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Supplemental Information

Neuroglobin Can Prevent or Reverse Glaucomatous Progression in DBA/2J Mice

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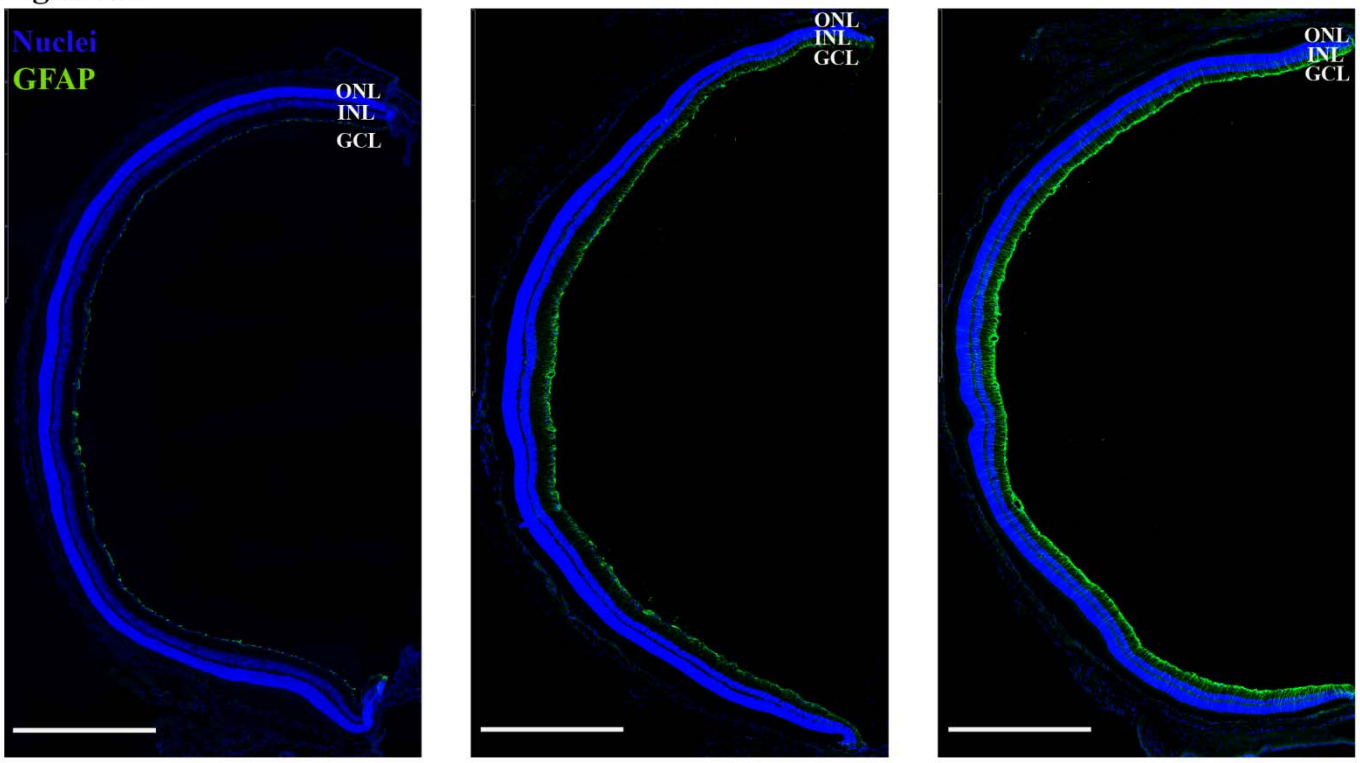
Figure S1



Untreated retina, 2-month-old DBA/2J | Untreated retina, 8-month-old DBA/2J | Untreated retina, 12-month-old DBA/2J

Figure S2

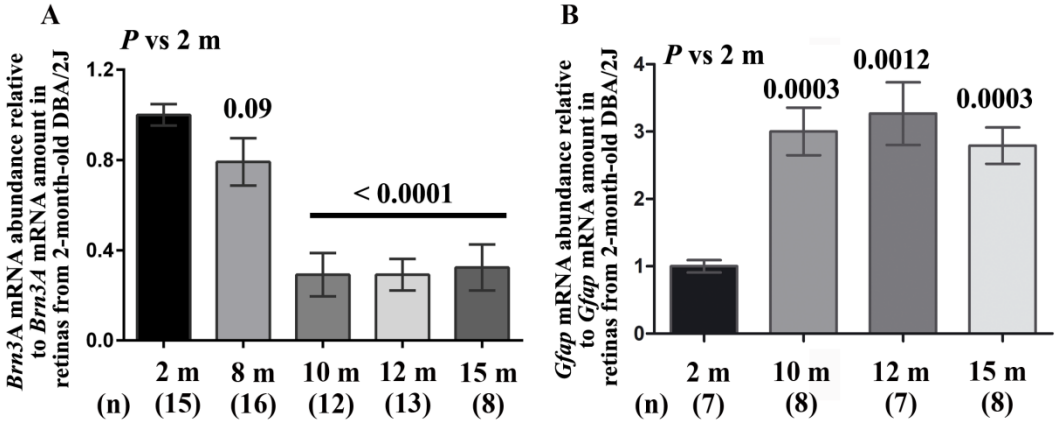


Figure S3

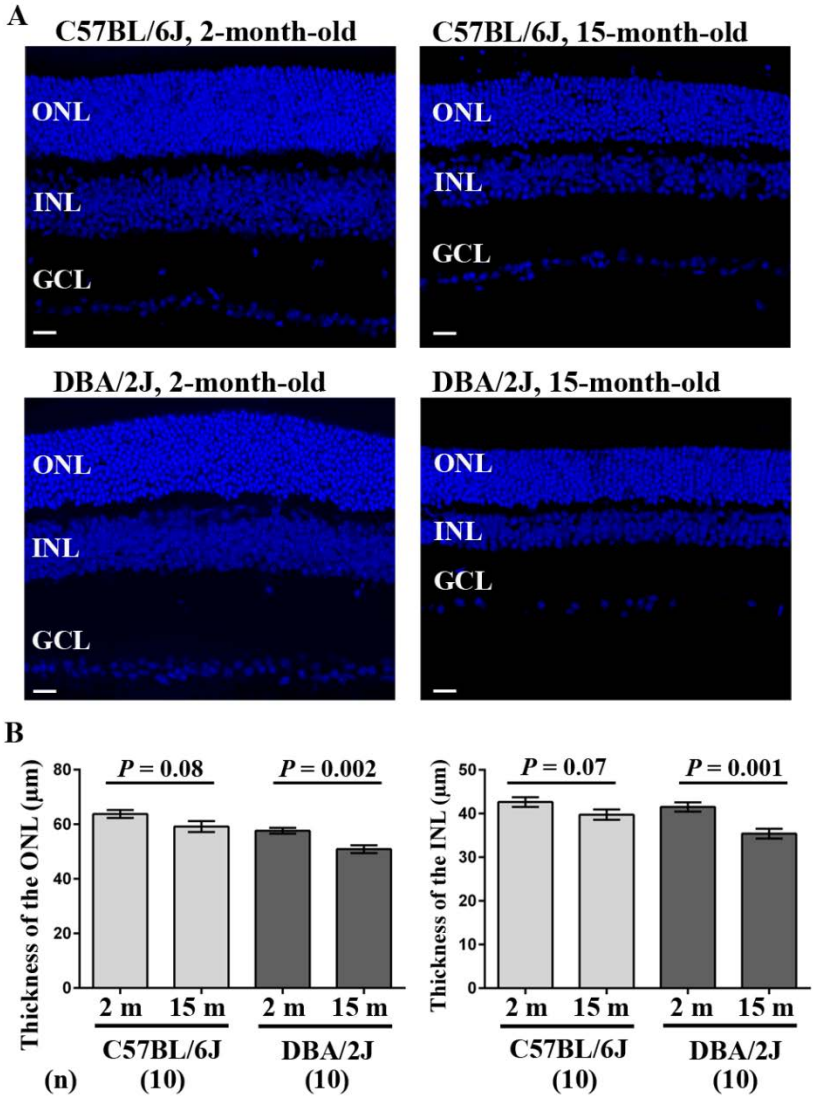

A
C57BL/6J, 2-month-old
C57BL/6J, 15-month-old
DBA/2J, 2-month-old
DBA/2J, 15-month-old
ONL
INL
GCL
B
Thickness of the ONL (μm)
P = 0.08
P = 0.002
Thickness of the INL (μm)
P = 0.07
P = 0.001
2 m
15 m
C57BL/6J
DBA/2J
(n)
(10)

Figure S4

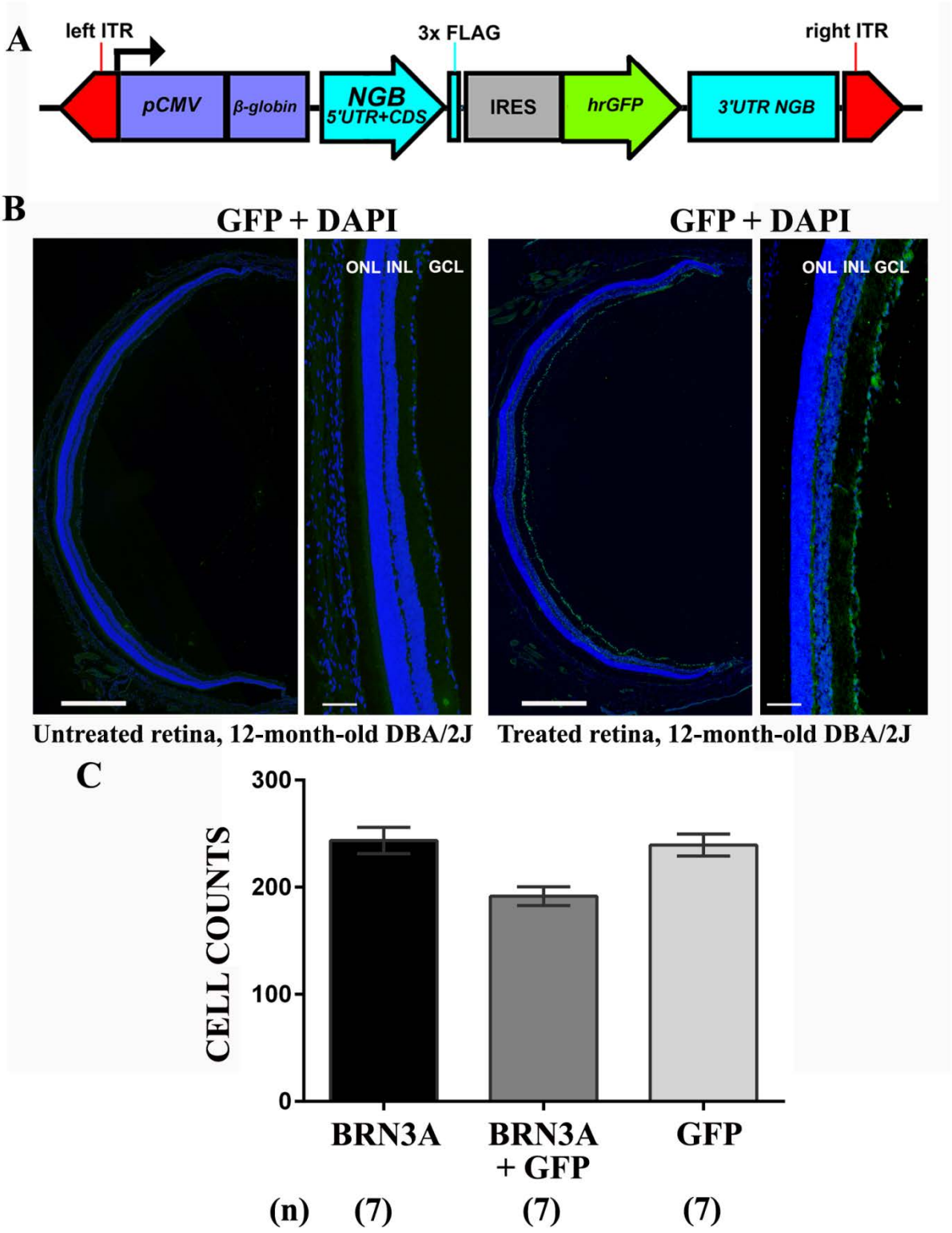

A
left ITR
3x FLAG
right ITR
pCMV
β-globin
NGB
5'UTR+CDS
IRES
hrGFP
3'UTR NGB
B
GFP + DAPI
GFP + DAPI
ONL INL GCL
ONL INL GCL
Untreated retina, 12-month-old DBA/2J
Treated retina, 12-month-old DBA/2J
C
CELL COUNTS
300
200
100
0
BRN3A
BRN3A + GFP
GFP
(n) (7) (7) (7)

Figure S5

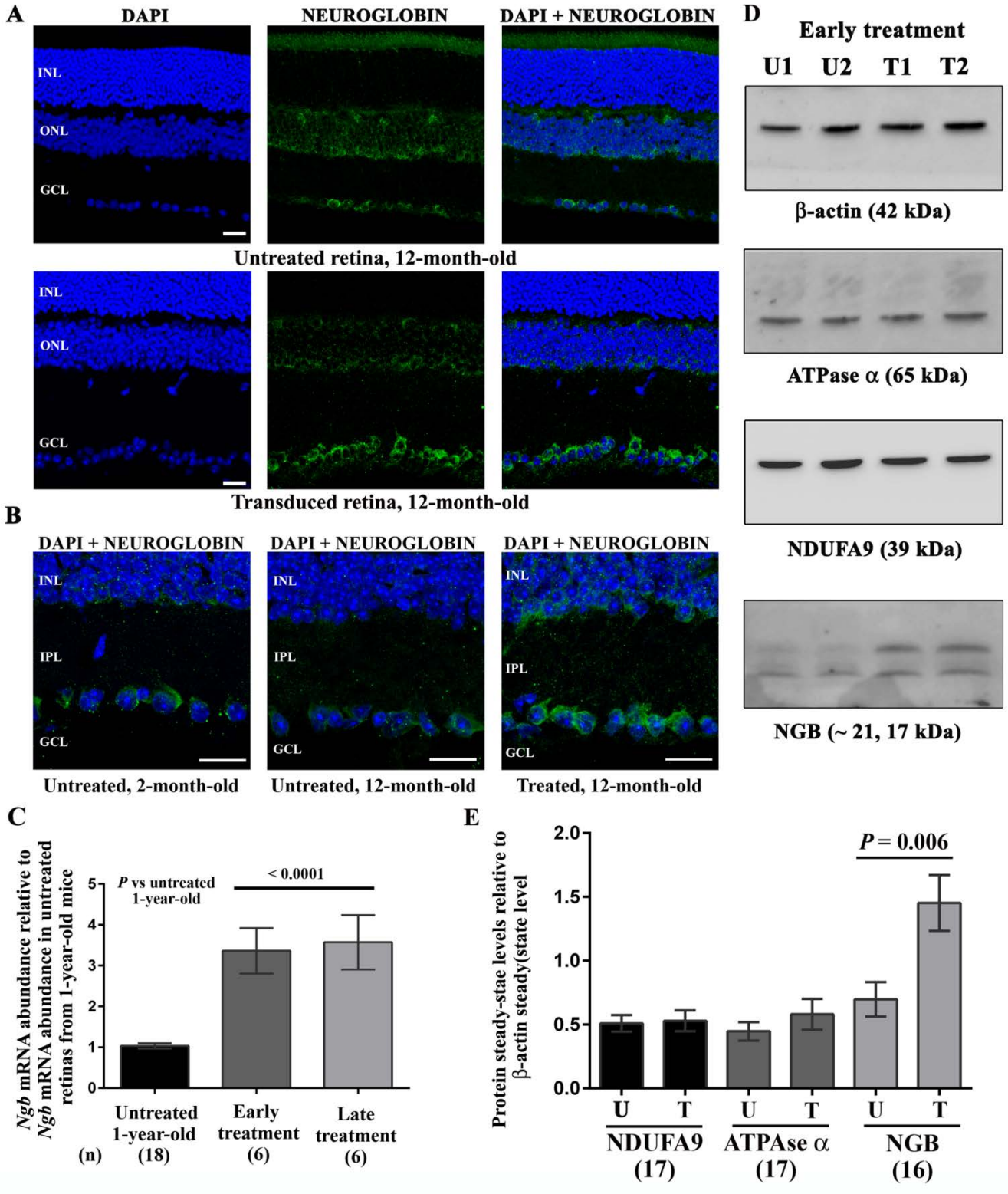

A
DAPI
NEUROGLOBIN
DAPI + NEUROGLOBIN
INL
ONL
GCL
Untreated retina, 12-month-old
Transduced retina, 12-month-old
B
DAPI + NEUROGLOBIN
IPL
Untreated, 2-month-old
Untreated, 12-month-old
Treated, 12-month-old
C
Ngb mRNA abundance relative to Ngb mRNA abundance in untreated retinas from 1-year-old mice
P vs untreated 1-year-old
< 0.0001
Untreated 1-year-old
Early treatment
Late treatment
(n)
(18)
(6)
(6)
D
Early treatment
U1
U2
T1
T2
β-actin (42 kDa)
ATPase α (65 kDa)
NDUFA9 (39 kDa)
NGB (~ 21, 17 kDa)
E
Protein steady-stae levels relative to β-actin steady(state level
P = 0.006
U
T
NDUFA9 (17)
ATPase α (17)
NGB (16)

Figure S6

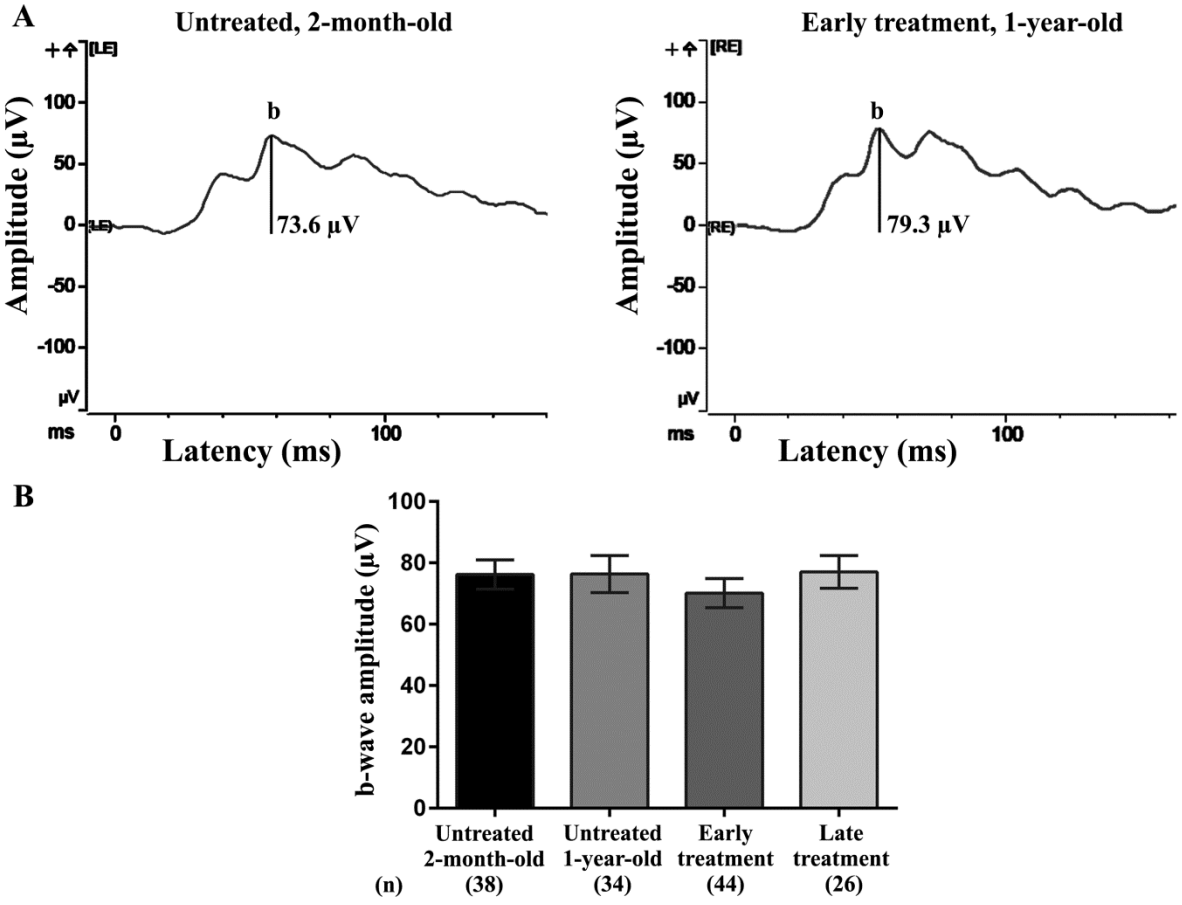

A
Untreated, 2-month-old
Early treatment, 1-year-old
[LE]
[RE]
b
73.6 µV
79.3 µV
Amplitude (µV)
100
50
0
-50
-100
µV
ms
0
100
Latency (ms)
B
b-wave amplitude (µV)
100
80
60
40
20
0
Untreated 2-month-old
Untreated 1-year-old
Early treatment
Late treatment
(n)
(38)
(34)
(44)
(26)

FIGURE LEGENDS

Figure S1: Glial fibrillary acidic protein abundance in retinas of DBA/2J mice at different ages.

The results of immunohistochemical staining for GFAP (green) and the reconstruction of whole retinal sections from untreated mice aged 2, 8, and 12 months are shown. The reconstruction of retinal sections was performed with the NDP 2.0 HT scanner. Cell nuclei were stained with DAPI (blue). The scale bars correspond to 5 mm.

Figure S2: *Brn3A* and *Gfap* expression in retinas of DBA/2J mice at different ages.

RT-qPCR assays were performed using total RNA extracted from retinas isolated from DBA/2J mice aged between 2 and 15 months. The histograms show the steady-state levels of *Brn3a* and *Gfap* mRNAs as the means ± SEMs after normalization of the signals against the mean signals for *Brn3a* and *Gfap* mRNAs in retinas from 2-month-old mice. The number of independent RNAs assessed per group is indicated in brackets below each bar. The primers used are shown in Table S4. The *P*-values shown were calculated with respect to data collected from untreated 2-month-old DBA/2J mice and plotted using GraphPad Prism 6.

Figure S3: Assessment of the thickness of neuronal cell layers in retinas of C57BL/6J and DBA/2J mice.

(A) Confocal images of retinal sections stained with DAPI from C57BL/6J and DBA/2J mice aged 2 months (2 m) or 15 months (15 m). The scale bars correspond to 20 μm. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

(B) The thickness of the ONLs and INLs was estimated using the NDP 2.0 HT scanner and the associated software after reconstructing the entire retinal sections from five C57BL/6J mice aged 2 months, five C57BL/6J mice aged 15 months, five DBA/2J mice aged 2 months, and five DBA/2J mice aged 15 months. The measurements were performed at two central points for each section, and two or three sections were evaluated for each animal. A total of 10 independent retinas were analyzed for each group. Bar graphs of the

thickness in μm for the ONLs and INLs were produced with GraphPad Prism 6, with the values plotted as means ± SEMs. *P*-values were calculated by comparing the values obtained for mice aged 2 and 15 months for the two strains evaluated.

Figure S4: AAV2/2-*NGB* vector map and transduction yield after a single intravitreal injection.

A) Physical map of the AAV2/2-*NGB* vector genome (7255 bp), encompassing mouse *NGB* sequences inserted into the *pAAV-IRES-hrGFP* plasmid: the NGB ORF (453 bp), encoding 151 amino acids (CDS), is in frame with three FLAG epitopes and is transcribed under the control of the cytomegalovirus promoter (pCMV) and the β-globin intron. The construct contains the untranslated regions at the 5′ (279 bp) and 3′ (895 bp) ends of the mouse *Ngb* mRNA (NM_022414.2). The plasmid also contains a cassette that allows the expression of the recombinant humanized green fluorescent protein (GFP) translated from the encephalomyocarditis virus internal ribosome entry site.

(B) Transduction efficiency was evaluated by immunohistochemical staining for GFP in sections of retinas from DBA/2J mice euthanized 10 months after undergoing a single intravitreal injection of AAV2/2-*NGB* (2 × 109 VG in one eye). Labeling for GFP is shown in green; cell nuclei were stained with DAPI (blue). The scale bars correspond to 2.5 mm and (in the magnification) 50 μm. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

(C) The histogram shows the numerical evaluation of the data illustrated in (B). Mice underwent intravitreal injection of AAV2/2-*NGB* at the age of 2 months (early treatment) and were euthanized 10 months later. Three or four independent sections from each of seven treated eyes were examined to estimate the number of (a) RGCs (BRN3A-positive cells), (b) transduced cells in the GCL (GFP-positive cells), and (c) transduced RGCs (BRN3A and GFP positive cells). The histogram, prepared using GraphPad Prism 6, shows the means ± SEMs obtained for the seven mice.

Figure S5: *Ngb* expression in retinas of mice treated with AAV2/2-*NGB*.

(A) The results of immunofluorescence analysis using the anti-NGB antibody (green), showing retinal sections from a single 1-year-old DBA/2J mouse that underwent AAV2/2-*NGB* intravitreal injection in one eye at 2 months of age while the contralateral eye remained untreated. The nuclei were stained with DAPI (blue). The scale bars correspond to 20 μm. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

(B) Confocal images of retinal sections subjected to immunohistochemical staining for NGB are shown as composites of NGB (green) and DAPI (blue) signals. They correspond to retinal sections from two untreated mice aged 2 and 12 months, respectively, and a treated animal that received early treatment and was euthanized at 12 months of age. The scale bars correspond to 20 μm. Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer.

(C) RT-qPCR assays were performed with total RNA extracted from 17 retinas from 1-year-old untreated mice, six retinas from the treated eyes of mice that received early treatment, and six retinas from mice that received late treatment. The primers used are shown in Table S4. The bar chart shows the steady-state levels of *Ngb* mRNA relative to the values assessed in retinal RNAs purified from 1-year-old untreated mice. The *P*-values were calculated with GraphPad Prism 6.

(D) Representative Western blots obtained from two pairs of retinas isolated from 1-year-old mice that received early treatment in only one eye (T) and from their contralateral untreated counterparts (U). The membranes were successively incubated with antibodies against NGB, NDUFA9, ATP synthase α, and β-actin (as the loading control). Seven pairs of retinas from mice that were euthanized at 1 year of age and in which only one eye underwent vector administration at the age of 2 months were assessed. As seen in Figure 5, staining with antibody against NGB revealed two main signals, with apparent molecular masses of 21 and 17 kDa, and also a faint band between the two main bands.

(E) Bar chart showing the relative amounts of NGB, NDUFA9, and ATP synthase α in retinas from untreated and treated eyes. The intensities of the NGB signals were normalized against β-actin signals; the number of individual signals is indicated in brackets (n). No treatment-linked difference was noticed in the abundance

of NDUFA9 or ATP synthase α, whereas there was a twofold increase in NGB abundance in retinas from the treated eyes of mice. This difference was significant when the results were compared to those obtained in age-matched untreated retinas ($P = 0.006$).

Figure S6: Light-adapted electroretinograms recorded in mice treated with AAV2/2-*NGB*.

(A) Plots of light-adapted ERG responses recorded in two DBA/2J mice: one 2-month-old untreated mouse and 12-month-old mouse that had undergone an injection of AAV2/2-*NGB* in one eye at the age of 2 months. (B) ERG responses are represented in the bar chart as means ± SEMs for each group evaluated. There were no statistically significant differences between the data for the different groups. The number of individual responses recorded for each group is indicated in brackets below each bar (n).

Table S1: Electroretinogram and flash visual evoked potential components in DBA/2J mice

	Untreated 2-month-old mice ± SEM (n = 38)	Untreated 12-month-old mice ± SEM (n = 34)	Treated mice at 2 months ± SEM (n = 44)	Treated mice at 8 months ± SEM (n = 26)
Amplitude of b-wave (µV)	76.22 ± 4.8	76.37 ± 6.03	70.11 ± 4.73	77.02 ± 5.32
Latency of b-wave (ms)	69.72 ± 2.66	61.21 ± 1.86	61.76 ± 1.59	59.42 ± 1.45
Amplitude of N1 wave (µV)	46.95 ± 4.1	27.1 ± 4.51	50.9 ± 4.96	51.4 ± 4.86
Latency of N1 wave (ms)	68.04 ± 2.2	69.9 ± 2.7	72.7 ± 2.2	68.1 ± 1.7
Amplitude of P1 wave (µV)	58.6 ± 6.4	52.6 ± 8.9	46.9 ± 3.5	48.1 ± 6.13
Latency of P1 wave (ms)	111.2 ± 4.5	122.3 ± 4.7	116.2 ± 3.13	113.3 ± 2.84

The b-wave values shown correspond to photopic ERG recordings.

The N1 and P1 waves correspond to F-VEP responses.

The number of individual recordings is indicated in brackets.

Table S2: Antibody descriptions

Antibody target or reagent	Type	Assay: concentration	Supplier, catalog no.
BRN3A	Monoclonal	IIF: 8 µg/mL	Chemicon, MAB1585
GFP	Polyclonal	IIF:1.5 µg/mL	Torrey Pines Biolabs, TP401
NGB	Polyclonal	IIF: 5 µg/mL	Sigma-Aldrich, N-7162
NGB	Polyclonal	Western: 2 µg/mL	BioVendor, RD181043050
NGB	Polyclonal	Western: 1 µg/mL	Santa Cruz, Sc-30144
ATP synthase subunit α	Monoclonal	IIF: 2 µg/mL Western: 0.5 µg/mL	Thermo Fisher, 459240
β-actin	Monoclonal	Western: 0.2 µg/mL	Sigma-Aldrich, A5316
SOD2	Polyclonal	Western: 0.5 µg/mL	Abcam, ab13534
OPA1	Polyclonal	IIF: 5 µg/mL Western: 0.5 µg/mL	Abcam, ab42364
TOMM20	Monoclonal	IIF: 5 µg/mL Western: 1.25 µg/mL	Abcam, ab56783
NDUFA9	Monoclonal	IIF: 10 µg/mL Western: 1 µg/mL	Life Technologies, 459100
HSP60	Polyclonal	Western: 0.05 µg/mL	Abcam, ab46798

CytC	Monoclonal	IIF: 5 µg/mL Western: 2 µg/mL	Abcam, ab13575
AIF	Polyclonal	IIF: 2.5 µg/mL Western: 0.05 µg/mL	Abcam, ab32516
GFAP	Polyclonal	IIF: 3 µg/mL	Sigma-Aldrich, G3893
NF200	Polyclonal	IIF: 1 µg/mL	Sigma-Aldrich, N4142
IBA1	Polyclonal	IIF: 5 µg/mL	Wako, 019-19741
Vimentin	Monoclonal	IIF: 2.5 µg/mL	BD Pharmingen, 550513
Alexa 488	Anti-IgG, rabbit	IIF: 4 µg/mL	Life Technologies, A11008
Alexa 594	Anti-IgG, mouse	IIF: 4 µg/mL	Life Technologies, A11005
Goat anti-rabbit IgG	Goat anti-rabbit IgG, horseradish peroxidase conjugate	Western: 0.05 µg/mL	Jackson ImmunoResearch Laboratories, 111-035-144
Goat anti-mouse IgG	Goat anti-mouse IgG, horseradish peroxidase conjugate	Western: 0.05 µg/mL	Jackson ImmunoResearch Laboratories, 115-035-003
DAPI (4',6-diamidino-2-phenylindole, dihydrochloride)	Nucleic acid stain	IIF: 2 µg/mL	Life Technologies, D1306

Abbreviations: IIF, indirect immunofluorescence in retinal or optic-nerve sections.

Table S3: Comparison of retinal lengths estimated in reconstructed scanned images

Mouse age groups (no. of individual samples)	2 months (20)	8 months (18)	10 months (16)	12 months, untreated (20)	12 months, treated (20)
Retinal length normalized against the mean for the 2-month-old group	1 ± 0.015	1.117 ± 0.018	1.197 ± 0.02	1.185 ± 0.016	1.192 ± 0.015
P-value compared to 2-month-old mice		< 0.0001	< 0.0001	< 0.0001	< 0.0001
P-value for 12-month-old untreated vs 12-month-old treated mice					0.878

Retinal lengths are expressed as the means ± SEMs obtained for independent eyes in each group.

Table S4: Pairs of primers used in the RT-qPCR assays

Gene	Forward 5′–3′	Reverse 5′–3′
Ngb	CTCAGGCAAGGGAAGCATAG	CAGTTAGGTTTCCCCCAAAA
AAV-*NGB*	AGGCTATGTCACGAGGTTGG	GGGTAACCCTATGCAGTCGT
Atp6	CGTAATTACAGGCTTCCGACA	AGCTGTAAGCCGGACTGCTA
Brn3A	GAGGCCTATTTTGCCGTACA	CAGTAAGTGGCAGAGAATTTCA
Gfap	CCCGTTCTCTGGAAGACACT	CTTCAGGGCTGAGAGCAGTC