1   **Title:** Systemic Treatment with Nicotinamide Riboside is Protective in Three

2   Mouse Models of Retinal Degeneration

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Abstract

Purpose: The retina is highly metabolically active, suggesting that metabolic dysfunction could underlie many retinal degenerative diseases. Nicotinamide adenine dinucleotide (NAD^+) is a cofactor and a co-substrate in several cellular energetic metabolic pathways. Maintaining NAD^+ levels may be therapeutic in retinal disease since retinal NAD^+ levels decline with age and during retinal damage or degeneration. The purpose of this study was to investigate whether systemic treatment with nicotinamide riboside (NR), a NAD^+ precursor, is protective in disparate models of retinal damage or degeneration.

Methods: Three mouse models of retinal degeneration were tested: an albino mouse model of light-induced retinal degeneration (LIRD) and two models of retinitis pigmentosa (RP), including a mouse line deficient in interphotoreceptor binding protein (IRBP) gene expression (IRBP KO), and a naturally-occuring cGMP phosphodiesterase 6b mutant mouse model of RP (the Pde6b^{rd10} mouse). Mice were intraperitoneally (IP) injected with PBS or NR at various times relative to damage or degeneration onset. One to two weeks later, retinal function was assessed by electroretinograms (ERGs) and retinal morphology was assessed by optical coherence tomography (OCT). Afterwards, retina sections were H&E stained for morphological analysis or by terminal deoxynucleiotidyl transferase dUTP nick and labeling (TUNEL). Retinal NAD^+/NADH levels were enzymatically assayed.

47   Results: The retinal degeneration models exhibited significantly suppressed

48   retinal function, and where examined, severely disrupted photoreceptor cell layer

49   and significantly decreased numbers of nuclei and increased accumulation of

50   DNA breaks as measured by TUNEL-labeled cells in the outer nuclear layer

51   (ONL). These effects were prevented by various NR treatment regimens. IP

52   treatment with NR also resulted in increased levels of NAD$^+$ in retina.

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54   Conclusions: This is the first study to report protective effects of NR treatment in

55   mouse models of retinal degeneration. The positive outcomes in several models,

56   coupled with human tolerance to NR dosing, suggest that maintaining retinal

57   NAD$^+$ via systemic NR treatment should be further explored for clinical relevance.

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69   **Introduction**

70　　　　Retinal diseases are a leading cause of blindness globally, accounting for

71　　more than 25% of all vision impairment (over 55 million people).[1] Macular

72　　degenerations such as age-related macular degeneration (AMD), inherited retinal

73　　degenerations such as retinitis pigmentosa (RP), and several other retinopathies,

74　　syndromes, and dystrophies are linked to mutations in over 300 different genes

75　　or loci (Retnet; sph.uth.edu/retnet/disease.htm; update 10/29/2019). In addition

76　　to heterogeneous heritable risk factors, several retinal diseases such as AMD

77　　have strong environmental risk factors such as smoking, or the case of retinal

78　　detachments with degenerative outcome, extreme myopia.[2-5] Although treatment

79　　strategies based on specific genetic mutations, risk factors, or combinations

80　　thereof are being developed (one gene therapy study has already entered the

81　　clinic[6]), approaches with targets that are common to many diseases may have

82　　greater impact.

83　　　　　　Though of varied etiology, the outcome that is common to retinal

84　　degenerative diseases is the progressive loss of rod and cone photoreceptor

85　　cells and retinal pigment epithelial (RPE) cells. The retina is the most

86　　metabolically demanding tissue in the body, and experiences significant,

87　　persistent oxidative stress arising from its primary function, visual transduction.[7, 8]

88　　Photoreceptor cells in particular are highly metabolically active as their normal

89　　function includes maintenance of the "dark current" and continuous production of

90　　proteins that support their specialized function,[8] many of which are lost daily due

91　　to outer segment disc shedding.[9] Because of this, photoreceptors are thought to

92　　be especially sensitive to dysregulation of bioenergetic metabolism.[10-12] Indeed,

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93 genetic mutations that degrade photoreceptor bioenergetic metabolism cause

94 blindness, including mutations of Krebs cycle enzymes that cause forms of RP[13]

95 and mutations in mitochondrial DNA (mtDNA) that cause Leber Hereditary Optic

96 Neuropathy (LHON).[14] Also, as photoreceptor and RPE cells exist within a

97 metabolic symbiosis,[15] functional compromise in one cell type may negatively

98 affect function in the other.[10] Thus, targeting metabolic pathways of

99 photoreceptor or RPE cells may be therapeutic across a variety of retinal

100 diseases.

101   Nicotinamide adenine dinucleotide (NAD^+) is a cofactor in glycolysis and

102 the Krebs cycle and a co-substrate for NAD^+-consuming enzymes.[8, 16] NAD^+

103 levels decline with age and in neurodegeneration, possibly in response to

104 increased activity of NAD^+-consuming enzymes involved in DNA repair, such as

105 poly (ADP-ribose) polymerase (PARP).[16] Maintenance of NAD^+ levels is critical to

106 retinal health. Mutations in nicotinamide mononucleotide adenylyltransferase-1

107 (NMNAT1), a NAD^+ salvage pathway enzyme, causes Leber Congenital

108 Amaurosis Type 9 (LCA9).[17-19] Experimentally inhibiting nicotinamide

109 phosphoribosyltransferase (NAMPT), a key enzyme in a NAD^+ salvage

110 biosynthesis pathway, with pharmacological agents causes retinal

111 degeneration.[20] In a seminal series of experiments, Apte and colleagues

112 established the importance of NAD^+ in retinal health and the role of NAD^+

113 deficiency in retinal degeneration.[8] They found that retinal NAD^+ levels decline in

114 models of retinal degeneration and that mice made deficient in NAMPT have

115 diminished retinal NAD^+ and develop retinal degeneration.[21] Further, they

116   demonstrated that systemic treatment with the NAD^{+} precursor nicotinamide

117   mononucleotide (NMN) was protective in mouse models of retinal

118   degeneration.[21] Similarly, John and colleagues have demonstrated that NAD^{+} is

119   critical to retinal ganglion cell (RGC) health, that NAD^{+} levels decline in models of

120   glaucoma, and that treatment of these models with NAM maintains or increases

121   NAD^{+} in RGCs via the NAMPT pathway, and that this protects function and optic

122   nerve morphology.[22, 23]

123   Brenner and colleagues discovered an alternative NAD^{+} salvage

124   biosynthesis pathway in which nicotinamide riboside (NR), a form of vitamin B3

125   that is found in milk and other foods,[24, 25] can be taken up from oral dosing in

126   humans.[26] NR enters cells through nucleotide transporters and is then converted

127   to NMN then to NAD^{+} by NR kinases (NMRK1 and NMRK2) and NMNAT

128   isozymes.[27, 28] They and others find that administration of NR to rodents prevents

129   cognitive decline and amyloid-beta peptide aggregation,[29] prevents noise-

130   induced neurite degeneration and hearing loss,[30] protects in several models of

131   neuropathies,[31, 32] and protects against excitotoxicity-induced or axotomy-

132   induced axonal degeneration.[33, 34] The NMRK kinases are expressed in many

133   neuronal subtypes, and may be upregulated following neuronal injury or during

134   extreme energetic stress.[16] NR treatment also protects in neurodegeneration

135   models. NR treatment increases NAD^{+} and protects mitochondrial function in

136   cultured neurons from Parkinson Disease (PD) patients and prevents age-related

137   dopaminergic neuronal loss and motor decline in Drosophila models of PD.[35] In

138   a new mouse model of Alzheimer Disease (AD), NR provided in drinking water

139   improved learning, memory, and motor function. In addition, NR

140   supplementation led to normalized NAD^+/NADH ratios, better synaptic

141   transmission, and decreased DNA damage.[36]

142   Given this background supporting a neuroprotective role of NR in several

143   neuronal degenerations, we hypothesized that NR would be effective in more

144   than one retinal degeneration model including both inherited retinal

145   degenerations and experimentally induced retinal degenerations. In this study,

146   we tested whether systemic delivery of NR is protective in three disparate mouse

147   models of retinal degeneration: a mouse model of light-induced retinal

148   degeneration (LIRD),[37-39] and two models of autosomal recessive retinitis

149   pigmentosa (arRP), the IRBP knock-out (KO) mouse[40, 41] and the rd10 mouse.[42]

150   NR treatment increased retinal NAD^+ and the NAD^+/NADH ratio and protected

151   retinal morphology and function in all three models. This is the first study to

152   report protective effects of NR treatment in models of retinal degeneration and

153   suggests that maintaining retinal NAD^+ via systemic NR treatment should be

154   further explored for clinical relevance.

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156   **Materials and Methods**

157   *Animal models* – All mouse procedures were approved by the Emory Institutional

158   Animal Care and Use Committee and followed the ARVO Statement for the Use

159   of Animals in Ophthalmic and Vision Research. For the LIRD model, adult (3

160   months old) male Balb/c mice were obtained from Charles River Laboratory

161   (Wilmington, MA, USA) and were housed under a 12:12-hour light-dark cycle (7

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162 AM on and 7 PM off). During the light cycle, light levels measured at the bottom

163 of mouse cages ranged from 5 to 45 lux. Induction of LIRD was previously

164 described.[37-39] Briefly, Balb/c mice were placed individually into white, opaque,

165 standard-sized and -shaped housing cages. LED light panels were placed on top

166 of the cages. Mice were exposed to 3,000 lux light for 4 hours. After this

167 exposure, mice were returned to home cages under normal lighting conditions for

168 the remainder of the experiment. Mice had access to standard mouse chow (Lab

169 Diet 5001; LabDiet, Inc., St. Louis, MO, USA) ad libitum throughout the study

170 except during bright light exposure. At the time of light induction they weighed 22

171 to 28 g. NR treatment did not significantly alter weight (data not shown). Mice

172 were euthanized by asphyxiation with CO_2 gas for all experiments.

173

174 IRBP KO mice[43] used in these experiments were originally created from 129/Ola

175 embryonic stem cells and were backcrossed against C57BL/6J for 10

176 generations.[44] Food and water were provided ad libitum, with 12:12 light-dark

177 cycling. Genotypes were verified with specific primers[45] in all strains. The ERG

178 phenotype in the IRBP KO mouse was reduced by 40% of the wild type (WT) a-

179 wave magnitude at postnatal 30 (P30) as previously shown,[43] validating use of

180 the current KO mice in this study. Male and female mice from each litter were

181 randomly divided at P18 to receive NR treatment (1000 mg/kg) or PBS.

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183 rd10 mice on a C57BL/6J background[46, 47] were obtained from Jackson

184 Laboratories (Bar Harbor, Maine). In the rd10 retina, rod cell death begins at

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185   about P15, and by P25 almost all of the rod photoreceptors have been lost.[48] In

186   our experiments, male and female mice from each litter were randomly divided at

187   P6 to receive subcutaneous NR (1000 mg/kg) or PBS treatment until P14 and

188   then received IP NR or PBS treatment until P27.

189

190

191   *Drug Administration* – NR was kindly provided by Dr. Charles Brenner

192   (ChromaDex, Item #ASB-00014332-101, Lot# 40C910-18209-21). NR and

193   vehicle (phosphate buffered saline, PBS, VWRVK813, Cat#97063-660, 1X

194   solution composition:137 mM NaCl, 2.7 mM KCl, 9.5 mM phosphate buffer.)

195   Solutions were made fresh each day. Balb/c mice received IP injections of either

196   PBS alone or NR (1000 mg/kg dose in PBS; specified by experiment as reported

197   in Results) using an injection volume of 10 µl of solution per gram of mouse body

198   weight in accordance with in vivo rodent experiments of Yen et al.[49] For LIRD

199   experiments, two injections were administered before a toxic light exposure. One

200   injection was performed the day before (at 4 pm), and the other injection was

201   performed at 9 am the morning of light insult. Toxic light exposure was from 10

202   am to 2 pm. The IRBP KO mice received treatment 5 days per week from P18 to

203   P33. rd10 mice were injected 5 days per week from P6 to P27. Injections from

204   P6-P14 were subcutaneous, and from P15-P27 were IP.

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206   *Electroretinograms (ERG)* – The complete ERG protocol was previously

207   detailed.[50] Briefly, mice were dark-adapted overnight. In preparation for ERGs,

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208   mice were anesthetized with IP injections of ketamine (10 mg/ml: AmTech Group

209   Inc.) and xylazine (100 mg/ml; AKORN Animal Health, Lake Forest, IL).[50]

210   Proparacaine (1%; AKORN, Lake Forest, IL) and tropicamide (1%; AKORN, Lake

211   Forest, IL) eye drops were administered to reduce eye sensitivity and dilate

212   pupils. Once anesthetized, mice were placed on a heating pad inside a Faraday

213   cage in front of a UBA-4200 Series desktop Ganzfeld stimulator (LKC

214   Technologies, MDIT-100). A DTL fiber active electrode was placed on top of

215   each cornea. A drop of Refresh Tears (Allergan, Dublin, Ireland) was added to

216   each eye to maintain conductivity with the electrode fibers. The reference

217   electrodes was a 1-cm needle inserted into the cheeks, and the ground electrode

218   placed in the tail. ERGs were recorded for the scotopic condition (0.00039-25.3

219   cd s/m^2 with increasing flash stimulus intervals from 2 to 62.6 s). Mice recovered

220   from anesthesia individually in cages placed partly on top of heated water pads

221   (39 °C). For the LIRD model, ERGs were performed 1 week after toxic light

222   exposure and again at 2 weeks following light exposure. As for the two inherited

223   RP models, ERGs were performed after 10 or 20 injections of NR.

224

225   *In vivo ocular imaging* – Spectral domain optical coherence tomography (SD-

226   OCT) was conducted immediately after ERG measurement, when mice were still

227   anesthetized and their pupils were still dilated. A Micron IV SD-OCT system with

228   fundus camera (Phoenix Research Labs, Pleasanton, CA) and a Heidelberg

229   Spectralis HRA+OCT instrument with +25D lens (Heidelberg Engineering,

230   Heidelberg, Germany) were used in tandem sequentially to assess ocular

231   posterior segment morphology in section and *en face.* Using the Micron IV

232   system, image-guided OCT images were obtained for the left and right eyes after

233   a sharp and clear image of the fundus (with the optic nerve centered) was

234   obtained. SD-OCT imaging was a circular scan about 100 μm from the optic

235   nerve head. Fifty scans were averaged. The retinal layers were identified

236   according to published nomenclature.[51] Total retinal thickness and thickness of

237   the individual retinal layers were analyzed using Photoshop CS6 (Adobe

238   Systems Inc., San Jose, CA). The number of pixels was converted into

239   micrometers by multiplying by the micrometers/pixel conversion factor (1.3

240   microns = 1 pixel). Immediately after imaging on the Micron IV system, a rigid

241   contact lens was placed on the eye (Back Optic Zone Radius: 1.7 mm, diameter:

242   3.2 mm, power: PLANO) and blue autofluorescence imaging at the layer of the

243   photoreceptor-RPE interface was conducted using the Heidelberg Spectralis

244   HRA+OCT instrument. During imaging and afterwards through anesthesia

245   recovery, mice were kept on a water circulating heat pad set to 39 °C to maintain

246   body temperature.

247

248   Histology and Morphometrics

249   The Balb/c mice were sacrificed the same day after in-vivo measurments. We

250   followed the recommendations of Howland and Howland[52] for nomenclature of

251   axes and planes in the mouse eye, as indicated in Figure 1. Histologic and

252   morphometric procedures followed standard techniques.[46, 53] Eyes were

253   dehydrated, embedded in paraffin, and sectioned through the sagittal plane on a

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254   microtome at 5 μm increments. Sections were cut on a vertical (sagittal) plane

255   through the optic nerve head (ONH) and the center of the cornea to ensure that

256   consistent regions were examined between animals. Slides were de-paraffinized

257   across five Coplin jars with 100 ml of xylene for two minutes each, consecutively.

258   Then slides were rehydrated in a series of 100 ml ethanol solutions for two

259   minutes each: 100%, 90%, 80%, 70%, 60%, 50%. Slides were immersed in PBS

260   for five minutes twice. After rehydration, a TUNEL assay was performed on

261   some sections according to the protocol for the DeadEnd Fluorometric TUNEL

262   Kit (Promega, Fitchburg, WI). Stained sections were imaged using fluorescent

263   microscopy and TUNEL-positive cells in the outer-nuclear layer (ONL) were

264   manually counted for each whole retina using Adobe Photoshop Creative Suite 6

265   (Adobe Systems Inc. San Jose, CA). Some sections were used for hematoxylin

266   and eosin (H&E) staining. ONL nuclei were counted within 100 μm-wide

267   segments spaced at 250, 750, 1250, 1750 μm from the optic nerve head in both

268   the inferior and superior directions. Mean counts of n = 3-6 retinas per group

269   were plotted as a spidergram diagram.

270

271   NAD$^+$/NADH measurements

272   Levels of NAD$^+$ in retina homogenates were measured using a commercially

273   available kit by following manufacturer's instructions (Abcam; ab. 65348; #Lot:

274   GR3226737-3; San Francisco, CA). In brief, retina samples were homogenized in

275   extraction buffer. Extracted samples were separated in two aliquots. One was

276   used to measure total NAD (NADt). The other was heated to 60℃ for 30 minutes

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277   to convert NAD^+ to NADH. Samples were placed in a 96-well plate. Then, NADH

278   developer was added into each well and incubated at room temperature for 1-4

279   hours. The plate was placed into a hybrid reader and read every half hour at OD

280   450nm while the color was still developing. We used the 2-hour data, at which

281   the reaction was well developed. NADt and NADH concentration was quantified

282   comparing with NADH standard curve data. In the end, NAD^+ was calculated with

283   the equation $NAD^+ = NADT - NADH$.

284

285   *Statistical Analyses* – Statistical analyses were conducted using Prism 8.1.1

286   Software (GraphPad Software Inc. La Jolla, CA, USA). One-way ANOVA with

287   Tukey's post-hoc test, two-way ANOVA with Sidak's post-hoc test, and Student's

288   *t*-tests were performed for ERG, biochemical, and morphometric data. Tukey

289   post-hoc test is considered to be more powerful in comparing every mean with

290   every other mean, and so is most appropriate for our use of the one-way

291   ANOVA. Sidak's test is more generalized, allowing for comparisons of just

292   subsets of means, and thus recommended for our use of the two-way ANOVA.

293   For all analyses, results were considered statistically significant if *p < 0.05*. All

294   graphs display data as mean ± SEM. The stated *n* is the number of animals or

295   individual eye used in each group.

296

297   **Results**

298   **NR treatment preserves retina function in three mouse models of retinal**

299   **degeneration**

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301   ERG a- and b-wave mean amplitudes of PBS-treated Balb/c mice were

302   significantly reduced one week after exposure of to 3,000 lux light for 4 hours.

303   Representative ERG waveforms of one eye of a single mouse from each

304   treatment group are shown in Fig. 2. This functional loss was entirely prevented

305   in mice treated with NR (Fig. 3A, 3B). Functional loss was diminished

306   significantly, but not entirely, in the two genetic mouse models of RP. The IRBP

307   KO mouse degeneration starts around P24 with a burst of apoptosis in the outer

308   nuclear layer, followed by a very gradual loss of photoreceptor cells over the

309   lifespan of the animals.[40] This gradual loss is accompanied by a gradual decline

310   of ERG waveform amplitudes.[54] Scotopic and photopic ERG a- and b-wave

311   amplitudes were greater following daily NR versus PBS injections (Fig. 4). The

312   rd10 mouse has an early onset and rapid photoreceptor degeneration due to a

313   mutation in the beta subunit of rod phosphodiesterase, with significant

314   morphological and functional losses apparent at P16 that are nearly complete by

315   P30.[42, 50] Daily IP injections of NR significantly preserved scotopic ERG b-wave

316   amplitudes and photopic ERG a- and b-wave amplitudes (Fig 5). This protective

317   effect persisted at P38, 10 days after treatment had been halted (Fig 6). These

318   data suggest that NR treatment protects against of retinal damage and

319   degeneration.

320

321   **NR treatment preserves retinal morphology in LIRD mouse**

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322   As imaged *in vivo* by fundus photography and SD-OCT one week after

323   degeneration induction, the retinas of LIRD mice treated with PBS exhibited

324   significant damage (Fig. 7). The representative fundus image of Fig. 7A shows

325   the region being measured. Corresponding SD-OCT images show considerable

326   thinning of the photoreceptor layer that is prevented by NR treatment (Fig. 7B).

327   Quantification of total retina and photoreceptor layer thicknesses show that mice

328   undergoing LIRD had significantly thinner retinas compared to non-induced mice,

329   largely due to nearly complete loss of the photoreceptor layer. This was entirely

330   prevented with NR treatment (Fig. 7C, 7D).

331

332   Photomicroscopy of H&E-stained sections of eyes harvested one week after

333   LIRD induction showed marked degradation of morphology in the outer retina in

334   LIRD mice treated with PBS compared to non-induced mice (Fig.8A, 8B, and Fig.

335   S1). In induced mice treated with PBS, photoreceptor cell inner and outer

336   segments and most of the nuclei of the ONL were eliminated, with loss

337   predominantly centrally, (Fig. 8A-D, and Fig. S1). Nearly all of this degeneration

338   was prevented in mice treated with NR (Fig. 8C and Fig. S1). Quantification of

339   ONL nuclei counts across retinal sections confirmed significant losses due to

340   degeneration in PBS-treated mice and confirmed nearly-complete preservation,

341   in NR-treated mice (Fig. 8D and Fig. S1).

342

343   **NR treatment prevents accumulation of TUNEL signal in photoreceptor**

344   **cells of LIRD mice**

345   Paraffin-embedded ocular sections from mice euthanized a week after toxic light

346   induction were stained for TUNEL and DAPI to label nuclei that contained

347   double-stranded DNA breaks (a marker of programmed cell death [apoptosis] or

348   other forms of cell death).[55] ONL TUNEL signal was high in retinas from LIRD

349   mice treated with PBS compared to retinas from uninduced mice (Fig. 9A, 9B

350   and Fig. S2). Induced mice treated with NR exhibited significantly less TUNEL

351   signal (Fig. 9C and Fig. S2). These data suggest that NR treatment diminished or

352   delayed apoptosis in photoreceptor cells.

353

354   **NR treatment prevents an inflammatory response following induction of**

355   **LIRD**

356   Subretinal autofluorescent spots observed *in vivo* by fundus examination in

357   patients and in animal models are considered diagnostic markers for

358   inflammatory responses in retinal damage and disease.[56-60] To test whether NR

359   treatment alters inflammatory responses of the LIRD mice, *in vivo* fundus

360   examination at the level of the subretinal space was conducted one week

361   following induction of retinal degeneration. Uninduced eyes showed few blue

362   autofluorescent spots at the level of the subretinal space in vivo (Fig. 10A). In

363   eyes of induced mice treated with PBS, numerous and widespread

364   autofluorescent spots were observed (Fig. 10B). This was prevented in mice

365   treated with NR, which exhibited fewer autofluorescent spots (Fig. 10C), similar

366   in number and pattern to the uninduced group, confirmed by statistical testing on

367   counts of these spots across several autofluorescent fundus images (Fig. 10D).

16

368   These data suggest that LIRD leads to an inflammatory response that is largely

369   prevented by NR treatment.

370

371   **NR elevates retinal NAD$^+$ levels in mice**

372   NR is a NAD$^+$ precursor that when given systemically increases NAD$^+$ levels in

373   many tissues, including CNS structures,[29, 35, 36] and its protective effects are

374   ascribed in part to local NAD$^+$ increases in target tissue.[30-32, 34, 36] As an initial test

375   of whether this may be the case in NR-induced retinal neuroprotection, we used

376   C57Bl/6J mice to test whether NR treatment could increase NAD$^+$ levels in retina.

377   We IP-injected mice with either NR or PBS daily for 5 days. One hour after the

378   last treatment, retinas were harvested and assayed for NAD$^+$/NADH content,

379   revealing that NR treatment increased retinal NAD$^+$ (Fig 11A.) and NAD$^+$/NADH

380   ratio (Fig 11B) compared to the PBS-treated group.

381

382   **Discussion**

383   In this study, we found that systemic treatment with NR protected photoreceptors

384   from toxic light damage and delayed inherited retinal degeneration. NR has been

385   shown to be protective in many models of neurodegenerative diseases, like

386   Parkinson's disease[35] and Alzheimer's disease.[29] Our study is the first to

387   demonstrate NR's protective effects in retinal degeneration models.

388

389   Light-induced retinal damage is an acute model of retinal degeneration with a

390   well-described oxidative stress response.[61] We assessed by ERG the efficacy of

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391   NR treatment in preserving phoreceptor function in mice undergoing LIRD (Fig.

392   3). Remarkably, just two NR IP injections completely preserved scotopic a- and

393   b- wave amplitudes. *In vivo* OCT imaging and fundus imaging showed that

394   systemic NR treatment protected photoreceptors and whole retina from

395   degeneration (Fig 7). Postmortem H&E staining provided more detailed

396   information on retinal morphology, indicating that NR treatment protected retinal

397   structure, both inferior and superior (Fig. 8). Retinas of mice undergoing LIRD

398   exhibited marked TUNEL staining, especially in the ONL, suggesting massive

399   apoptosis of photoreceptor cells (Fig. 9, compare Panel A with Panel B). NR

400   treatment largely prevented this, suggesting that apoptosis was suppressed (Fig

401   9).

402

403   Autofluorescent spots similar to those observed here are postulated to be

404   components of inflammatory responses, like activated microglial cells, lipofuscin

405   in RPE cells, bisretinoids in the photoreceptors,[62-65] and RPE cells undergoing

406   epithelial-mesenchymal transition (EMT) that are migrating into the neural

407   retina.[66, 67] The present data indicate that NR treatment prevented the

408   appearance of these autofluorescing entities (Fig 10), and thus, may be

409   preventing retinal inflammation, possibly indirectly by partially preventing early

410   rod photoreceptor injury, or more directly by some as-yet undefined action.

411

412   Although the LIRD mouse model of retinal degeneration has been extremely

413   valuable in increasing our understanding of photoreceptor and retinal

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414   degeneration,[37, 68, 69] it is a damage model and not a disease model. The rd10

415   mouse and the IRBP KO mouse are considered models of arRP.[42, 46, 47, 50, 70-76]

416   We found that retinal NR protection extends to these two arRP models (Fig 4-6),

417   with significant increases of ERG amplitudes well after photoreceptor

418   degeneration onset and retinal function loss in untreated cohorts.[42, 46, 47, 50, 70-74,]

419   [76] The NR-induced increase in ERG mean amplitudes in IRBP KO mice, though

420   significant (Fig. 4), was modest most likely because retinal function has not yet

421   declined dramatically at this early stage in the degeneration.[40, 54] The NR-induced

422   increases in mean amplitudes of scotopic b-waves and photopic a- and b-waves

423   in P28 rd10 mice were more dramatic and persisted at least 10 days after

424   injections were halted (Figs. 5 and 6), demonstrating that NR treatment is

425   capable of preserving cone photoreceptor and inner retinal function even at a

426   stage at which extensive degeneration and function loss otherwise occurs.[42, 72, 75]

427   Rod photoreceptor function was not protected, unsurprising given that the

428   mutation underlying the degeneration is in a rod-specific phototransduction

429   gene.[42, 72] Overall, it appears that systemic treatment with NR protects against

430   both retinal damage and disease.

431

432   Photoreceptors and RPE have high metabolic demands.[15] It may be that the

433   observed increase in NAD^+ and NAD^+/NADH ratio following NR treatment (Fig.

434   11) allowed increased retinal metabolic capacity that contributed to protection.[77]

435   Recent work investigating the effects of NR in yeast and mammals established

436   that its metabolic pathway involves kinases NMRK1 and NMRK2, which were

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437   first described by Brenner and colleagues in 2004.[27] Apte and collegues

438   demonstrated that an intact nicotinamide-NAMPT-NAD+ pathway is requisite for

439   retinal health, but whether the same holds true for an NR-NMRK1/2-NAD+

440   pathway has not been explored. Additionally, it may be useful to compare the

441   efficacy and potency between NR and nicotinamide treatment in retinal

442   degeneration. Nicotinamide treatment has been well-investigated in

443   neurodegenerative diseases, in mouse glaucoma models,[22, 23, 78 79] and in a rat

444   LIRD model,[80] but has yet to be test in a mouse retinal degeneration model.

445

446   In summary, this is the first study to demonstrate that systemic treatment with

447   nicotinamide riboside is protective for photoreceptors undergoing stress in

448   damage and in genetic degeneration. The protection is significant, and may

449   support the proposition for prospective human subject studies.

450

20

460 **Figures:**

461

462 **Figure 1. Naming conventions recommended by Howland and Howland for**

463 **planes and axes of the vertebrate eye regardless of species.** A histologic

464 section cut on the vertical plane through the great meridian is illustrated.[40] The

465 horizontal, vertical, and frontal planes are marked, as are the anterior-posterior

466 (A-P) axis (also known as optic axis), the nasal-temporal (N-T) axis, and the

467 superior-inferior (S-I; vertical) axis. Republished from Wisard at el. 2011 (citation

468 40), with permission.

469

470 **Figure 2. NR (1000 mg/kg) treatment preserves a- and b-wave amplitudes 1**

471 **week following toxic light exposure.** Representative ERG waveforms of one

472 eye of a single mouse from each treatment group. Mice treated with PBS and

473 exposed to 3,000 lux for 4 hours show the most diminished a- and b-wave

474 amplitudes. Treatment with NR protected against the loss of a- and b-wave

475 amplitudes. The ERG flash intensity was 24.9 cd-s/m^2 and was the same for all

476 four mice.

477

478 **Figure 3. NR treatment preserves retinal function in LIRD mouse.** Scotopic

479 ERG a-wave (A) and b-wave (B) mean amplitudes from LIRD mice at 1 week

480 after degeneration induction. Mice treated with PBS and exposed to 3,000-lux

481 light for 4 hours to induce degeneration exhibited a- and b-wave mean

482 amplitudes (red) that were statistically significantly diminished compared to those

483 of dim groups (blue and black). However, the mean ERG amplitudes of mice

484　undergoing retinal degeneration and treated with NR (green) were statistically

485　indistinguishable from those treated with PBS and exposed to bright light. **P <

486　0.01, ***P < 0.001, ****P < 0.0001 versus other groups by two-way ANOVA with

487　Tukey's multiple comparisons test. n=8-12 eyes per group. Error bars represent

488　SEM.

489

490　**Figure 4. NR treatment preserves retinal function in IRBP KO mice.** Scotopic

491　ERG a-wave (A) and b-wave (B) and photopic a-wave (C) and b-wave (D) mean

492　amplitudes from IRBP KO mice which were IP injected 5 days a week from P18

493　to P33. The mean ERG amplitudes of mice treated with NR (green) were

494　statistically greater than those treated with PBS. *P<0.05, **P < 0.01, ***P <

495　0.001, ****P < 0.0001 by two-way ANOVA with Sidak's multiple comparisons

496　non-parametric test; For each group, n=10-12 eyes. Error bars represent SEM.

497

498　**Figure 5. NR treatement preserves retinal function in PDE6brd10 mice at**

499　**P28.** Scotopic ERG a-wave (A) and b-wave (B) and photopic a-wave (C) and b-

500　wave (D) mean amplitudes from rd10 mice which were IP injected 5 days a week

501　from P6 to P28. The mean ERG amplitudes of rd10 mice treated with NR (green)

502　were statistically greater than those treated with PBS. *P<0.05, ****P < 0.0001 by

503　two-way ANOVA with Sidak's multiple comparisons non-parametric test; For

504　each group, n=6 eyes. Error bars represent SEM.

505

506 **Figure 6. NR preserves retinal function in PDE6b^{rd10} mice P38 (10 days after**

507 **cessation of treatment).** Scotopic ERG a-wave (A) and b-wave (B) and

508 photopic a-wave (C) and b-wave (D) mean amplitudes from rd10 mice at P38,

509 following 21 treatments ending 10 day prior (P28). The mean ERG amplitudes of

510 rd10 mice treated with NR (green) were statistically greater than those treated

511 with PBS. *$P<0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by two-way ANOVA

512 with Sidak's multiple comparisons non-parametric test; For each group, n=6

513 eyes. Error bars represent SEM.

514

515 **Figure 7. NR treatment preserves photoreceptor layer thickness and total**

516 **retinal thickness as assessed in vivo following light-induced retinal**

517 **degeneration.** (A). Representative fundus image (B). OCT images from each

518 group. The OCT image is a circular scan about 100 µm from the optic nerve

519 head. Photoreceptor thickness (C) and retinal thickness (D) from Balb/c mice at

520 one week after degeneration induction. Mice treated with NR and exposed to

521 3,000-lux light for 4 hours (red bar) exhibited great losses in thickness of the

522 photoreceptor and retina layers, whereas induced mice treated with NR (green

523 bar) exhibited statistically significant preservation of layer thickness. ****$p<0.0001$

524 versus all other group means; one-way ANOVA with Tukey's multiple

525 comparisons non-parametric test. n = 3-6 mice/group. Error bars represent SEM.

526 Size marker represents 200 µm.

527

528 **Figure 8. NR treatment preserves nuclei of the outer nuclear layer in the**

529 **LIRD mice.** (A)-(C). Representative H&E images of retina sections from each

530 group at the region of 250-750 µm from optic nerve. Complete sections are

531 shown in Supplemental Figure S1. (D). One week after degeneration induction,

532 nuclei were counted in eight discrete regions of retinal sections starting at 250

533 µm from the optic nerve head and extending every 500 µm outward along both

534 the dorsal/superior (positive values on abscissa) and ventral periphery/inferior

535 (negative numbers on abscissa). 'PBS, bright' treated mice (red) showed

536 significant loss of nuclei at six distances from the optic nerve head compared to

537 the control group (black). However, NR treated mice (green), exhibited mean

538 nuclei counts statistically indistinguishable from that of the control group (black)

539 throughout the length of the retina. ***$P < 0.001$, ****$P < 0.0001$ versus other

540 groups by two-way ANOVA with Tukey's multiple comparisons non-parametric

541 test. n = 3-6 retinal images/group. Error bars represent SEM. Size marker

542 represents 50 µm.

543

544 **Figure 9. NR treatment prevents increases TUNEL-positive cells following**

545 **induction of LIRD.** Both PBS and NR treated Balb/c mice received toxic light

546 exposure and were euthanized 1 week after exposure. Retinas from mice treated

547 as described in text were fixed, sectioned, and used in a TUNEL assay. (A)-(C).

548 Representative morphological images of each group. Complete sections are

549 shown in Supplemental Figure S2. (D). TUNEL positive nuclei in ONL were

550 counted from the entire retina. NR treated mice (red bar) exhibited significantly

24

551 fewer TUNEL-positive cells compared to the PBS treated group (green bar) . *P <

552 0.05, **P<0.01 by one-way ANOVA with Tukey's multiple comparisons non-

553 parametric test. n = 4 retinal images/group. Error bars represent SEM. Size

554 marker represents 50µm.

555

556 **Figure 10. NR treatment prevented subretinal autofluorescence observed in**

557 **vivo in LIRD mice.**(A)-(C). Representative morphology image from each group

558 at the level of the photoreceptor-RPE interface. In vivo Spectralis HRA+OCT

559 images (with blue autofluorescence detection) were taken one week following

560 induction of degeneration. (D). Autofluorescent white spots were counted across

561 the fundus image FIELD. Few were detected in uninduced mice (black). Bright

562 light exposed mice treated with NR exhibited significantly fewer white dots

563 compared to the PBS treated group. ****P < 0.0001 one-way ANOVA with

564 Tukey's multiple comparisons non-parametric test. n = 3-6 mice/group. Error bars

565 represent SEM. Size marker represents 200µm.

566

567 **Figure 11. NR treatment increased retinal NAD$^+$ levels in C57Bl/6J mice.**

568 Mice were IP injected with 1000 mg/kg NR or PBS for 5 consecutive days. Two

569 hours after the last injection, they were sacrificed and retinas harvested. NAD$^+$

570 and NAD(total) were assessed by colorimetric assay. Retinal NAD$^+$ levels (A)

571 and NAD$^+$/NADH ratio (B) from mice treated with NR were statistically

572 significantly increased compared to vehicle (PBS) treated group. **P<0.01 by

573 Student t-test. n = 6 retinas per group. Error bars represent SEM.

25

574

575 **Figure S1. NR treatment preserves nuclei of the outer nuclear layer in the**

576 **LIRD mice.** A week after toxic light exposure, mice were euthanized and ocular

577 sections stained with hematoxylin and eosin (H&E). Representative H&E images

578 of complete retina sections from each treatment group are shown. **Panel A**:

579 Section from a PBS-treated mouse exposed to dim light shows unperturbed ONL

580 across the span of the retina. **Panel B**: Section from a PBS-treated mouse with

581 toxic light exposure shows extreme thinning in the central, superior (right side of

582 section) ONL. **Panel C**: Section from a NR-treated mouse exposed to toxic light

583 shows similar thickness ONL as that in control mouse.

584

585 **Figure S2. NR treatment prevents increases TUNEL-positive cells following**

586 **induction of LIRD.** A week after toxic light exposure the mice were euthanized

587 and ocular sections stained for TUNEL (green) and DAPI (blue). Representative

588 images of complete retina sections from each treatment group are shown. The

589 right half of each section is dorsal/superior and the left half is ventral

590 periphery/inferior. **Panel A**: Section from a PBS-treated mouse exposed to dim

591 light shows little to no TUNEL-positive ONL nuclei across the span of the retina.

592 **Panel B**: Section from a PBS-treated mouse with toxic light exposure shows

593 numerous TUNEL-positive ONL nuclei in both superior and inferior halves. **Panel**

594 **C**: Section from a NR-treated mouse exposed to toxic light shows much fewer

595 TUNEL-positive ONL nuclei relative to PBS-treated mouse section.

596

26

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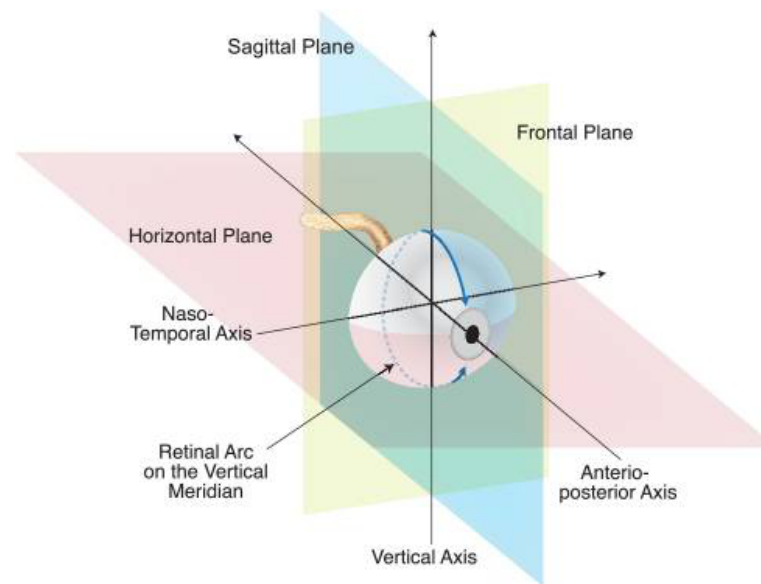818   *Mol Vis* 2010;16:2639-2652.

819

Figure 1. Naming conventions recommended by Howland and Howland for planes and axes of the vertebrate eye regardless of species. A histologic section cut on the vertical plane through the great meridian is illustrated. The horizontal, vertical, and frontal planes are marked, as are the anterior-posterior (A-P) axis (also known as optic axis), the nasal-temporal (N-T) axis, and the superior-inferior (S-I; vertical) axis. Republished from Wisard at el. 2011 (citation 40), with permission.
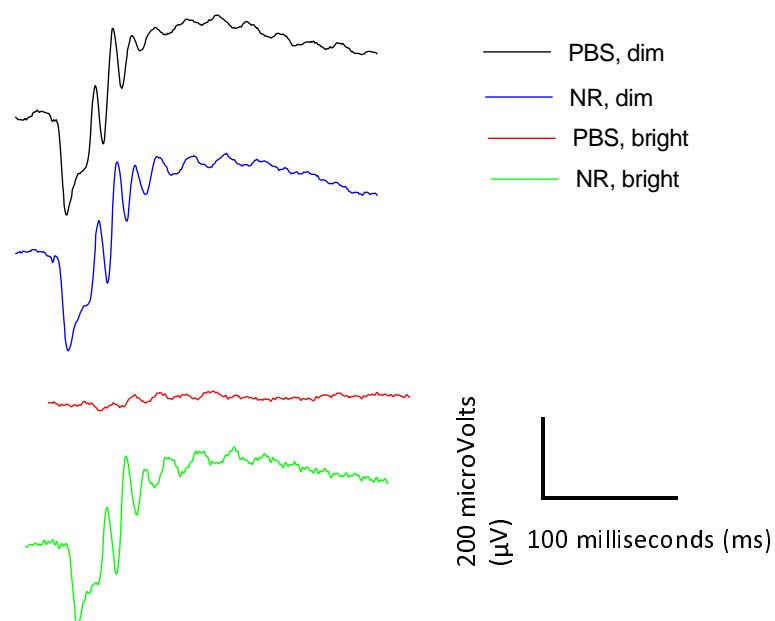
Figure 2. NR (1000 mg/kg) treatment preserves a- and b-wave amplitudes 1 week following toxic light exposure. Representative ERG waveforms of one eye of a single mouse from each treatment group. Mice treated with PBS and exposed to 3,000 lux for 4 hours show the most diminished a- and b-wave amplitudes. Treatment with NR protected against the loss of a- and b-wave amplitudes. The ERG flash intensity was 24.9 cd-s/m^2 and was the same for all four mice.
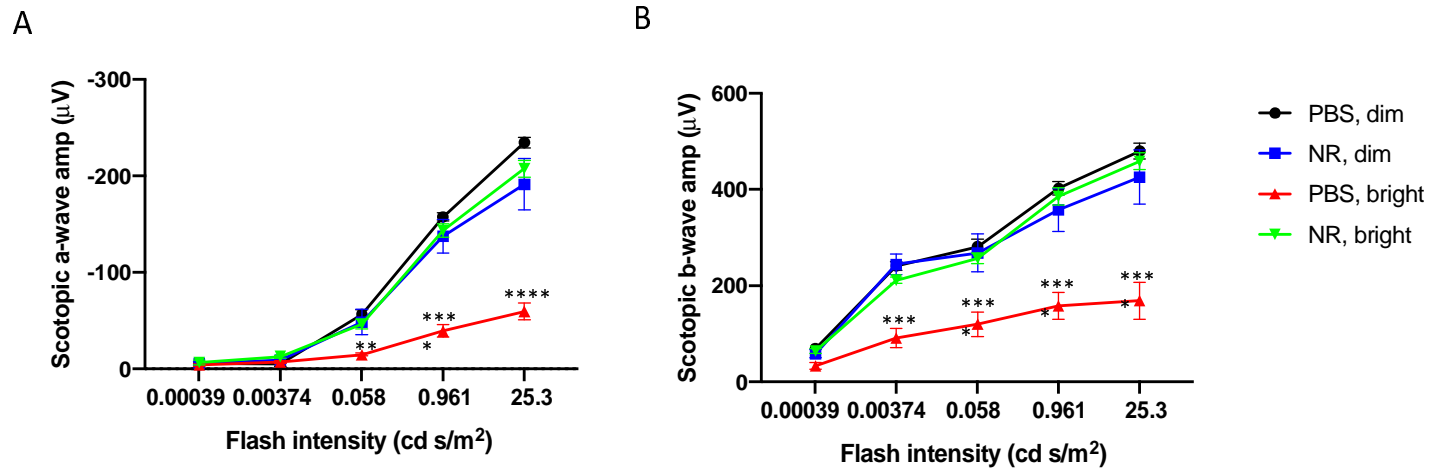
FIGURE 3. NR treatment preserves retinal function in LIRD mouse. Scotopic ERG a-wave (A) and b-wave (B) mean amplitudes from LIRD mice at 1 week after degeneration induction. Mice treated with PBS and exposed to 3,000-lux light for 4 hours to induce degeneration exhibited a- and b-wave mean amplitudes (red) that were statistically significantly diminished compared to those of dim groups (blue and black). However, the mean ERG amplitudes of mice undergoing retinal degeneration and treated with NR (green) were statistically indistinguishable from those treated with PBS and exposed to bright light . **P < 0.01, ***P < 0.001, ****P < 0.0001 versus other groups by two-way ANOVA with Tukey's multiple comparisons non-parametric test. N=8-12 eyes per group. Error bars represent SEM.
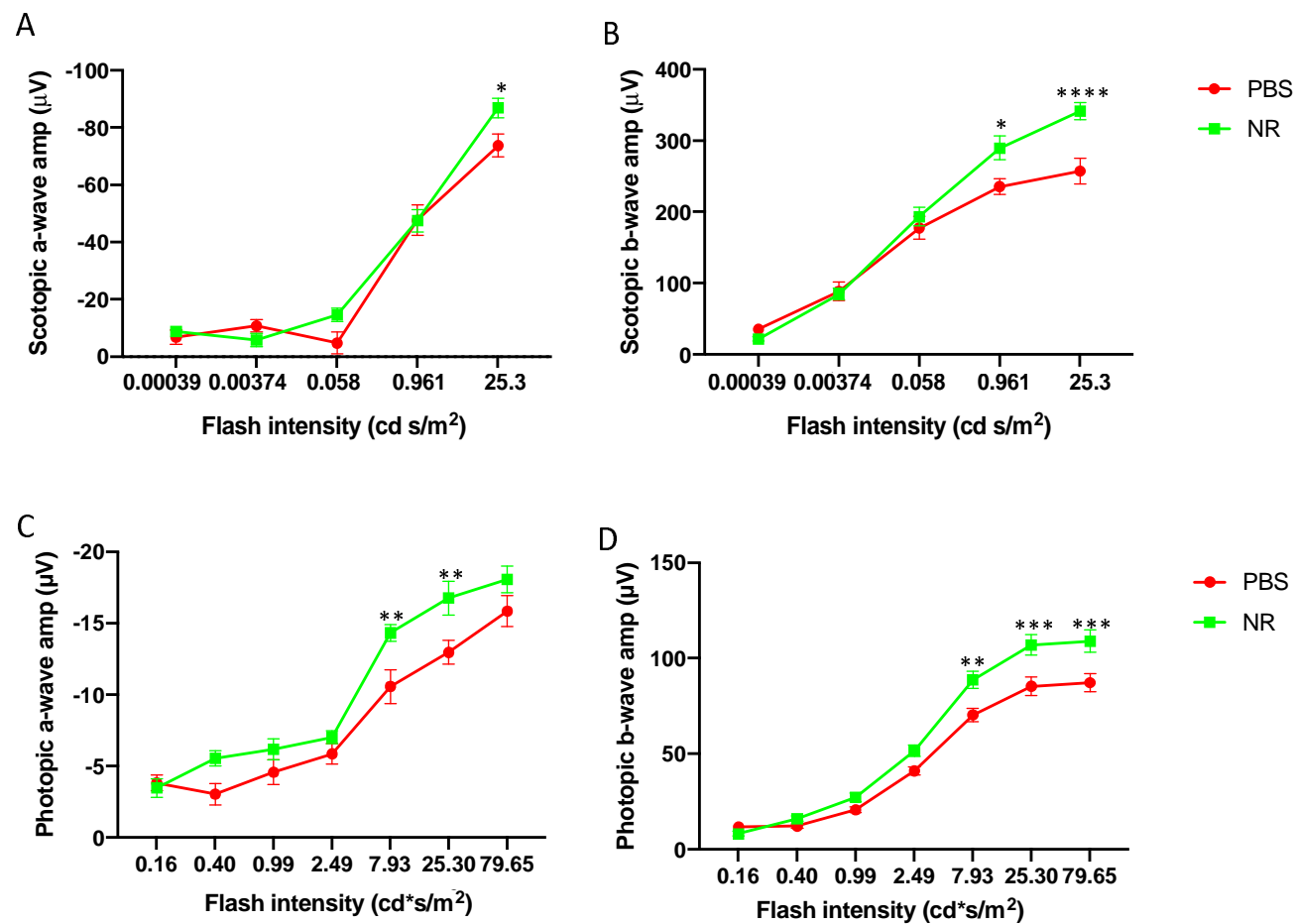
FIGURE 4. NR treatment preserves retinal function in IRBP KO mice. Scotopic ERG a-wave (A) and b-wave (B) and photopic a-wave (C) and b-wave (D) mean amplitudes from IRBP KO mice which were IP injected 5 days a week from P18 to P33. The mean ERG amplitudes of mice treated with NR (green) were statistically greater than those treated with PBS. *P<0.5, **P < 0.01, ***P < 0.001, ****P < 0.0001 by one-way ANOVA with Sidak's multiple comparisons non-parametric test; For each group, n=10-12 eyes. Error bars represent SEM.
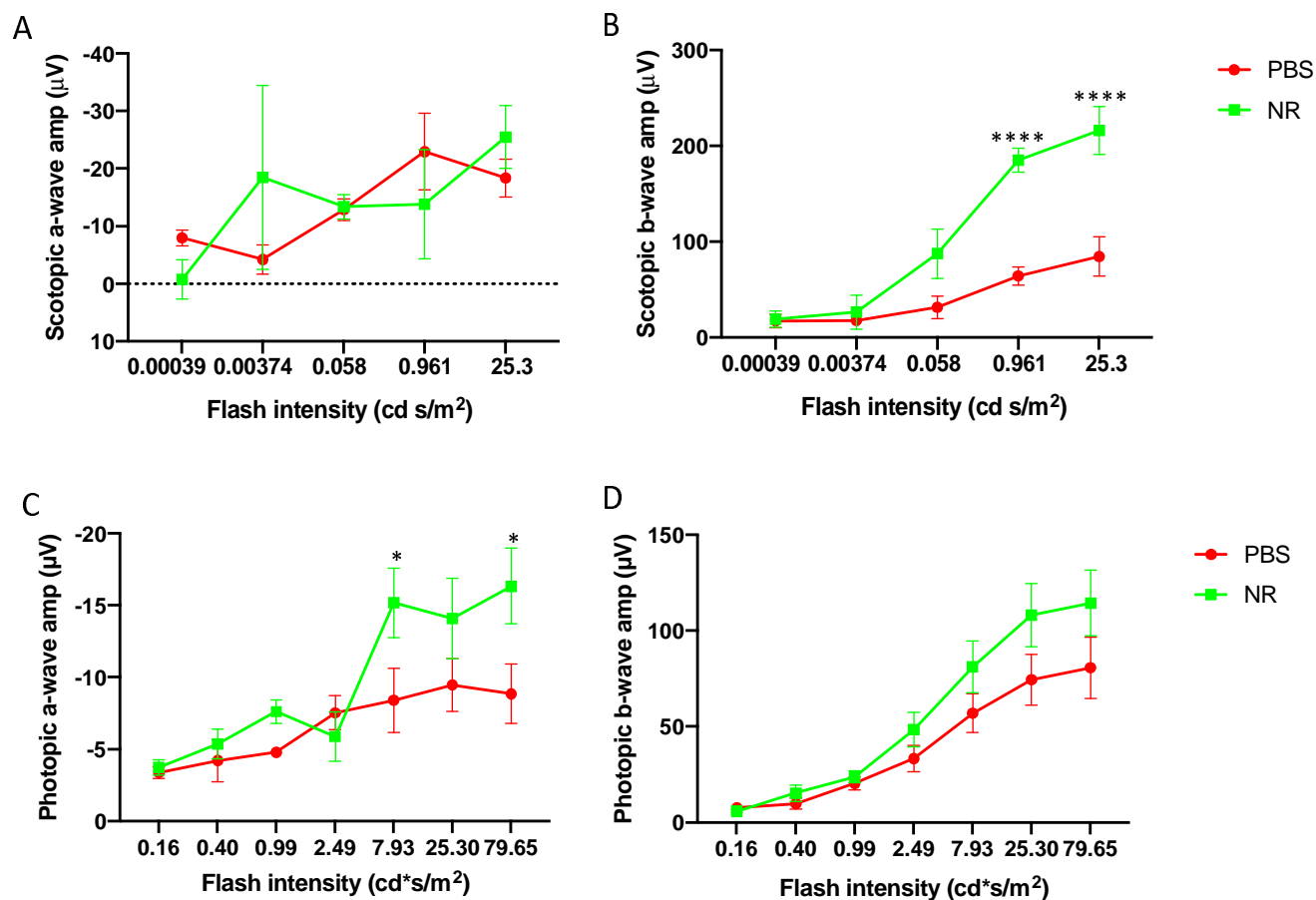
FIGURE 5. NR treatment preserves retinal function in PDE6b[rd10] mice at P28. Scotopic ERG a-wave (A) and b-wave (B) and photopic a-wave (C) and b-wave (D) mean amplitudes from rd10 mice which were IP injected 5 days a week from P6 to P28. The mean ERG amplitudes of rd10 mice treated with NR (green) were statistically greater than those treated with PBS. *P<0.5, ****P < 0.0001 by one-way ANOVA with Sidak's multiple comparisons non-parametric test; For each group, n=6 eyes. Error bars represent SEM.
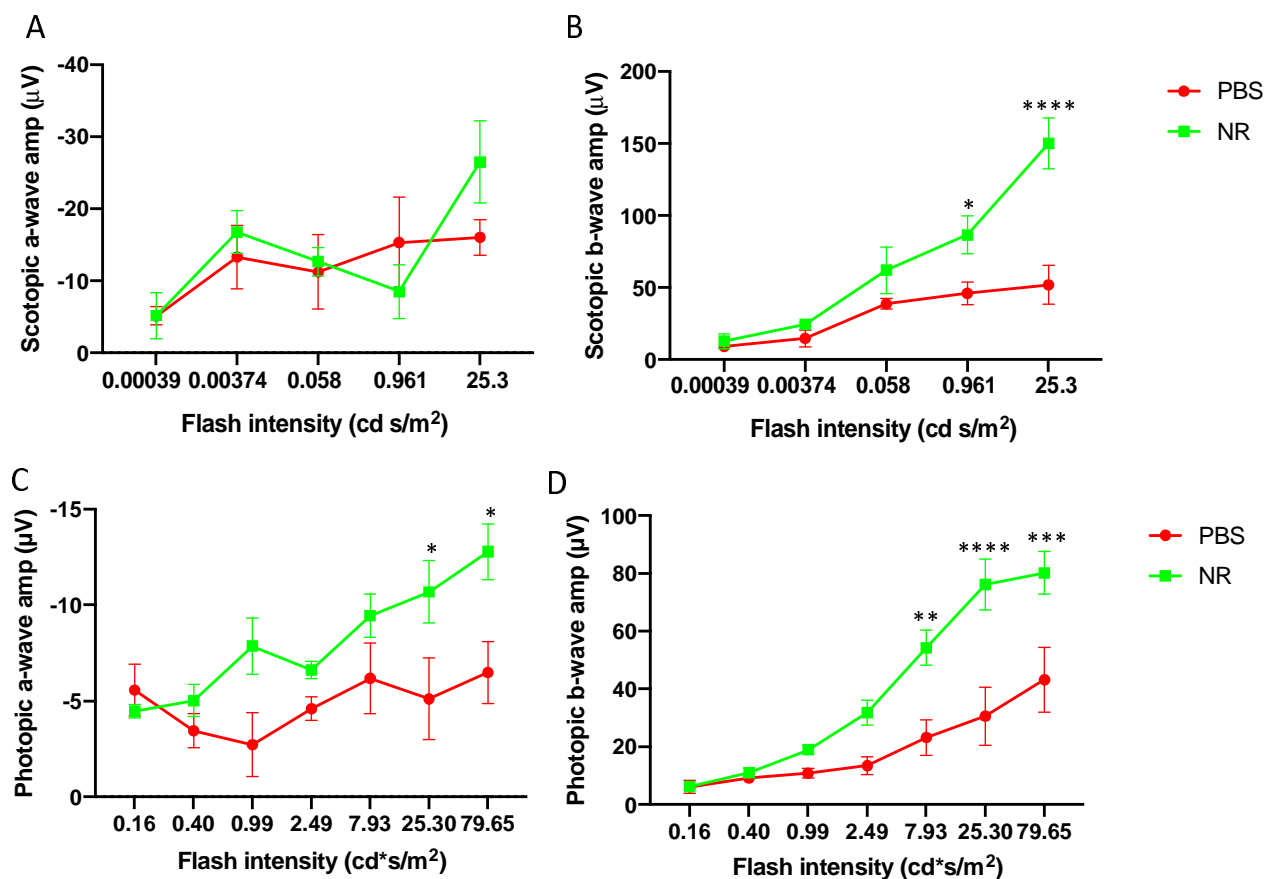
FIGURE 6. NR preserves retinal function in PDE6b[rd10] mice P38 (10 days after cessation of treatment). Scotopic ERG a-wave (A) and b-wave (B) and photopic a-wave (C) and b-wave (D) mean amplitudes from rd10 mice at P38, following 21 treatments ending 10 day prior (P28). The mean ERG amplitudes of rd10 mice treated with NR (green) were statistically greater than those treated with PBS. *$P<0.5$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by one-way ANOVA with Sidak's multiple comparisons non-parametric test; For each group, n=6 eyes. Error bars represent SEM.
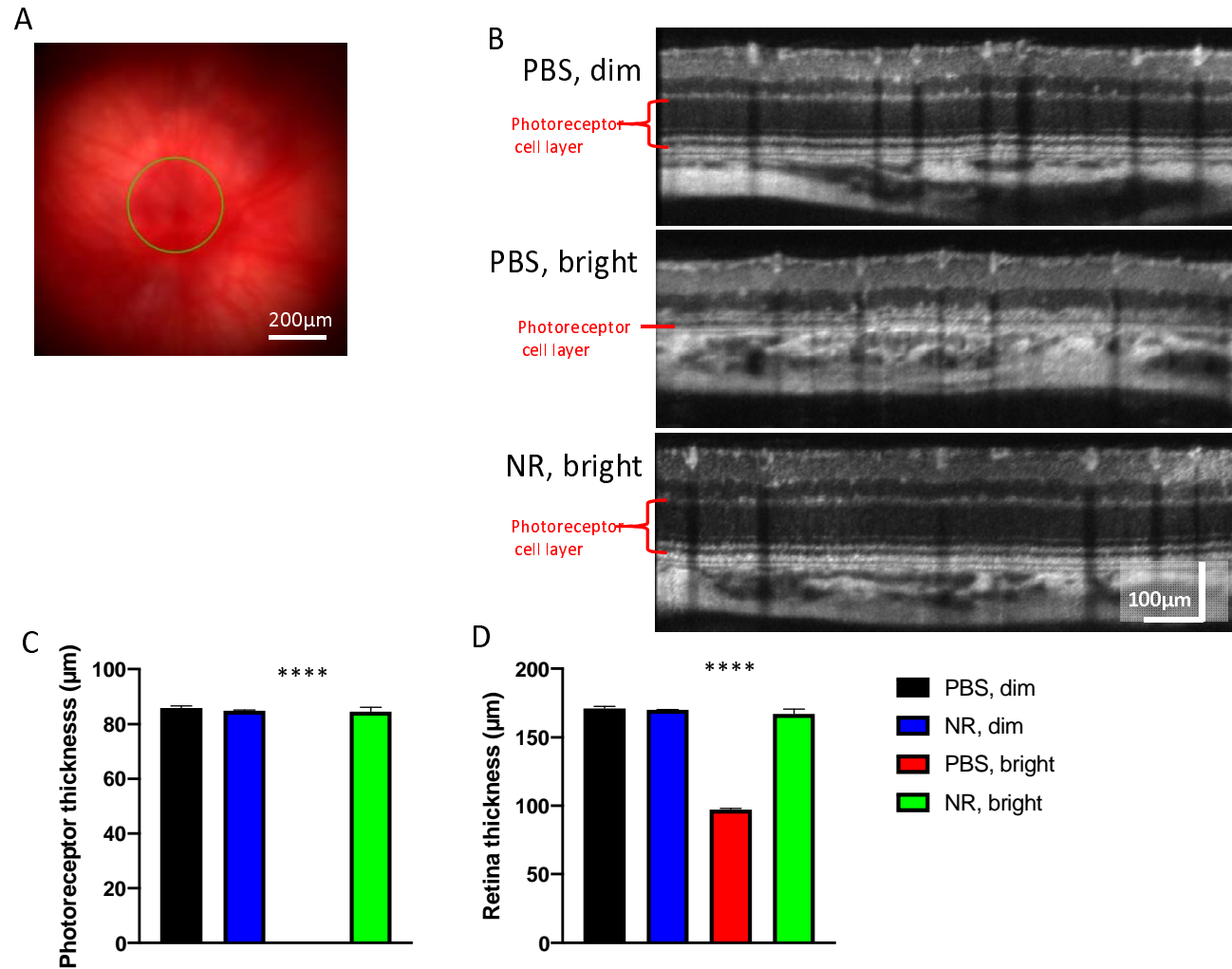
FIGURE 7. NR treatment preserves photoreceptor layer thickness and total retinal thickness as assessed in vivo following light-induced retinal degeneration. (A). Representative fundus image (B). OCT images from each group. The OCT image is a circular scan about 100 μm from the optic nerve head. Photoreceptor thickness (C) and retinal thickness (D) from Balb/c mice at one week after degeneration induction. Mice treated with NR and exposed to 3,000-lux light for 4 hours (red bar) exhibited great losses in thickness of the photoreceptor and retina layers, whereas induced mice treated with NR (green bar) exhibited statistically significant preservation of layer thickness. ****p<0.0001 versus all other group means; one-way ANOVA with Tukey's multiple comparisons non-parametric test. N = 3-6 mice/group. Error bars represent SEM. Size marker size as shown in images .

A PBS, dim

RGC
INL
ONL

50μm

B PBS, bright

C NR, bright

D

- PBS, dim
- PBS, bright
- NR, bright

FIGURE 8. NR treatment preserves nuclei of the outer nuclear layer in the LIRD mice. (A)-(C). Representative H&E images of retina sections from each group at the region of 250-750 μm from optic nerve. Complete sections are shown in Supplemental Figure S1. (D). One week after degeneration induction, nuclei were counted in eight discrete regions of retinal sections starting at 250 μm from the optic nerve head and extending every 500 μm outward along both the dorsal/superior (positive values on abscissa) and ventral periphery/inferior (negative numbers on abscissa). 'PBS, bright' treated mice (red) showed significant loss of nuclei at six distances from the optic nerve head compared to the control group (black). However, NR treated mice (green), exhibited mean nuclei counts statistically indistinguishable from that of the control group (black) throughout the length of the retina. ***P < 0.001, ****P < 0.0001 versus other groups by two-way ANOVA with Tukey's multiple comparisons non-parametric test. N = 3-6 retinal images/group. Error bars represent SEM. Size marker represents 50μm.

A PBS, dim

B PBS, bright

C NR, bright

RGC

INL

ONL

50μm

D

FIGURE 9. NR treatment prevents increases TUNEL-positive cells following induction of LIRD. Both PBS and NR treated Balb/c mice received toxic light exposure and were euthanized 1 week after exposure. Retinas from mice treated as described in text were fixed, sectioned, and used in a TUNEL assay. (A)-(C). Representative morphological images of each group. Complete sections are shown in Supplemental Figure S2. (D). TUNEL positive nuclei in ONL were counted from the entire retina. NR treated mice (red bar) exhibited significantly fewer TUNEL-positive cells compared to the PBS treated group (green bar) . *P < 0.05, **P<0.01 by one-way ANOVA with Tukey's multiple comparisons non-parametric test. N = 4 mouse retinal images/group. Error bars represent SEM. Size marker represents 50μm.

A PBS, dim

B PBS, bright

C NR, bright

200µm

D

FIGURE 10. NR treatment prevented subretinal fluorescent spots observed in vivo in LIRD mice. (A)-(C). Representative morphology image from each group at the level of the photoreceptor-RPE interface. *In vivo* Spectralis HRA+OCT images (with blue autofluorescence detection) were taken one week following induction of degeneration. (D). Autofluorescent white spots were counted on each FIELD of the same size centered on the ONH. Few were detected in uninduced mice (black). There is a greatly increased number of white dots (on average about 280 white dots) per FIELD one week after bright light damage. Bright light exposed mice treated with NR exhibited significantly fewer white dots compared to the PBS treated group. ****P < 0.0001 one-way ANOVA with Tukey's multiple comparisons non-parametric test. N = 3-6 mice/group. Error bars represent SEM. Size marker represents 200µm.
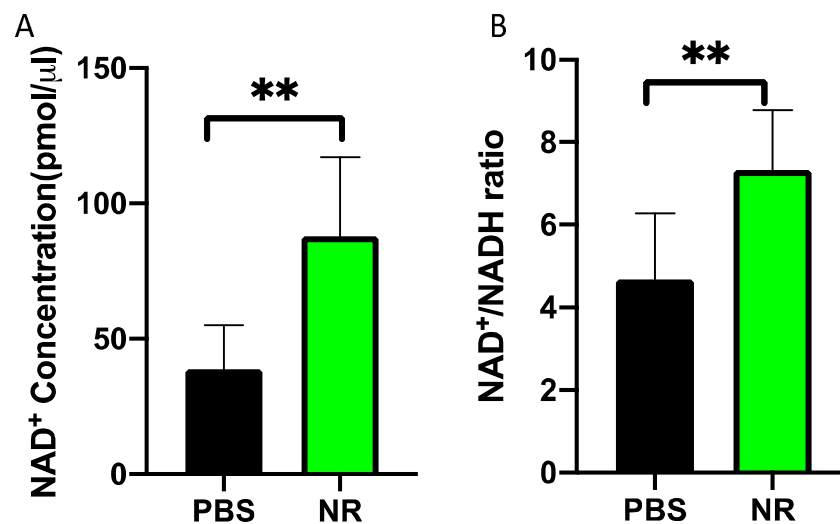
FIGURE 11. NR treatment increased retinal NAD+ levels in C57Bl/6J mice. Mice were IP injected with 1000 mg/kg NR or PBS for 5 consecutive days. Two hours after the last injection, they were sacrificed and retinas harvested. NADH and NAD(total) were assessed by colorimetric assay. Retinal NAD+ levels (A) and NAD+/NADH ratio (B) from mice treated with NR were statistically significantly increased compared to vehicle (PBS) treated group. **P<0.01 by Student *t*-test. N = 6 mouse retinas/ group. Error bars represent SEM.

Figure S1. NR treatment preserves nuclei of the outer nuclear layer in the LIRD mice. A week after toxic light exposure, mice were euthanized and ocular sections stained with hematoxylin and eosin (H&E). Representative H&E images of complete retina sections from each treatment group are shown. **Panel A**: Section from a PBS-treated mouse exposed to dim light shows unperturbed ONL across the span of the retina. **Panel B**: Section from a PBS-treated mouse with toxic light exposure shows extreme thinning in the central, superior (right side of section) ONL. **Panel C**: Section from a NR-treated mouse exposed to toxic light shows similar thickness ONL as that in control mouse.

Figure S2. NR treatment prevents increases TUNEL-positive cells following induction of LIRD. A week after toxic light exposure the mice were euthanized and ocular sections stained for TUNEL (green) and DAPI (blue). Representative images of complete retina sections from each treatment group are shown. The right half of each section is dorsal/superior and the left half is ventral periphery/inferior. **Panel A**: Section from a PBS-treated mouse exposed to dim light shows little to no TUNEL-positive ONL nuclei across the span of the retina. **Panel B**: Section from a PBS-treated mouse with toxic light exposure shows numerous TUNEL-positive ONL nuclei in both superior and inferior halves. **Panel C**: Section from a NR-treated mouse exposed to toxic light shows much fewer TUNEL-positive ONL nuclei relative to PBS-treated mouse section.