA tissue (Baltan et al., 2010). Since there is no known single mutation that results in glaucoma, particularly one that is associated with mitochondrial genes, we analyzed the frequency of mtDNA mutations in glaucomatous DBA mice. No significant differences in mtDNA mutation burden were observed between D2G control and DBA mouse tissues, indicating that mitochondrial mutation frequency is not a contributing factor to declining mitochondrial function in the DBA mouse model of

glaucoma. These data suggest that mtDNA mutations, thought to result from increased oxidative stress or inflammation, do not underlie mitochondrial dysfunction in glaucoma. Hence, we focused our subsequent investigations on analyzing key mitochondrial regulatory proteins important to mobility, biogenesis, and repair.

The outer mitochondrial membrane family of fusion and fission proteins has recently garnered significant interest in multiple aspects of mitochondrial function (Chen et al., 2010; Cho et al., 2010; Elgass et al., 2013; Huang et al., 2007; Peng et al., 2017; Poole et al., 2008; Youle and van der Bliek, 2012; Yue et al., 2014). There has been a much greater focus on fission proteins and their role in disease than on fusion proteins (Corazao-Rozas et al., 2016; Elgass et al., 2013; Youle and van der Bliek, 2012). Previous studies have shown that mutations of Opa1 disrupt mitochondrial inner membrane fusion, resulting in DOA (Amati-Bonneau et al., 2008; Davies et al., 2007; Nguyen et al., 2011). We chose to focus on the outer membrane fusion proteins, Mfn1 and Mfn2, known to be important in mitochondrial morphology, docking, and transport (Chen et al., 2003; Huang et al., 2011; Mishra et al., 2015; Misko et al., 2010; Mourier et al., 2015), as their role in glaucoma and other neurodegenerative diseases is not yet fully understood. In this study, we focused on both RNA and protein levels of Mfn1 and Mfn2 to determine whether they are damaged or dysregulated in glaucoma.

RNA and protein analyses using pooled tissues demonstrated that changes in levels of the mitofusins occur as disease progresses. However, these methods only allowed for observation of overall trends associated with age and disease. We hypothesized that small differences could be masked by both inter-sample variability as well as the complex architecture and multiple cell types of the visual system, which could obscure key mechanistic findings.

To solve this problem, we developed the NICIF assay which is sensitive enough to evaluate very small quantities of protein (Frogne et al., 2012; O'Neill et al., 2006). This was essential for detecting low abundance proteins in minute samples of tissue, such as the ON, while still allowing single-animal assessments. The additional sensitivity showed that Mfn2 is more dynamic than Mfn1 in the DBA mouse model of glaucoma, perhaps because of Mfn2's additional role in mitochondrial transport (Misko et al., 2010). Our assay also showed that Mfn1 and Mfn2 were both less dynamic in the superior colliculus (data not shown) than in retinal or ON tissue.

We have previously reported differences by Western blot in mitochondrial protein levels in the retina, ON, and superior colliculus of the DBA mice (Uo et al., 2009). These data indicated that Mfn1 and Mfn2 levels increased significantly with age compared to age-matched B6 controls. We also observed an age-related decline for both mitofusins in the B6 control mice. In this study, we sought to reassess these data using tissue from D2G mice, the optimal genetic control. The D2G mouse does not develop glaucoma, but is in all other ways genetically similar to the DBA model. The similarity in findings between the previous and current studies of the B6 and the D2G mice confirms the appropriateness of using the D2G as controls, eliminating any question that genetic variability alone is the cause of observed protein changes. In using the D2G genetic controls, we were able to confirm elevated levels

of Mfn2 in aged, diseased (DBA) retina and slightly elevated levels in aged controls, which indicates that retinal Mfn2 is increasing with age, thereby suggesting an increased demand for mitochondrial fusion or transport.

Mfn1 has been shown to have a rescue effect in circumstances in which Mfn2 is compromised (Chen et al., 2003; Chen et al., 2007; Misko et al., 2010). In this compensatory role, one would expect to see an upregulation of Mfn1 in instances where Mfn2 is not present or present at diminished levels. As is shown in Fig. 8, Mfn1 does not compensate for the loss of Mfn2 seen in the aged diseased ON. The significant changes found in Mfn1 occur in retina, and suggest that protein levels decrease in normal aging, while increasing during disease progression. However, because Mfn2 also increases in the retina during disease progression, the increase in Mfn1 is not compensatory. Importantly, in the ON, in contrast to the stable Mfn1 levels, we found Mfn2 levels were significantly different between the healthy and diseased animals at both young and older ages. Thus, the dynamics between Mfn1 and Mfn2 are not solely of a compensatory nature, with Mfn2 levels changing more significantly, particularly in the ON where transport is ongoing.

In glaucoma, RGC axons are compromised in many ways, including decreases in ATP production and disruptions of mitochondrial transport (Baltan et al., 2010; Knott et al., 2008; Lee et al., 2011). In our lab, we have previously shown that the axons of DBA mice are more susceptible to metabolic challenges, as their ability to recover is severely compromised (Baltan et al., 2010). Mfn1 and Mfn2 proteins are directly involved in mitochondrial fusion, as well as transport of the mitochondria up and down the axons. We have shown previously that mitochondria in normal RGCs are short and punctate, while those in glaucomatous neurons are elongated or fused and tend to cluster more tightly around the nucleus (Uo et al., 2009). Our current data indicate that as RGCs are degenerating, Mfn2 protein is accumulating within the perinuclear space of the surviving nuclei at a higher rate. The colocalization of Mfn2 with mitochondria near the nuclei implies decreased degradation or dysfunctional circulation of mitochondria.

We have also previously detected a decrease in mitochondrial number in glaucomatous axons, potentially indicating a failure of transport (Uo et al., 2009). In the current study, image analysis showed that Mfn2 accumulation is localized to the perinuclear space of glaucomatous RGCs (see Fig. 2). We also demonstrate that, while overall Mfn2 levels decrease, the amount of Mfn2 in surviving RGCs increases. Together with our observation of decreased Mfn2 expression and protein in the aged DBA ON (Fig. 5E), we postulate that adequate Mfn2 protein exists in the retina, but the protein is either damaged or fails to transport to the ON. Lower levels of Mfn2 in the ON could indicate that Mfn2 protein is not being transported down the axon to where it normally functions in mitochondrial transport and repair. If this is confirmed to be a transport dysfunction for mitochondria, it could have a significant impact on the energy state of the cells (Rangaraju et al., 2014). A better understanding of this mechanism could be pivotal for establishing the role of Mfn2 function in the axon transport deficits and metabolic crisis that coincides with glaucoma.

Mfn2 exists in both active and inactive states that are phosphorylation-dependent. Mfn2 is phosphorylated by PINK1 at T111 and S442 in models of Parkinson's disease (Chen and

Dorn, 2013), and mutations at these sites alter mitochondrial dynamics (Shiba-Fukushima et al., 2014). Recent work in heart tissue has uncovered two phosphorylation sites on Mfn2 (Eschenbacher et al., 2012). Phosphorylated Mfn2 is a receptor for Parkin on depolarized mitochondria in cardiac tissue (Chen et al., 2011). In the current study, we demonstrate for the first time that Mfn2 has at least one active phosphorylation site in the CNS, and that it is reversibly phosphorylated in neuronal tissue. In addition, we detected two separate Mfn2 protein peaks that are distinguished by their isoelectric points with the ~6.2 isoelectric point corresponding to the phosphorylated Mfn2 present in the tissue. We found the initial amounts of phosphorylated Mfn2 in young DBA mice were greater than in the age-matched controls. Interestingly, we detected a significant shift to the non-phosphorylated form of Mfn2 during disease progression with no similar shift occurring in controls. This implies that the loss of phosphorylation seen is not due to aging (Fig. 4C), but rather, is likely brought about by the disease process (Fig. 4D; see also Fig. 5). These data indicate that the phosphorylation state of Mfn2 may be a novel indicator of disease.

Decreased and delayed axon transport has been observed in cultured dorsal root ganglions that lack Mfn2 (Misko et al., 2010) and is not a part of normal aging. Since Mfn2 requires active phosphorylation to bind to kinesin, an essential protein for organelle transport, impaired phosphorylation could result in fewer mitochondria transporting along the axon during disease progression. Alternatively, lowered ATP availability or damage to Mfn2 would prevent its phosphorylation and subsequent interaction with kinesin. The end result would be similar to the transport deficits that arise in glaucoma. That the transport malfunction can be attributed to Mfn2 dysregulation is further strengthened by our findings that Mfn2 is localized most heavily around the nuclei of the RGCs, and not widely diffused throughout the axonal processes. Because mitochondrial fusion also consumes ATP, this process would be compromised in the presence of unphosphorylated Mfn2, low Mfn2 availability, or damaged Mfn2 protein. Deficits in mitochondrial fusion impede mitochondrial repair and function (Chan, 2006a, 2006b, 2012; Chen et al., 2011; Cho et al., 2010). Future research is needed to understand the temporal dynamic whereby unphosphorylated Mfn2 does not function properly, and contributes to transport disruption, mitochondrial dysfunction and underlying neurodegeneration progression (Rangaraju et al., 2014).

We next questioned whether ubiquitin is recruited to Mfn2, where it could interfere with Mfn2's role in transport. It is known that ubiquitin is recruited to the mitochondrial outer membrane in drosophila via the PINK/Parkin pathway when membrane depolarization occurs (Glauser et al., 2011; Poole et al., 2008; Poole et al., 2010), with translocation of Parkin to the mitochondria for degradation (Gegg et al., 2010; Gong et al., 2015; Tanaka et al., 2010). In the present study, we found that ubiquitin does colocalize with Mfn2, but not with Mfn1 (data not shown), and that it is most highly present in young DBA retinas. Therefore, we hypothesize that the loss of ubiquitination of Mfn2 prevents repair and recycling of the protein which could interfere with both mitochondrial fusion and transport. However, determining the role of ubiquitin and its interference with the function of Mfn2 in ON is challenging as ubiquitin was only detectable by Western blot in pooled samples, suggesting that levels are low enough in individual animals to make it problematic to

determine whether ubiquitin is absent in most mice or just appearing at levels below the assay's detection limit.

5. Conclusions

Mitochondrial disruption is an early event in neurodegeneration, with impaired bioenergetics and migration acting as neurodegenerative triggers (Knott et al., 2008). As mitochondria play so many key functional roles in the cell, the decrease of ATP production and disruption of mitochondrial transport can readily be viewed as pivotal causes underlying disease (Baltan et al., 2010; Rangaraju et al., 2014). Our data implicate a loss of function of Mfn2 and its altered post-translational states in the metabolic dysfunction, transport, and mitochondrial fusion changes observed in many forms of glaucoma. Conversely, disease may interfere with the cellular infrastructure, disrupting the ability of mitochondria to function in many necessary locations throughout a cell. In either case, properly functioning mitochondria and disease are incontrovertibly linked (Detmer and Chan, 2007). Demonstration of the presence of phosphorylated Mfn2 is a novel finding in the nervous system. The potential significance of our observations of the change in Mfn2 phosphorylation state is still speculative and warrants continued exploration.

In the future, we can begin to address potential targets for correcting mitochondrial behavior and health and also explore the exciting possibility that changes in fusion protein post-translational status could serve as a useful biomarker for axonal degeneration (Zhou et al., 2016). Therapeutically manipulating the balance between fusion and fission proteins may improve recovery or delay axonal degeneration. In addition, what we can learn about mitochondrial compromise and Mfn2 in this specific neurodegenerative disease may well translate to other “long axon” diseases (Chan, 2006b).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.expneurol.2017.07.001>.

Abbreviations

CMT Charcot-Marie-Tooth

DBA	DBA/2J
DOA	dominant optic atrophy
D2G	DBA/2J ^{Gpnmb+}
IOP	intraocular pressure
Mfn1	mitofusin 1
Mfn2	mitofusin 2
NICIF	nanoimmuno capillary isoelectric focusing
NP	NanoPro
ON	optic nerve
Opa1	optic atrophy 1
pI	isoelectric point
RGC	retinal ganglion cell

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Fig. 1.

Protein and RNA levels change with disease progression. (A) Overall Mfn2 RNA expression in retina is significantly greater in the controls when compared to the DBAs (** $p < 0.005$). Mfn2 expression is significantly lower in the DBA animals at both 3 months (* $p < 0.02$) and 9 months (* $p < 0.05$). (B) In ON, Mfn1 RNA trends toward increased expression with age in D2Gs, while decreasing with age in DBAs, with the decrease reaching significance between 6 month cohorts ($p < 0.02$). (C) Mfn2 protein levels were higher overall in DBA retinas, with levels being higher as the mice aged. (D) Mfn1 protein levels were lower in the ON of DBA mice when compared to D2G controls.

Fig. 2. Mfn2 is concentrated in RGCs, with additional accumulation in degenerating RGC somas. (A) Immunofluorescent image of 3-month-old DBA retina shows expression of Mfn2 throughout the retina. Mfn2 (green) can be seen to concentrate in NeuN⁺ RGCs (red), but is limited in other cells and layers of the retina. (B) Imaris imaging shows distribution of Mfn2 (green) is primarily localized to RGCs (red) of young DBA mice. (C) Image of a 10-month-old DBA retina shows fewer RGCs. (D) Imaris imaging of aged retinas show decreased NeuN (decreased RGC population), but greater accumulation of Mfn2 in the perinuclear space on a per-cell basis. Blue stain: GFAP. Scale bars: A, B, C, D = 10 μ m; insets = 5 μ m.

Fig. 3.

NICIF assay uncovers differences in protein expression based on age and disease. Replicate tracings reflect retinal protein levels in (A) young control (D2G), (B) aged D2G, (C) a comparison between the young and aged D2Gs, (D) young DBA, (E) aged DBA, and (F) a comparison between the young and aged DBAs. Representative average tracings (C, F) compare young to aged conditions on the same scale. The percentages given for each peak in A, B, D, and E reflect the average amount of the total protein per peak for each age/condition.

Fig. 4.

Mfn2 displays a selective loss of the phosphorylation-specific peak in diseased animals. A phosphatase-stripping assay performed on control tissues shows a near-complete depletion of the peak at 6.2 pI in (A) ON and (B) retina, with a concurrent increase in the peak at 6.5 pI. Blue tracing = untreated tissues; green tracing = tissues treated with lambda phosphatase; $n = 3$. This shift to the non-phosphorylated form of Mfn2 is not due to aging (C), but occurs with disease (D). Tracings are from one representative animal. Group data are quantified in Figs. 5–7. pI, isoelectric point.

Fig. 5.

Mfn2 protein levels decrease with age in the diseased ON, but increase with age in the diseased retina. In young mice, Mfn2 protein levels are higher in DBA mice (green) than in controls (blue) in both (A) ON tissue and (B) retina. (C) In aged mice, a three-fold decrease in Mfn2 is seen in diseased ON (green) when compared with controls (blue), while (D) aged, diseased retinal tissue shows a three-fold increase in Mfn2 when compared to age-matched controls (blue). In aged DBA mice, there was a significant increase in non-phosphorylated Mfn2 in both the ON and retina (C, D). Each tracing (A-D) is from one representative animal. (E) Quantification of Mfn2 in ON shows significantly higher levels of protein in young DBA mice ($n = 8$) compared to young controls ($n = 8$; $p < 0.004$), as well as a significantly lower levels of Mfn2 in aged DBA ($n = 64$) compared to age-matched controls ($n = 48$; $p < 0.0001$). Mfn2 increases significantly with age in controls ($p = 0.004$), while significantly decreasing with age in DBAs ($p = 0.002$). (F) In the retina, Mfn2 quantification shows no significant differences between young ($n = 22$) and aged ($n = 44$) control mice, however there are significantly higher levels of protein expression in young DBA mice ($n =$

30) compared to age-matched controls ($n = 22$; $p < 0.02$). Mfn2 expression levels are significantly higher in aged DBA retina ($n = 72$) compared to young DBAs ($n = 30$; $p < 0.004$). Also, there is significantly higher Mfn2 protein levels in aged DBA retina ($n = 72$) compared to age-matched controls ($n = 44$; $p < 0.003$). pI, isoelectric point; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Fig. 6.

Mfn1 expression in retina decreases with age in control mice, but increases with disease progression. In ON (A–C), Mfn1 shows similar expression patterns and levels in both young and aged mice (A). Blue and green tracings are young D2G and DBA, respectively; burgundy and pink tracings are aged D2G and DBA, respectively. (B) Quantification of total Mfn1 protein levels shows a significant drop with aging ($p < 0.02$), with no difference between control and disease. (C) Comparison of individual peak areas shows a major shift from the phosphorylated peak (P1) to the non-phosphorylated peak (P2) that occurs with age

($p < 0.02$). In retina (D–G), control animals have a significant drop in Mfn1 levels with age (D; $p < 0.004$), while, conversely (E) DBA mice show a significant increase with age ($p < 0.003$) (D and E, blue tracings = young animals; green tracings = aged animals). (F) Aged DBA mice (green) express significantly higher levels of Mfn1 than aged controls (blue) ($p < 0.004$). (G) Quantification of Mfn1 protein levels in retina reflects the inverse expression pattern between control and DBA animals with age ($p \leq 0.002$). pI, isoelectric point; each tracing (A, D, E, F) is from one representative animal; graphs (B, C, G) are derived from representative average data from 3 samples.

Fig. 7.

Ubiquitin levels are highest in the retinas of young DBA mice. (A) In control D2G mice, ubiquitin levels in the retina remain stable during aging, but (B) decline significantly with disease progression. Each tracing is from one representative animal. (C) Quantification shows significantly higher ubiquitin expression in 3-month-old DBA when compared to controls of either age or to aged DBA ($p = 0.001$; $n = 3$). Western blots show (D) retinal ubiquitin levels drop with disease progression while remaining stable in control mice, but ON ubiquitin drops with age in both D2G and DBA. (D') Quantification, using ImageJ, is normalized to young controls in both retina and ON. (E) Immunoprecipitation with Mfn2 shows ubiquitin colocalizes with Mfn2. Lane markers represent varying urea concentrations used: L1 = 0 M, L2 = 5 M, L3 = 7 M.

Fig. 8.

Summary of fold changes of Mfn1 and Mfn2 protein levels in all cohorts (see Table 1). All fold changes are in comparison to young controls in each group.

Table 1

Number of individual mice^a per strain and tissue type used in NICIF assays.

		Mfn1/Mfn2		
		3 mo	6 mo	9 mo
Retina	DBA	15	17	36
	D2G	11	11	27/22 ^b
ON	DBA	4	21	32
	D2G	4	12	24

^a Each mouse provided two retina and two ON samples.

^b 27 mice were probed for Mfn1 and 22 for Mfn2.

Table 2

Intracellular distribution of Mfn2 protein.

	NeuN ⁺ cells/retina (<i>n</i> = 3)	Mfn2 tags/RGC	Nuclear proximity (4 μm) of Mfn2
Young DBA ^a	54 ± 6.2	768 ± 87.8	14.2 ± 1.6/nucleus
Aged DBA	14 ± 1.6 ^{**}	406 ± 47.6 [*]	29.0 ± 3.4/nucleus [*]
Aged D2G (controls)	48 ± 11.6	565 ± 135.8	11.8 ± 2.8/nucleus

^aData for the young D2G controls are not shown as they were virtually identical to that found in the young DBAs.^{*}*p* < 0.05.^{**}*p* < 0.01.