GLAUCOMA

Vitamin B3 modulates mitochondrial vulnerability and prevents glaucoma in aged mice

Pete A. Williams, Jeffrey M. Harder, Nicole E. Foxworth, Kelly E. Cochran, Vivek M. Philip, Vittorio Porciatti, Oliver Smithies, Simon W. M. John

Glaucoma is a group of complex, multifactorial diseases characterized by the progressive dysfunction and loss of retinal ganglion cells (RGCs), leading to vision loss. Glaucoma is one of the most common neurodegenerative diseases worldwide, affecting more than 70 million people (1). High intraocular pressure (IOP) and increasing age are important risk factors for glaucoma (2, 3). However, specific mechanisms rendering RGCs more vulnerable to damage with age are unknown. Here, we address how increasing age and high IOP interact to drive neurodegeneration using DBA/2J (D2) mice, a widely used model of chronic, age-related, inherited glaucoma (4).

We used RNA-sequencing (RNA-seq) to elucidate age and IOP-dependent molecular changes within RGCs that precede glaucomatous neurodegeneration. We analyzed RGCs of 9-month-old D2 mice (in a stage termed early glaucoma) and 4-month-old D2 mice (in a stage preceding high IOP); and age-, sex-, and strain-matched D2-Gmpnb+ controls (which do not develop high IOP or glaucoma (4)) (Fig. 1). RGCs were isolated (fig. S1), and their RNA was sequenced at a depth of 35 million reads per sample. Unsupervised hierarchical clustering (HC) allowed molecular definition of early glaucoma stages among samples that were still morphologically indistinguishable from age-matched D2-Gmpnb+ or young controls. HC identified four distinct groups of 9-month-old D2 samples (groups 1 to 4). Group 1 clustered with all of the control samples and represents D2 RGCs with no detectable glaucoma at a molecular level. Although groups 2 to 4 were all early stages, increasing group number reflects greater glaucoma progression at a transcriptomic level (Fig. 1A and fig. S2, A and B). As disease progressed, there was an increase in transcript abundance that was most pronounced for mitochondrial reads (Fig. 1B). Emerging evidence suggests that imbalances in the relative proportions of mitochondrial proteins encoded by nuclear and mitochondrial genomes negatively affect mitochondrial function (5). In D2 groups 2 to 4, differential expression of genes encoding mitochondrial proteins, as well as significant enrichment [false discovery rate (FDR) < 0.05] of differentially expressed (DE) genes in the mitochondrial dysfunction and oxidative phosphorylation pathways, further point to mitochondrial abnormalities (Fig. 1, B to G; fig. S2, C to G; and tables S1 to S3). Pathway analysis identified enrichment of eukaryotic initiation factor 2 (eIF2) and mammalian target of rapamycin (mTOR) signaling transcripts (Fig. 1C). eIF2 is a key regulator of redox homeostasis and cellular adaptations to stress and was the most enriched pathway in group 2 (first distinguishable stage from controls). Through mTOR inhibition, it promotes survival in the presence of oxidative stress (6–8) and likely protects from mitochondrial abnormalities in RGCs at this early disease stage. Mitochondrial fission (Fig. 1F) and mitochondrial unfolded protein response genes were also DE (Fig. 1G) (9–11). Electron microscopy (EM) revealed abnormal mitochondria with reduced cristae volume in the dendrites of D2 RGCs but not in those of control RGCs (Fig. 1, H and I). These mitochondrial EM findings coincide with synapse loss in 9-month-old D2 retinas (12), with early decreases in pattern electroretinogram amplitude (PERG) (13) and an increase in retinal cytochrome c levels (fig. S2, D and F). Extending previous studies (14, 15), our data demonstrate that mitochondrial perturbations are among the very first changes occurring within RGCs during glaucoma. Guided by the above data, we assessed metabolites in retinas with increasing age and disease (D2 and D2-Gmpnb+ at 4, 9, and 12 months). We detected early decreases in metabolites that are central to healthy mitochondrial metabolism and protection from oxidative stress: nicotinamide adenine dinucleotide (NAD+), and the reduced form of NAD+ (NADH) (total NAD, NADH)) and both the oxidized and reduced forms of glutathione (total glutathione, glutathione(t)) (Fig. 2A and fig. S2, H and I). These age-dependent decreases were not a response to IOP insult(s), as they also occurred in control D2-Gmpnb+ retinas (fig. S2I). These decreases are expected to sensitize retinal neurons to disease-related stresses and mitochondrial dysfunction. There are increased levels of hypoxia-inducible factor HIF-1α mRNA and protein [a key metabolic regulator during perturbed redox states (16)] in the ganglion cell layer early in glaucoma, which suggests that there is greater metabolic stress in RGCs than in other retinal neurons (fig. S3, A and B). Our data suggest that RGCs go through a period of mitochondrial stress and metabolite depletion, which potentially moves them toward fatty acid metabolism (fig. S4). Fatty acid β-oxidation can increase generation of free radicals and/or reactive oxygen species (ROS) (17). Both RNA-seq (fig. S2C) and γ-H2AX immunostaining (fig. S2, J and K) results support increased ROS and DNA damage within RGCs early in glaucoma. Providing a link between DNA damage and increased metabolic stress, poly(ADP-ribose) polymerase (PARP) activity (NAD consuming) is induced in RGCs with age (fig. S5, A and B).

Our data support a model where age-dependent declines of NAD+ and glutathione in the retina render RGCs vulnerable to damage from elevated IOP. Thus, increasing NAD levels would be predicted to protect IOP-insulted eyes from glaucomatous changes by decreasing the probability of metabolic and/or energetic failure and render the RGCs more resilient to IOP-induced stress. Oral supplementation of vitamin B3 [nicotinamide (NAM), a precursor of NAD+] has been successfully used to correct disturbances in NAD+ metabolism in two mouse models of preclampsia (18). Accordingly, we administered NAM to D2 mice, initially at the same dose (550 mg/kg of body weight per day, NAM150) (Fig. 2). NAM administration in drinking water prevented the decline of NAD levels up to 12 months of age (a standard end stage for assessing neurodegeneration in this glaucoma model) (Fig. 2A). The finding that NAM150 did not alter IOP (fig. S6) but protected from glaucoma supported our neuronal vulnerability hypothesis.

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NAM was protective both prophylactically (starting at 6 months, before IOP elevation in most eyes in our colony) and interventionaly (starting at 9 months, when the majority of eyes have had continuing IOP elevation) (Fig. 2B). NAM significantly reduced the incidence of optic nerve degeneration (Fig. 2, B and E), prevented RGC loss and retinal nerve fiber layer thinning (Fig. 2, C and E), and protected visual function, as described by Williams et al. (Science 355, 756–760 (2017) 17 February 2017).
Fig. 2. Vitamin B3 (NAM) supplementation protects against glaucoma development in mice. (A) NAD(t) levels were increased in NAM-treated D2 retinas as measured by colorimetric assay (n = 22 per group). Mo, months. (B and E) NAM intervention protected from optic nerve degeneration as assessed by PPD staining paraphenylenediamine, a sensitive stain for damaged axons. Green, no or early damage (<5% axon loss; no or early (NOE)); yellow, moderate damage (~30% axon loss; MOD); red, severe (>50% axon loss; SEV) damage. Early start indicates mice that started treatment at 6 months (before IOP elevation in most eyes in our colony and, thus, prophylactic). Late start indicates mice that started treatment at 9 months (when the majority of eyes have had continuing IOP elevation, thus interventional). **P < 0.01; ***P < 0.001 (Fisher’s exact test). (C and E) NAM protected from RGC soma loss [number of somas positive for RNA binding proteins with multiple splicing (RBPMS+ cells), n = 8 per group]; the density drop between D2 and D2-Gpnmb+ is due to pressure-induced stretching. (D) NAM protected from early loss in PERG amplitude (n > 20 per group). (E) NAM protected from RGC soma loss (n = 8 per group), retinal nerve fiber layer and inner plexiform layer (IPL) thinning (n = 8 per group), optic nerve degeneration (n > 50 per group), and loss of anterograde axoplasmic transport (n = 20 per group). DAPI, 4′,6-diamidino-2-phenylindole; GCL, ganglion cell layer; INL, inner nuclear layer. Corresponding markers and color keys are beneath each column. Scale bars: RBPMS (a specific marker of RGCs; immunofluorescence), 20 μm; Nissl (a pan-neuronal stain; light microscopy), 20 μm; PPD (light microscopy), 20 μm; CT-β, 100 μm (for retina; immunofluorescence); 200 μm (for LGN and Sup. col.). ONH, optic nerve head; LGN, lateral geniculate nucleus; Sup. col., superior colliculus. White asterisk denotes loss of axonal transport at the site of the ONH. (F) Heat map of gene expression (all expressed genes) shows that NAM-treated RGCs were molecularly similar to controls. (G) Individual gene expression plots show metabolic and DNA damage pathways were returned to normal in NAM-treated RGCs. Dots represent individual genes; gray, not differentially expressed; red, differentially expressed at q < 0.05 compared with the D2-Gpnmb+ 9-month-old control. (A), (C), and (D): *P < 0.05; **P < 0.01; ***P < 0.001 (Student’s t test). For box plots, center hinge represents the mean and the upper and lower hinges represent the first and third quartiles; whiskers represent 1.5 times the interquartile range; values beyond the whiskers are plotted as outliers. See also fig. S4 and tables S1 to S3.
Fig. 3. Gene therapy protected eyes from glaucomatous neuron degeneration. D2 eyes were intravitreally injected at 5.5 months with the adenovirus AAV2.2 carrying a plasmid to overexpress murine Nmnat1 under a CMV promoter. (A) Nmnat1 overexpression prevented RGC soma loss (red) (scale bar, 50 μm) and loss of anterograde axoplasmic transport (n = 10 per group) (as demonstrated in Fig. 2). (B) Soma loss (red). Scale bar, 100 μm LGN, lateral geniculate nucleus; Sup. Col., superior colliculus.

Nmnat1 gene therapy also protected D2 eyes with elevated IOP against (C) optic nerve degeneration (red; n > 40 per group; ***P < 0.001, Fisher’s exact test); (D) soma loss (n = 6 per group); and (E) PERG amplitude (n > 20 per group). Addition of NAMLo in drinking water afforded additional protection against optic nerve degeneration (Nmnat1 compared to Nmnat1 + NAMLo = P < 0.001, Fisher’s exact test). (C), (D), and (E): ***P < 0.001; ***P < 0.001 (Student’s t test).
through to the end-stage time point (12 months) (Fig. S10). Overexpression of Nmnat1 was sufficient to prevent axon and soma loss (Fig. 3, A to D), to preserve axoplasmic transport (Fig. 3B), and to preserve electrical activity in RGCs (PERG) (Fig. 3E). Glaucomatous nerve damage was absent in >70% of treated eyes. Because NMNAT1 catalyzes the terminal step in NAD production, the major protective effects of NAM treatment likely result from driving NAD production in neurons rather than other NAD-independent mechanisms (but partial contributions from other mechanisms cannot be completely excluded). We further assessed the effects of combinational therapy of Nmnat1 and NAM16. This combination afforded significant additional protection over Nmnat1 or NAM17 alone, with 84% of eyes having no detectable glaucoma (~4-fold decreased risk of developing glaucoma). Increasing the NAM dose combined with gene therapy may prove even more protective.

In conclusion, we show that dietary supplementation with a single molecule (vitamin B3 or NAM) or Nmnat1 gene therapy significantly reduces vulnerability to glaucoma by supporting mitochondrial health and metabolism. Combined with established medications that lower IOP, NAM treatment (and/or Nmnat1 gene therapy) may be profoundly protective. By providing a new molecular and metabolic link between increased neuronal vulnerability with age and neurodegeneration, these findings are of critical importance for glaucoma and possibly other age-related diseases.

REFERENCES AND NOTES
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SUPPLEMENTARY MATERIALS
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Materials and Methods
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Vitamin B₃ modulates mitochondrial vulnerability and prevents glaucoma in aged mice

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Vitamin B₃ protects mice from glaucoma

Glaucoma is the most common cause of age-related blindness in the United States. There is currently no cure, and once vision is lost, the condition is irreversible. Williams et al. now report that vitamin B₃ (also known as niacin) prevents eye degeneration in glaucoma-prone mice (see the Perspective by Crowston and Trounce). Supplementing the diets of young mice with vitamin B₃ averted early signs of glaucoma. Vitamin B₃ also halted further glaucoma development in aged mice that already showed signs of the disease. Thus, healthy intake of vitamin B₃ may protect eyesight.

Science, this issue p. 756; see also p. 688