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Vitamin B₃ modulates mitochondrial vulnerability and prevents glaucoma in aged mice

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Abstract

Glaucomas are neurodegenerative diseases that cause vision loss, especially in the elderly. The mechanisms initiating glaucoma and driving neuronal vulnerability during normal aging are unknown. Studying glaucoma-prone mice, we show that mitochondrial abnormalities are an early driver of neuronal dysfunction, occurring prior to detectable degeneration. Retinal levels of nicotinamide adenine dinucleotide (NAD⁺; a key molecule in energy and redox metabolism) decrease with age, and render aging neurons vulnerable to disease-related insults. Oral administration of the NAD⁺ precursor nicotinamide (vitamin B₃), and/or gene therapy (driving expression of *Nmnat1*, a key NAD⁺ producing enzyme), was protective both prophylactically and as an intervention. On our highest dose, 93% of eyes did not develop glaucoma. This supports therapeutic use of vitamin B₃ in glaucoma and potentially other age-related neurodegenerations.

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 over 70 million people (1). High intraocular pressure (IOP) and increasing age are important risk factors for glaucoma (2, 3). However, specific mechanisms rendering retinal ganglion cells 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).

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We utilized 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 (mo) D2 mice (termed early glaucoma - high IOP and molecular changes but lacking neurodegeneration (2)), 4 mo old D2 mice (precedes high IOP) and age, sex and strain matched D2-*Gpnmb*⁺ controls (do not develop high IOP or glaucoma (4)) (Fig. 1). RGCs were isolated (Fig. S1) and their RNA 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-*Gpnmb*⁺ or young controls. HC identified 4 distinct groups of 9 mo 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, Fig. S2A 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 impact mitochondrial function (5). In D2 Groups 2 to 4, differential expression of genes encoding mitochondrial proteins, and significant enrichment of differentially expressed (DE) genes in the *mitochondrial dysfunction* and *oxidative phosphorylation* pathways further point to mitochondrial abnormalities (Fig. 1B–G, Fig. S2C–G, Table S1–3). Pathway analysis identified enrichment of eIF2 and 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 (UPR^{mt}) 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. 1H and I). These mitochondrial EM findings coincide with synapse loss in 9 mo D2 retinas (12), with early decreases in pattern electroretinogram amplitude (PERG) (13), and an increase in retinal cytochrome c levels (Fig. S2D 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-*Gpnmb*⁺; 4, 9, and 12 mo). We detected early decreases in metabolites that are central to healthy mitochondrial metabolism and protection from oxidative stress (NAD⁺ and NADH [total NAD; NAD(t)], GSH and GSSG [total glutathione; glutathione(t)]) (Fig. 2A, Fig. S2H–I). These age-dependent decreases were not a response to IOP-insult(s) as they also occurred in control D2-*Gpnmb*⁺ retinas (Fig. S2I). These decreases are expected to sensitize retinal neurons to disease related stresses and mitochondrial dysfunction. Suggesting greater metabolic stress in RGCs than other retinal neurons, HIF-1 α (a key metabolic regulator during perturbed redox states (16)) is induced in the ganglion cell layer early in glaucoma (Fig. S3A and B). Our data suggest that RGCs go through a period of mitochondrial stress and metabolite depletion, potentially moving towards fatty acid

metabolism (Fig. S4). Fatty acid β -oxidation can increase generation of free radicals/reactive oxygen species (ROS) (17). Both RNA-seq (Fig. S2C) and γ -H2AX immunostaining (Fig. S2J and K) support increased ROS and DNA damage within RGCs early in glaucoma. Providing a link between DNA damage, and increased metabolic stress, PARP activity (NAD consuming) is induced in RGCs with age (Fig. S5A 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/energetic failure and rendering 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 pre-eclampsia (18). Accordingly, we administered NAM to D2 mice, initially at the same dose (550 mg/kg/d, NAM^{Lo}) (Fig. 2). NAM administration in drinking water prevented the decline of NAD levels through to 12 mo (a standard end stage for assessing neurodegeneration in this glaucoma model) (Fig. 2A). Supporting our neuronal vulnerability hypothesis, NAM^{Lo} did not alter IOP (Fig. S6), but protected from glaucoma. NAM was protective both prophylactically (starting at 6 mo, prior to IOP elevation in most eyes in our colony) and interventionally (starting at 9 mo, when the majority of eyes have had continuing IOP elevation) (Fig. 2B). NAM significantly reduced the incidence of optic nerve degeneration (Fig. 2B and E), prevented RGC soma loss and retinal nerve fiber layer thinning (Fig 2C and E), and protected visual function as assessed by PERG (13) (Fig. 2D, Fig. S3E). NAM prevented RGC axonal loss, and these axons continued to support anterograde axonal (Fig. 2E). NAM administration was sufficient to inhibit the formation of dysfunctional mitochondria with abnormal cristae (Fig. 2E, Fig. S3G and H) and also limited synapse loss that occurs in this model (12) (Fig. S3C and D). Lipid droplet formation was also prevented in aged D2 retinas (Fig. S3F). NAM also decreased PARP activation, limited levels of DNA damage, and transcriptional induction of HIF-1 α (Fig. S3A and B) reflecting less perturbed cellular metabolism. NAM prevented even the earliest molecular signs of glaucoma in most treated eyes as assessed by RNA-seq (Fig. 2F and G, Fig. S7) and prevented the majority of age-related gene expression changes within RGCs (Fig. S8). This highlights the unexpected potency of NAM in decreasing metabolic disruption and prevention of glaucoma.

Attempting to further decrease the probability of glaucoma, we administered a higher dose of NAM (2000mg/kg/d; NAM^{Hi}). NAM^{Hi} was extremely protective with 93% of treated eyes having no optic nerve damage (Fig. 2B). The degree of protection afforded by administering this single molecule is unprecedented and unanticipated. Although NAM^{Lo} demonstrates a clear neuroprotective effect (no effect on IOP), NAM^{Hi} lessens the degree of IOP elevation (Fig. S6). This indicates that NAM can protect against age-related pathogenic processes in additional cell types to RGCs (11, 16, 19). Therefore vitamin B₃/NAM, a single molecule that protects against both IOP elevation and neural vulnerability, has great potential for glaucoma treatment, however human studies are needed.

NMNAT2 is emerging as an important NAD producing enzyme in axons, protecting from axon degeneration (20). Ongoing stress negatively impacts *Nmnat2* expression in RGCs ($q <$

0.05 in D2 Group 4) (Fig. S7F). This decline of NMNAT2 may induce vulnerability to axon degeneration in glaucoma. *NMNAT2* expression is decreased in brains with Alzheimer's disease and is highly variable in aged postmortem human brains (21). Such variation in expression may contribute to individual differences in vulnerability to various neurodegenerations.

Glaucoma is a complex disease involving multiple insults. Mechanical axon damage and local inflammation are two important contributors in glaucoma (22–24). To more fully assess the general effectiveness of NAM treatment, we tested its efficacy in two models of RGC death that are used to model these glaucomatous insults. We used a tissue culture model of axotomy, and intravitreal injections of soluble murine TNF α which can drive local inflammation as well as mitochondrial dysfunction and is implicated in glaucoma (25). NAM robustly protected cultured retinas from RGC somal degeneration (Fig. S9AB). NAM also protected against a loss of PERG amplitude and cell loss in TNF α injected eyes (Fig. S9C–E). Given these protections against severe acute insults, NAM could have broad implications for treating glaucoma and potentially other age-related neurodegenerative diseases.

Gene therapy is an attractive approach for overcoming compliance issues and improving efficacy. In the eye, gene therapy has proven successful for rare human Mendelian disorders (26). Viral gene therapy allows a large number of cells to be transfected lifelong, delivering a targeted gene product. To date, gene therapy has not been successfully applied to complex human diseases. Given that age is a common risk factor for most glaucomas, protecting from age-related declines in NAD may generally protect many glaucoma patients. Thus, we sought to support NAD⁺ producing cellular machinery through the overexpression of *Nmnat1*, a terminal enzyme in NAD⁺ production, further testing our NAD hypothesis. (Using NAMPT, the rate-limiting enzyme in NAD synthesis, may be complicated both by its cytokine functions and over-production of NMN, which may participate in axon degeneration (27).) D2 eyes were injected once with AAV2.2 containing the *Nmnat1* gene and *GFP* under a CMV promoter expressed as a single transcript (Fig. 3). *Nmnat1* expression (as assessed by GFP expression) was robust in RGCs 2 weeks after injection (expressed in >83% of RGCs), and remained robust through to the end stage time point (12 mo) (Fig. S10). Overexpression of *Nmnat1* was sufficient to prevent axon and soma loss (Fig. 3A–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. Since NMNAT1 catalyzes the terminal step in NAD production, the major protective effects of NAM treatment likely results from driving NAD production in neurons rather than other NAD independent mechanisms (but partial contributions from other mechanism cannot be completely ruled out). We further assessed the effects of combinational therapy of *Nmnat1* and NAM^{Lo}. This combination afforded significant additional protection over *Nmnat1* or NAM^{Lo} 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/nicotinamide), 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The data reported in this paper are available in the Supplementary Materials. RNA-seq data are available through the Gene Expression Omnibus (accession number GSE90654).

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One Sentence Summary

Targeting NAD through dietary vitamin B₃ or gene therapy prevents IOP-induced metabolic dysfunction and glaucoma.

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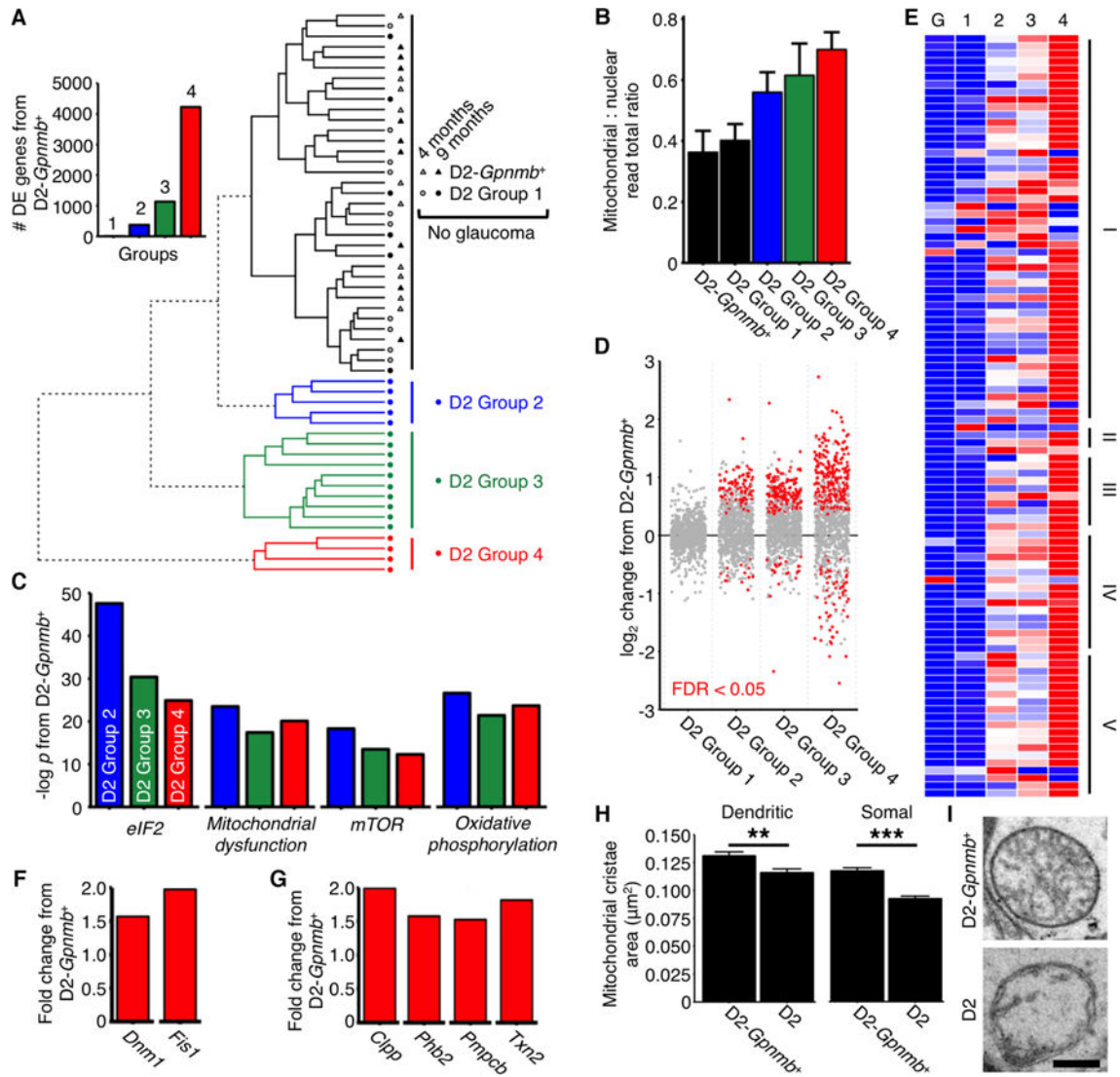


Fig. 1. Mitochondrial dysfunction is associated with progressive RGC damage in glaucoma (A) RGC samples were divided into molecularly distinct groups by HC of RNA-seq determined gene expression. Control (D2-Gpnm^{b+}) and young samples were molecularly similar (Spearman's ρ , $n = 63$ samples). Circles = samples from D2 RGCs, triangles = samples from D2-Gpnm^{b+} RGCs. Inset: number of DE genes ($q < 0.05$) between D2-Gpnm^{b+} and each group. D2 Group 1 and D2-Gpnm^{b+} represent no glaucoma at a molecular/transcriptomic level. (B) Mitochondrial:nuclear read total ratio increased with increasing HC distance from controls. Colors match key in A. (C) Top significantly enriched pathways between clusters and control based on IPA analysis (there are no differentially expressed pathways in D2 Group 1) (see also Table S3). (D) Transcript expression primarily increased for nuclear encoded mitochondrial proteins with increasing HC distance from controls. Dots represent individual genes, grey = not differentially expressed, red = differentially expressed at $q < 0.05$. Genes taken from mouse MitoCarta2.0 (28) (E) OXPHOS genes were differentially expressed across all groups. Red = highest expression, blue = lowest expression, I-V = mitochondrial complexes I-V (tabulated in Table S1), G =

D2-*Gpnmb*⁺, 1–4 = D2 Groups 1–4. **(F)** RNA-seq identified increased mitochondrial fission gene transcripts early in glaucoma and **(G)** suggests an early mitochondrial unfolded protein response compared to controls. Data shown is for D2 Group 4. **(H and I)** Mitochondria of D2 mice have decreased cristae volume (and cristae : total volume ratio, *not shown*) in RGC somal and dendritic mitochondria (there was no significant difference in total mitochondrial size/volume) ($n > 400$ mitochondria from 6 retinas/group). Scale bar = 350 nm. All data is at 9 mo unless otherwise stated. ** = $P < 0.01$, * = $P < 0.001$ (*Student's t* test). See also Table 1, Table 2.

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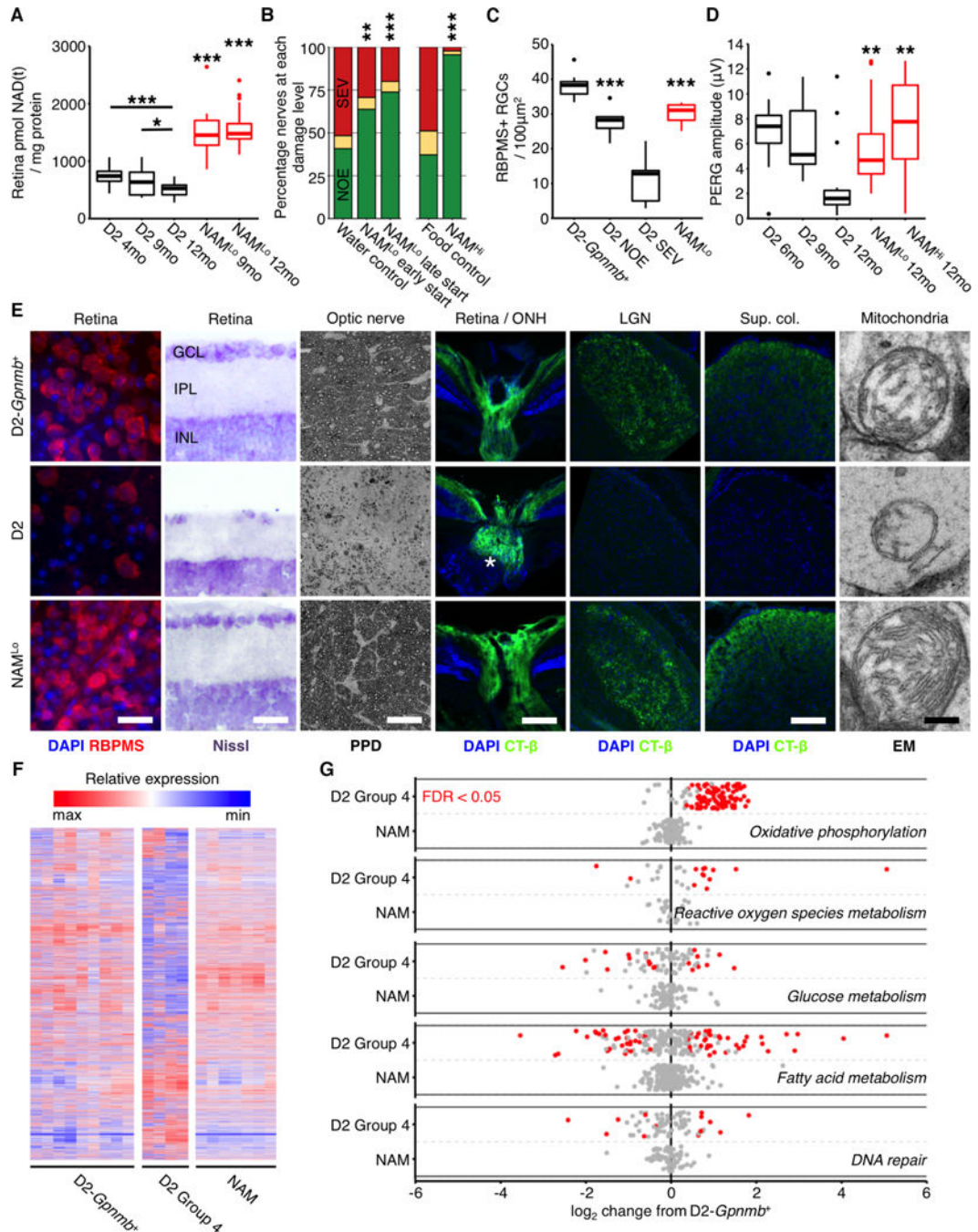


Fig. 2. Vitamin B₃/NAM supplementation protects against glaucoma development in mice
 (A) NAD(t) levels were increased in Vitamin B₃/NAM treated D2 retinas as measured by colorimetric assay ($n = 22$ /group). (B and E) NAM intervention protected from optic nerve degeneration as assessed by PPD staining (a sensitive stain for damaged axons). *Green* = no or early damage (<5% axon loss; NOE), *yellow* = moderate damage (~30% axon loss; MOD), *red* = severe (>50% axon loss; SEV) damage. Early start indicates mice that started treatment at 6 mo (prior to IOP elevation in most eyes in our colony, thus prophylactic). Late start indicates mice that started treatment at 9 mo (when the majority of eyes have had

continuing IOP elevation, thus interventional). Fisher's exact test: ** = $P < 0.01$, *** = $P < 0.001$. (**C and E**) NAM protected from RGC soma loss (number of RBPMS+ cells, $n = 8$ /group, the density drop between D2 and D2-*Gpnmb*⁺ is due to pressure induced stretching). (**D**) NAM protected from early loss in PERG amplitude ($n > 20$ /group). (**E**) NAM protected from RGC soma loss ($n = 8$ /group), retinal NFL and IPL thinning ($n = 8$ /group), optic nerve degeneration ($n > 50$ /group), and loss of anterograde axoplasmic transport ($n = 20$ /group). 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 (retina; immunofluorescence), 200 μm (LGN, 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**) Heatmap 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, *grey* = not differentially expressed, *red* = differentially expressed at $q < 0.05$ compared to D2-*Gpnmb*⁺ 9 mo control. (**B, D, and E**) * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ (*Student's t* test). For boxplots, center hinge represents the mean, and the upper and lower hinges represent the first and third quartiles, whiskers represent 1.5 * interquartile range, values beyond the whiskers are plotted as outliers. See also Fig. S4, Tables 1–3.

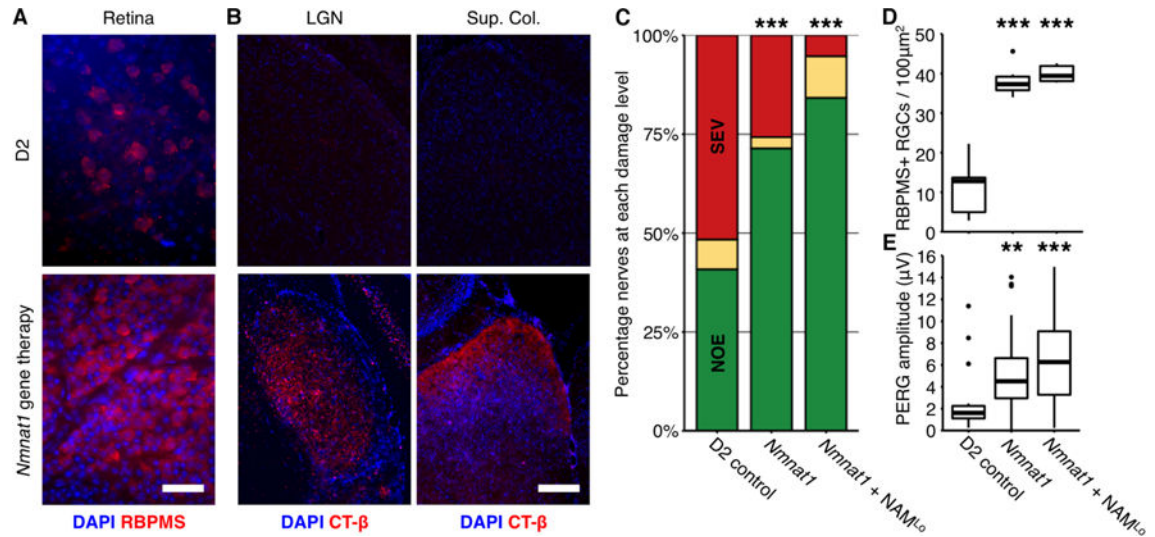


Fig. 3. Gene therapy protected eyes from glaucomatous neurodegeneration

D2 eyes were intravitreally injected at 5.5 mo with 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 ($n = 10/\text{group}$) (as demonstrated in Fig. 2.) (B; red, scale bar = 100 μm). *Nmnat1* gene therapy also protected D2 eyes with elevated IOP against optic nerve degeneration ($n > 40/\text{group}$; *** = $P < 0.001$, Fisher's exact test) (C), soma loss ($n = 6/\text{group}$) (D), and PERG amplitude ($n > 20/\text{group}$) (E). Addition of NAM^{Lo} in drinking water afforded additional protection against optic nerve degeneration (*Nmnat1* compared to *Nmnat1* + NAM^{Lo} = $P < 0.001$, Fisher's exact test) (C). (D and E) ** = $P < 0.01$, *** = $P < 0.001$ (Student's *t* test).