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Tyrosine triple mutated AAV2-BDNF gene therapy in an inner retinal injury model induced by intravitreal injection of *N*–methyl-D-aspartate (NMDA)

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Purpose: Glaucoma is a group of chronic optic neuropathies characterized by the degeneration of retinal ganglion cells (RGCs) and their axons, and they ultimately cause blindness. Because neuroprotection using neurotrophic factors against RGC loss has been proven a beneficial strategy, extensive attempts have been made to perform gene transfer of neurotrophic proteins. This study used the inner retinal injury mouse model to evaluate the neuroprotective effect of tyrosine triple mutated and self-complementary adeno-associated virus (AAV) encoding brain-derived neurotrophic factor (BDNF; tm-scAAV2-BDNF).

Methods: C57BL/6J mice were intravitreally injected with 1 µl of tm-scAAV2-BDNF and its control AAV at a titer of 6.6 E+13 genome copies/ml. Three weeks later, 1 µl of 2 mM *N*–methyl-D-aspartate (NMDA) was administered in the same way as the viral injection. Six days after the NMDA injection, we assessed the dark-adapted electroretinography (ERG). Mice were sacrificed at one week after the NMDA injection, followed by RNA quantification, protein detection, and histopathological analysis.

Results: The RNA expression of BDNF in retinas treated with tm-scAAV2-BDNF was about 300-fold higher than that of its control AAV. Meanwhile, the expression of recombinant BDNF protein increased in retinas treated with tm-scAAV2-BDNF. In addition, histological analysis revealed that tm-scAAV2-BDNF prevented thinning of the inner retina. Furthermore, b-wave amplitudes of the tm-scAAV2-BDNF group were significantly higher than those of the control vector group. Histopathological and electrophysiological evaluations showed that tm-scAAV2-BDNF treatment offered significant protection against NMDA toxicity.

Conclusions: Results showed that tm-scAAV2-BDNF-treated retinas were resistant to NMDA injury, while retinas treated with the control AAV exhibited histopathological and functional changes after the administration of NMDA. These results suggest that tm-scAAV2-BDNF is potentially effective against inner retinal injury, including normal tension glaucoma.

Glaucoma is a group of chronic optic neuropathies characterized by the degeneration of retinal ganglion cells (RGCs) and their axons [1]. These pathological changes are progressive and irreversible, and they ultimately cause blindness [2]. Tham et al. estimated that the number of 45 patients with glaucoma will increase from 64.3 million in 2013 to 111.8 million in 2040 due to the increase of aging populations and the lifestyle alternation [3]. Therefore, as glaucoma will have a significant influence on society, the development of effective therapies against this disease is an urgent need.

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As the most commonly identified risk factor for glaucoma is an elevated intraocular pressure (IOP), lowering IOP is believed important to prevent the progression of visual field loss [4-9]. However, it has been reported that normal tension glaucoma (NTG) patients, among others, derive few benefits from ocular hypertension treatments [10-13]. Moreover, glaucoma is often asymptomatic during the early stages [14,15]. On the other hand, micro changes, such as RGCs loss, occur in glaucomatous retinas even in the early stage [16-19]. For these reasons, although lowering IOP is certainly the first treatment choice, it is an insufficient treatment alone. Thus, direct intervention, such as providing neuroprotection to RGCs, is important to overcoming the glaucomatous pathology.

Previous attempts have been undertaken to protect RGCs by using neuroprotective substances [20]. Among these, brain-derived neurotrophic factor (BDNF) especially resulted

in beneficial effects. BDNF is a small protein purified from the mammalian brain [21] and is essential for RGC survival [22,23]. Gupta et al. reported that because $BDNF^{+/-}$ mice were vulnerable to chronic IOP increases, endogenous BDNF was important in maintaining the integrity of the inner retinas [24]. Furthermore, the protective effects of exogenous BDNF have been reported in several experimental or chronic glaucoma models [23,25-27].

However, due to the short half-life of neurotrophic factors, including BDNF [28], frequent administration of neurotrophic reagents is required clinically to maintain therapeutic drug levels. As a result, this increases the burden to the patient. Therefore, effective gene transfer and a stable expression of the neurotrophic factor within the target cells are necessary for safeguarding patient welfare.

Adeno-associated viruses (AAVs) have proven useful for gene therapy because of the long-lasting gene expression and lack of any apparent pathogenicity [29]. After many clinical trials using AAV vectors, the Food and Drug Administration (FDA) approved this gene therapy product for Leber congenital amaurosis in December 2017 [30,31].

AAV serotype 2 (AAV2) has been especially well studied, which has led to the extensive development of mutant AAVs. In particular, the self-complementary AAV (scAAV) has proven excellent in accelerating the protein expression [32]. In addition, the tyrosine triple mutant AAV (tmAAV) is also useful for increasing the efficacy of transduction into the retina [33,34]. In a subsequent study, we reported that the combination of these mutations led to a successful transduction into the inner retina of rodents [35]. In our current study, we tested the efficacy of our recombinant BDNF expression AAV, tm-scAAV2-BDNF, against inner retinal damage induced by an *N*-methyl-D-aspartate (NMDA) injection.

METHODS

Production of rAAV: An AAV packaging plasmid, pACG2–3M (pAAV2-Y730+500+444F; provided by Dr. Arun Srivastava) [33,34], and an adenovirus helper plasmid, pHelper (Agilent Technologies, Santa Clara, CA), were used in this study. The scAAV vector plasmid carrying cDNA encoding human BDNF (pdsCBA-BDNF) has been previously described [35]. For construction of the control vector, cDNA encoding the Emerald green fluorescent protein (Thermo Fisher Scientific, Waltham, MA), subsequently referred to as GFP, was inserted into the same plasmid backbone instead of BDNF. The recombinant scAAV vectors tm-scAAV2-GFP and tm-scAAV2-BDNF were produced by transient triple transfection of human embryonic kidney-derived 293 (HEK293) cells, as previously described [36] but with some modifications. Briefly, HEK293 cells were seeded in square culture dishes (Corning, New York, NY) and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (Biowest, Nuaillé, France), penicillin, and streptomycin (Sigma-Aldrich) at 37 °C in a 5% CO_2 incubator for 2 days. Authentication of the HEK 293 cell line was verified by the short tandem repeat (STR) analysis performed by the Certification of Cell Line Service (BEX Co., Ltd., Tokyo, Japan) using the GenePrint® 10 System (Promega, Madison, WI, USA). The STR analysis results are shown in Appendix 1. The cells were co-transfected with pACG2–3M, pHelper, and pdsCBA-BDNF or pdsCBA-GFP. Three days after the transfection, cells were collected and disrupted by four freezing and thawing cycles, treated with benzonase, and centrifuged at 18,800 ×*g* for 30 min. The supernatant was collected, heated for 30 min at 50 °C and centrifuged twice at 13,100 ×*g* for 10 min. After filtration through a 0.45-µm filter, rAAV was precipitated by half-saturated ammonium sulfate and dissolved in PBS. AAV particles were purified by a two-step density-gradient ultracentrifugation using 1.25 g/ml and 1.74 g/ml cesium chloride solutions. Recombinant AAV fractions with a refractive index of 1.368–1.376 were collected. The collected fractions were dialyzed against 3 mM $MgCl_2$ in PBS and concentrated using an Amicon Ultra 10 K device (Merck KGaA, Darmstadt, Germany). The viral titers were determined by quantitative real-time PCR as previously described [36] using specific primers for the inverted terminal repeat (ITR) sequence as follows: GGAACCCCTAGTGATGGAGTT (forward), GCCTCAGTGAGCGAGCGAGCG (reverse).

Animals: Eight-week-old male C57BL/6J mice were obtained from CLEA Japan, Inc. (Tokyo, Japan). Mice were maintained on a 14 h:10 h light-dark cycle at a constant 25 °C and given ad libitum access to food and water. All animal procedures were performed in accordance with the Experimental Ethical Review Committee of Nippon Medical School and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Intravitreal injection: Mice were anesthetized by intraperitoneal administration of ketamine/xylazine (Ketaral, 100 mg/kg, Daiichi Sankyo Co., Ltd., Tokyo, Japan; Selactar, 10 mg/kg, Bayer Medical, Ltd., Tokyo, Japan). After a topical application of 0.4% oxybuprocaine hydrochloride (Benoxil® ophthalmic solution 0.4%, Santen Pharmaceutical Co., Ltd., Osaka, Japan), tm-scAAV2-BDNF and its control AAV were injected intravitreally using 33-gauge Hamilton needles and syringes. Each eye received 1 µl of the vector at a titer of 6.6 E+13 genome copies/mL, as previously described [37]. Three

weeks later, 1 µl of 2 mM NMDA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was administered intravitreally in the same way as the viral injection.

Electroretinography (ERG): Six days after the NMDA injection, visual function was assessed by full-field ERG, as described in our previous report [35]. Mice were dark-adapted overnight and anesthetized with an intraperitoneal injection of the ketamine/xylazine cocktail. The cornea was anesthetized with a topical drop of 0.4% oxybuprocaine hydrochloride, and the pupils were dilated with a 0.05% tropicamide and phenylephrine hydrochloride solution, using a 1:10 dilution of Mydrin-P ophthalmic solution (Santen). Dark-adapted ERG responses were recorded using white light-emitting diode electrodes. Subcutaneous needle electrodes were placed in the forehead while the negative and ground electrodes were placed in the tail. All signals were recorded using LS-W (Mayo Corporation., Aichi, Japan) as a photostimulator, the PowerLab 2/26 (ADInstruments, Sydney, Australia) as an A/D converter, and the Bio Amp ML132 as amplifiers (ADInstruments).

RNA and protein quantification: One week after the NMDA injection, mice were sacrificed and the neural retina were collected. Total RNA was extracted from the neural retina using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Extracted RNA was reverse transcribed with the TaKaRa RNA PCR™ Kit (AMV) Ver. 3.0 (Takara Bio Inc., Shiga, Japan), and quantitative real-time PCR was performed on cDNA as previously described [38]. The relative expression of the target gene was quantified using the comparative threshold cycle method, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a reference gene. Primer sequences were as follows: BDNF-F, 5′-CCCTTACCATGGATAGCAAA-3′; BDNF-R, 5′-ATTTGCTGCCAGATCCTCT-3′; GAPDH-F, 5′-CATCACTGCCACCCAGAAGA-3′; GAPDH-R, 5′-ATGTTCTGGGCAGCC-3′.

For protein quantification, retinas were immersed in PBS and homogenized by sonication on ice. Homogenized retinas were examined using the Human Free BDNF Quantikine ELISA Kit (R&D Systems, Inc., MN) in accordance with the product protocol. The amount of total protein in retinas was determined using the DC Protein Assay Kit II (Bio-Rad Laboratories, Inc., Hercules, CA) and the amount of BDNF protein was corrected according to the total protein amount. When the expression level of BDNF protein was lower than the measurable limits of the kits, a value of 20 pg/ml was applied in the statistical analysis, as the manufacturer's protocol states that this is the minimum detectable dose.

Histopathological evaluation: One week after NMDA injection, mice were anesthetized with the ketamine/xylazine cocktail and perfused by a cardiac infusion of PBS followed by 4% paraformaldehyde in a 0.1 M phosphate buffer, as previously described [39]. Eyeballs were enucleated and then the anterior segments were removed. After the eye cups were postfixed overnight at 4 °C, they were sequentially transferred in a stepwise manner to sucrose/PBS solution. First, the eyes were incubated in 10% sucrose for 3 h. Second, they were sunk in 20% sucrose overnight. Finally, they were transferred in 30% sucrose and incubated overnight. The eyes were frozen in an optimum cutting temperature compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) using a dry ice/ethanol bath. The embedded samples were dissected into 6-µm sections along the vertical meridian through the optic nerve head (ONH). When the inner retinal thickness was analyzed, three cryosections were obtained from each eye and stained with hematoxylin and eosin. Images were obtained by BX60 (Olympus Corporation, Tokyo, Japan).

Fluorescent immunohistochemistry: For Brn3a immunostaining, sections were incubated with HistoVT One (Nacalai Tesque, Inc., Kyoto, Japan) at 70 °C for 20 min and then with 10% donkey serum and 1% BSA mixture in PBS containing 0.1% Triton X-100 at room temperature (RT) for 2 h. Goat anti-Brn3a antibody (1:400; sc-31984, Santa Cruz Biotechnology, TX) was applied at RT over a two-night period. After three washes in PBS that contained 0.05% Triton X-100, the Alexa Fluor 568 donkey anti-goat IgG conjugate was applied at RT for 1 h.

For glial fibrillary acidic protein (GFAP) immunostaining, sections were incubated with 10% skim milk in PBS at RT for 2 h and then with rabbit anti-GFAP antibodies (1:500; Z0334, Dako, Glostrup, Denmark) overnight at 4 °C. After three washes in PBS, biotinylated goat anti-rabbit IgG antibody (1:200; BA-1000, Vector Laboratories Inc., Burlingame, CA) was applied for 1 h and followed by Cy3 Streptavidin (1:2000; 016–160–084, Jackson ImmunoResearch Laboratories, Inc., PA) for 1 h.

All sections were mounted in the Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories Inc.). Images were obtained by BX60 (Olympus Corporation). Three sections from each eye were observed.

Statistics: All results are expressed as the mean ± standard error of the mean (SEM) in this report. Histological images were examined using the GIMP 2 (The GIMP Team) and ImageJ (NIH, Bethesda, MD, USA) software. RNA and protein expression levels of all groups were statistically evaluated using a Mann–Whitney U test. When electrophysiological and histopathological examinations were performed, control and experimental groups were compared using a one-way ANOVA followed by a Student–Newman–Keuls test

(SNK test). Statistical analysis was performed using Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA). A p value of <0.05 was considered statistically significant.

RESULTS

Intravitreal injection of tm-scAAV2-GFP induced a strong expression of GFP in the inner retina: Petrs-Silva et al. reported finding a much higher transduction potency of the triple mutated scAAV2 vector in the inner retina as compared to the wild AAV2 [34]. This mutated vector possesses mutations in tyrosine 444, 500, and 730 to phenylalanine (Y-F) and enhances the transduction efficiency into ganglion cells by >30-fold after an intravitreal injection.

To confirm the distribution of GFP after an intravitreal injection of tm-scAAV2-GFP, transverse sections were examined by fluorescence microscopy. Results revealed there was a robust expression of GFP, even in the NMDA-treated retina, especially in the ganglion cell layer and the inner nuclear layer (Figure 1A). Therefore, the tm-scAAV2 vector was used to express BDNF in the mouse retina.

Retinas treated with tm-scAAV2-BDNF showed high mRNA and protein expression levels of BDNF: RNA expression levels of BDNF were examined by real-time reverse transcription PCR and analyzed using the comparative threshold cycle method. After treating tm-scAAV2-BDNF or tm-scAAV2-GFP intravitreally, retinas were collected and total RNA was extracted from the retinal samples. Results showed that the RNA expression in retinas treated with tm-scAAV2-BDNF was about 300-fold higher than that observed for tm-scAAV2-GFP (Figure 1B, tm-scAAV2-BDNF + NMDA, 326.75±84.98-fold, Mann–Whitney U test, $p<0.05$). Furthermore, BDNF protein was detected using the ELISA assay. Results showed that the expression of recombinant BDNF protein increased in the retinas treated with tm-scAAV2-BDNF (Figure 1C). These findings indicated that tm-scAAV2-BDNF successfully induced the BDNF expression in the mouse retina despite the NMDA injury.

Injection of tm-scAAV2-BDNF suppressed histological damage caused by NMDA: Three weeks after the intravitreal injection of the tm-scAAV2 vectors, NMDA solution was administered into the vitreous cavity. One week later, eyes were collected and examined histopathologically.

The layers from the retinal nerve fiber layer (RNFL) to the inner nuclear layer (INL) were compared (Figure 2, Appendix 2). The thickness was analyzed at individual points that were 250 µm from the ONH. The NMDA injections led to a drastic reduction in the inner retinal thickness in the control vectors-treated group compared to the PBS-treated group. On the other hand, there was a preservation of the thickness of the BDNF vectors-treated group, especially in the central and middle retinas.

We additionally counted the number of Brn3a-positive RGCs in the ganglion cell layer (GCL) from one ora serrata through the ONH to the other ora serrata. Results showed that the loss of Brn3a-positive cells was prevented by the administration of tm-scAAV2-BDNF (Figure 3). These findings demonstrate that the injection of tm-scAAV2-BDNF prevented histological changes in NMDA-injured retinas.

Injection of tm-scAAV2-BDNF saved visual function from NMDA injury: To confirm the protective effect of tm-scAAV2-BDNF, visual function was assessed by scotopic ERG. After the NMDA treatment, b-wave amplitudes of the group treated with tm-scAAV2-GFP showed notable decreases compared to the PBS-injected control group for the three intensities evaluated. In contrast, b-wave amplitudes of the tm-scAAV2-BDNF group were significantly higher than those of the control vector group (Figure 4, Appendix 3).

Injection of tm-scAAV2-BDNF reduced GFAP positive activation induced by NMDA: To evaluate the effect of tm-scAAV2-BDNF against retinal stress induced by NMDA, we examined GFAP activation of the cross sections using immunohistochemistry. It has been widely reported that GFAP upregulation is an indicator of retinal stress during various conditions, including NMDA injury (e.g., [40]).

As described in Figure 5, NMDA injections did induce the upregulation of GFAP in the tm-scAAV2-GFP group. On the other hand, the activation of GFAP was significantly reduced in the retinas treated with tm-scAAV2-BDNF compared to those in the tm-scAAV2-GFP group. These results suggest that tm-scAAV2-BDNF reduces retinal damage (Figure 5).

DISCUSSION

The current study demonstrated that tm-scAAV2-BDNF-treated retinas were resistant to NMDA injury while retinas treated with control AAV exhibited histopathological and functional changes after NMDA administration. These results suggested that our tm-scAAV2-BDNF was potentially effective against inner retinal injury, including NTG.

The NMDA injection induced a significant reduction of the inner retinal thickness in the control vectors-treated group, while there was preservation of the thickness of the BDNF vectors-treated group, especially in the central and middle retinas. In previous reports, when using a fundus scope, several groups showed a higher fluorescent protein expression in the central area around the ONH rather than

in the peripheral area with regard to the intravitreal injection of AAV2 [41,42]. Thus, it is thought that tm-scAAV2-BDNF might also have a similar distribution pattern. As a result, the protective effect of tm-scAAV2-BDNF was more prominent in the central and middle retinas than in the peripheral retina.

In our study, the b-wave amplitudes of the tm-scAAV2-BDNF group were significantly higher than those of the control vector group. This finding suggests that tm-scAAV2-BDNF prevents functional loss of the retina induced by NMDA. Moreover, the a-wave amplitudes of tm-scAAV2-BDNF were significantly higher than those of

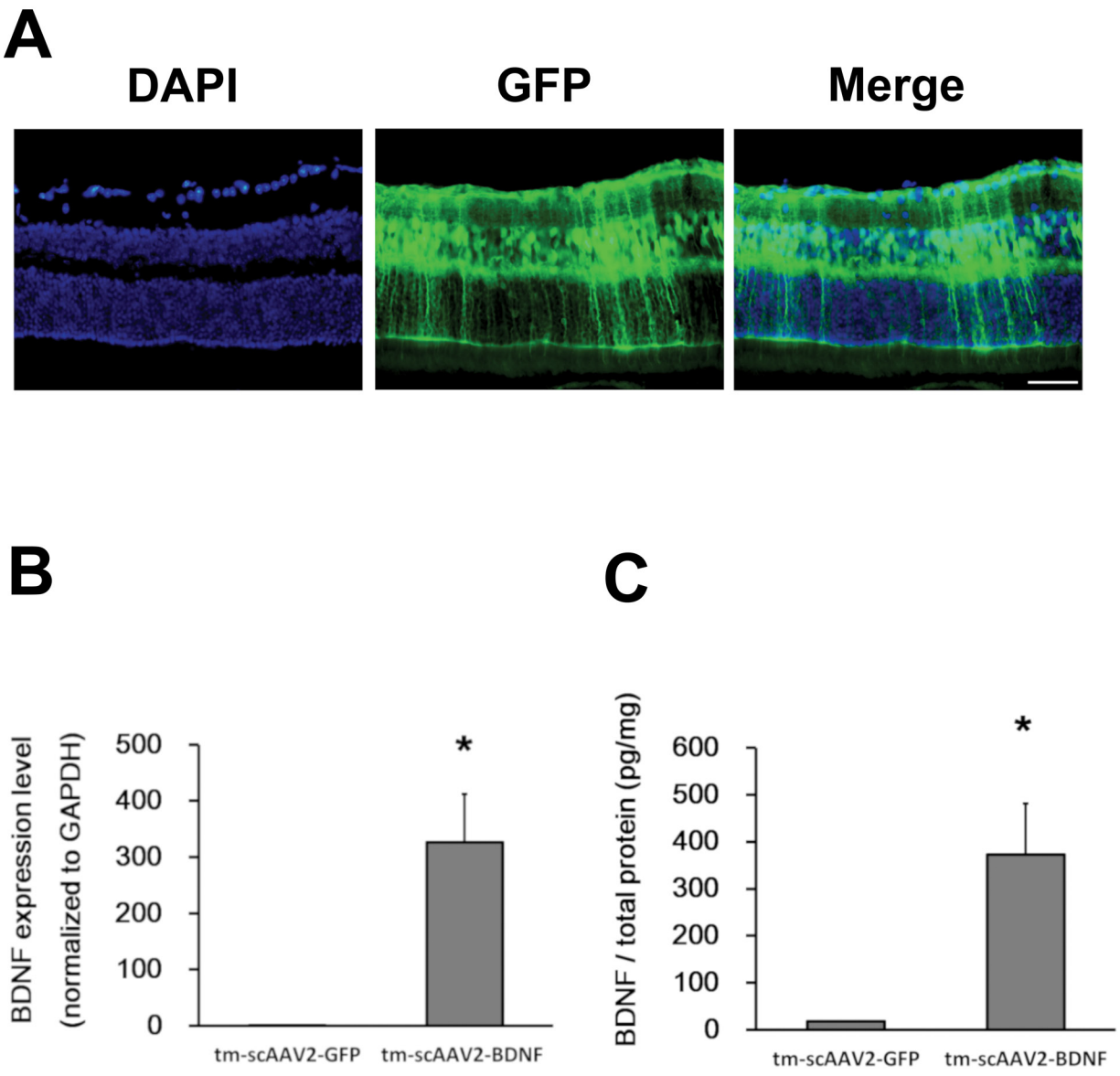

Figure 1. Retinal GFP and BDNF expression after intravitreal injection of tm-scAAV2-GFP and tm-scAAV2-BDNF. **A**: Fluorescence microscopic images of cross sections treated with tm-scAAV2-GFP and NMDA. Green: GFP, Blue: DAPI. Scale bar represents 50 µm. **B**: Relative RNA expression levels of BDNF analyzed by the comparative threshold cycle method. GAPDH was used to normalize gene expressions (n = 6 in each group, *p<0.05 for a two-tailed Mann–Whitney U test). **C**: Protein expression levels of BDNF after intravitreal injection of tm-scAAV2-BDNF, as analyzed by ELISA (n = 4 in each group, *p<0.05 for a two-tailed Mann–Whitney U test).

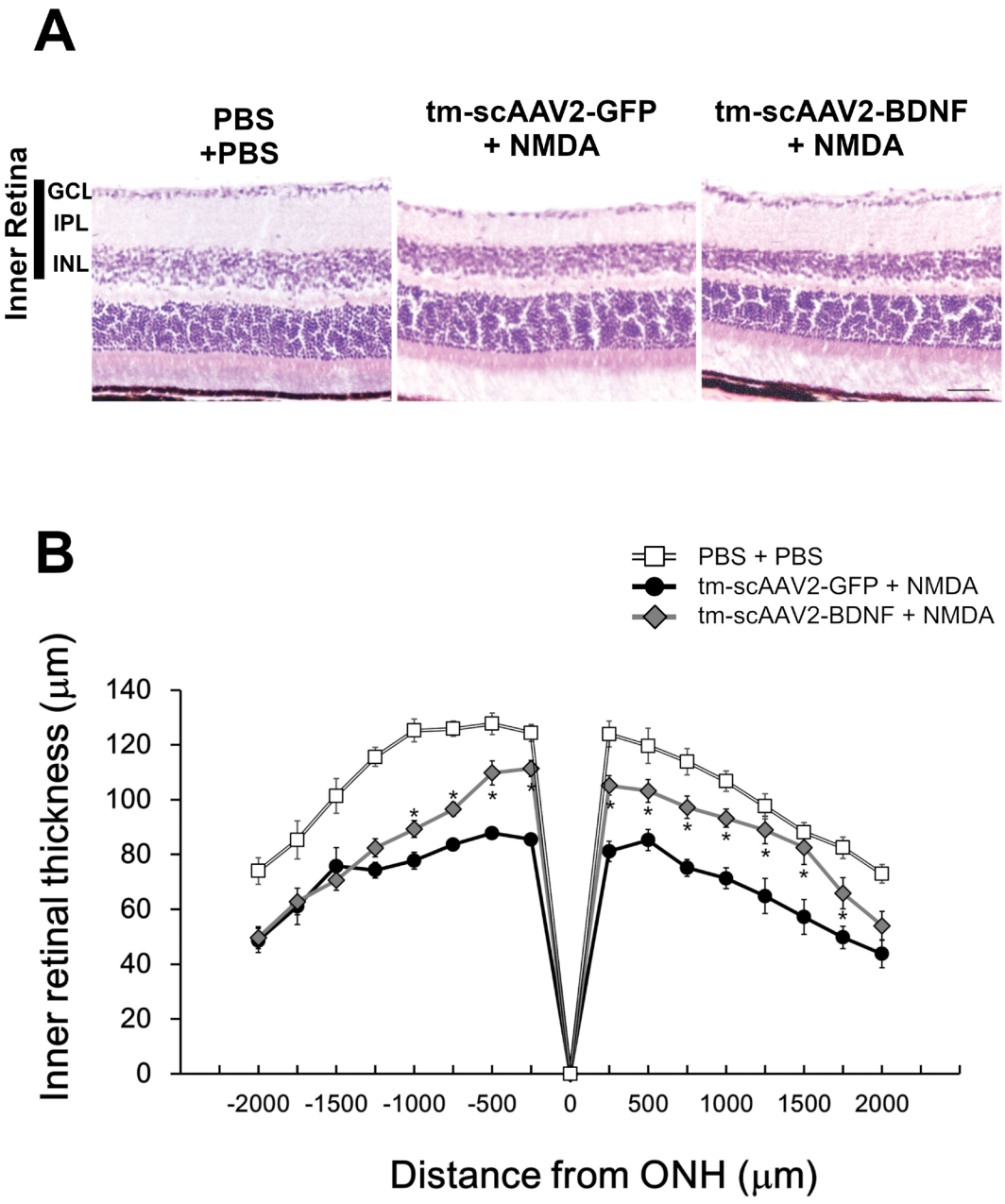

Figure 2. Histological analysis. **A**: Exemplary images of hematoxylin and eosin-stained cross sections obtained from the buffer-treated (left), control vector-NMDA-treated (middle), and therapeutic vector-NMDA-treated (right) groups. Images were captured at a distance of 1,000 µm from the ONH. Scale bar represents 50 µm. GCL: ganglion cell layer, IPL: inner plexiform layer, INL: inner nuclear layer. **B**: Comparison of inner retinal thickness at individual points that were 250 µm from the ONH in the three groups (buffer only, n = 6; control vector-NMDA, n = 6; therapeutic vector-NMDA, n = 9, *p<0.05 in SNK test).

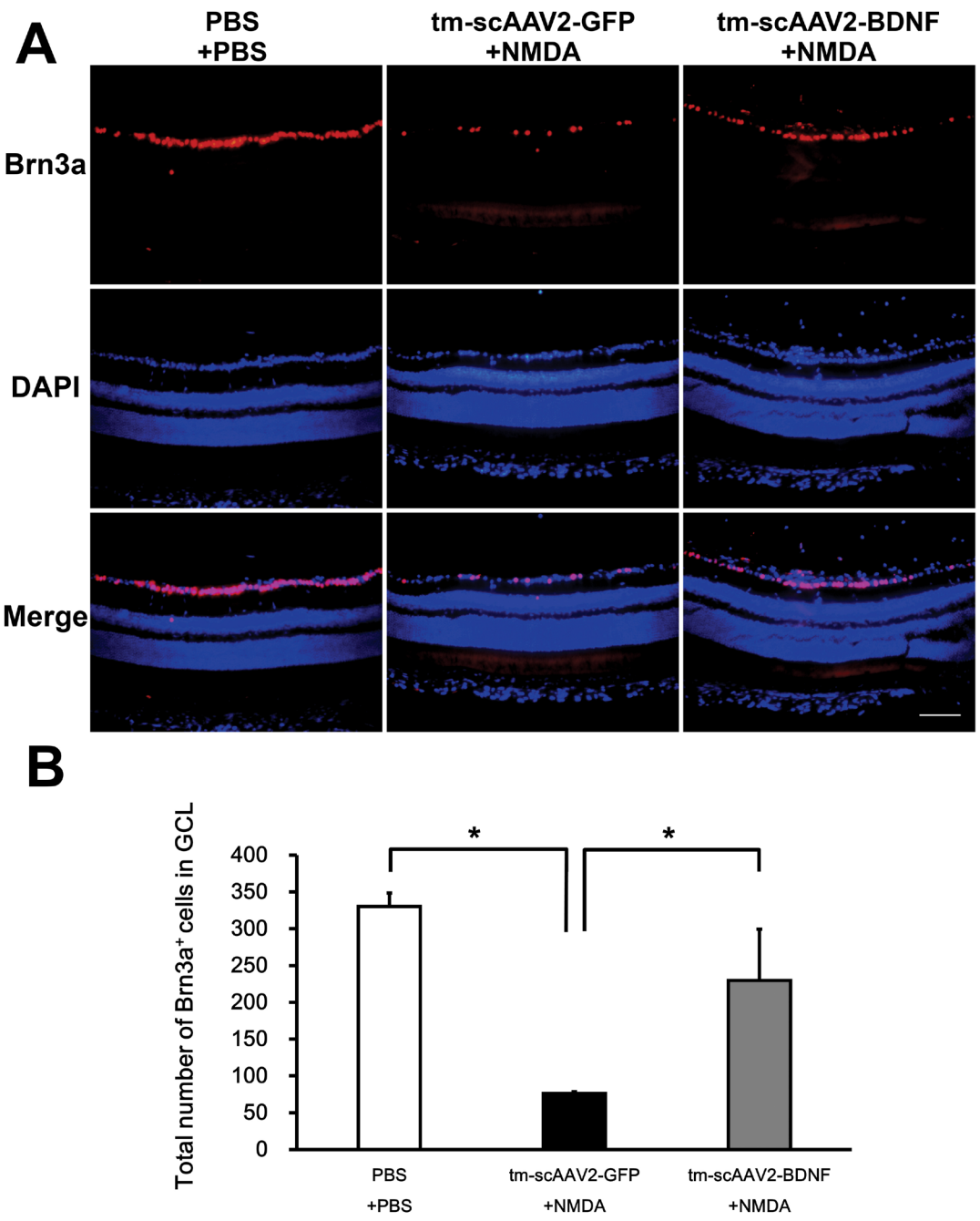

Figure 3. Immunofluorescence analysis on cross sections. **A**: Exemplary images of cross sections obtained from the buffer-treated (left), control vector-NMDA-treated (middle), and therapeutic vector-NMDA-treated (right) groups. Red: Brn3a, Blue: DAPI. Images were captured at a distance of 1,000 µm from the ONH. Scale bar represents 100 µm. **B**: Total number of Brn3a-positive cells in GCL from one ora serrata to the other (n = 3 in each group, *p<0.05 in SNK test).

tm-scAAV2-GFP. In addition, some studies have reported that an NMDA injection induced a reduction in a-wave amplitudes similar to that described in [43]. Thus, it is believed that the oxidative damage of the inner retina can spread over the outer retina. This may affect outer retinal function and lead to a reduction in a-wave amplitude. Therefore, we believe tm-scAAV2-BDNF indirectly protects from outer retinal dysfunction by rescuing the inner retina that was injured by NMDA.

Dreyer et al. reported finding an elevation in glutamate levels in the vitreous body of glaucomatous eyes [44]. Furthermore, excitatory amino acid transporter 1, also

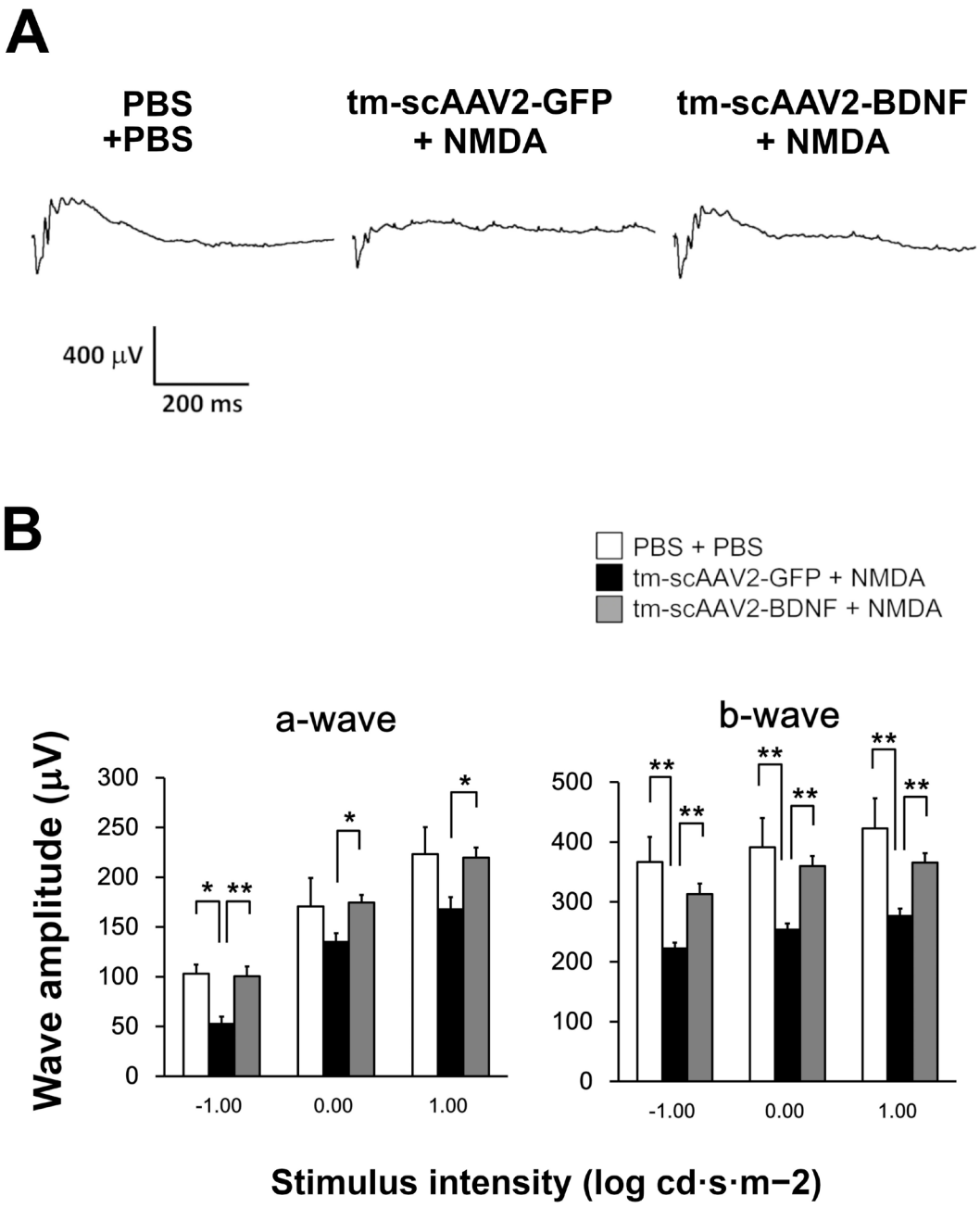

Figure 4. ERG. **A**: Representative ERG waveforms from the buffer-treated (left), control vector-NMDA-treated (middle), and therapeutic vector-NMDA-treated (right) groups. **B**: Statistical analysis of the a- and b-waves of each group (buffer only, n = 4; control vector-NMDA, n = 8; therapeutic vector-NMDA, n = 12, *p<0.05 and **p<0.01 in SNK test).

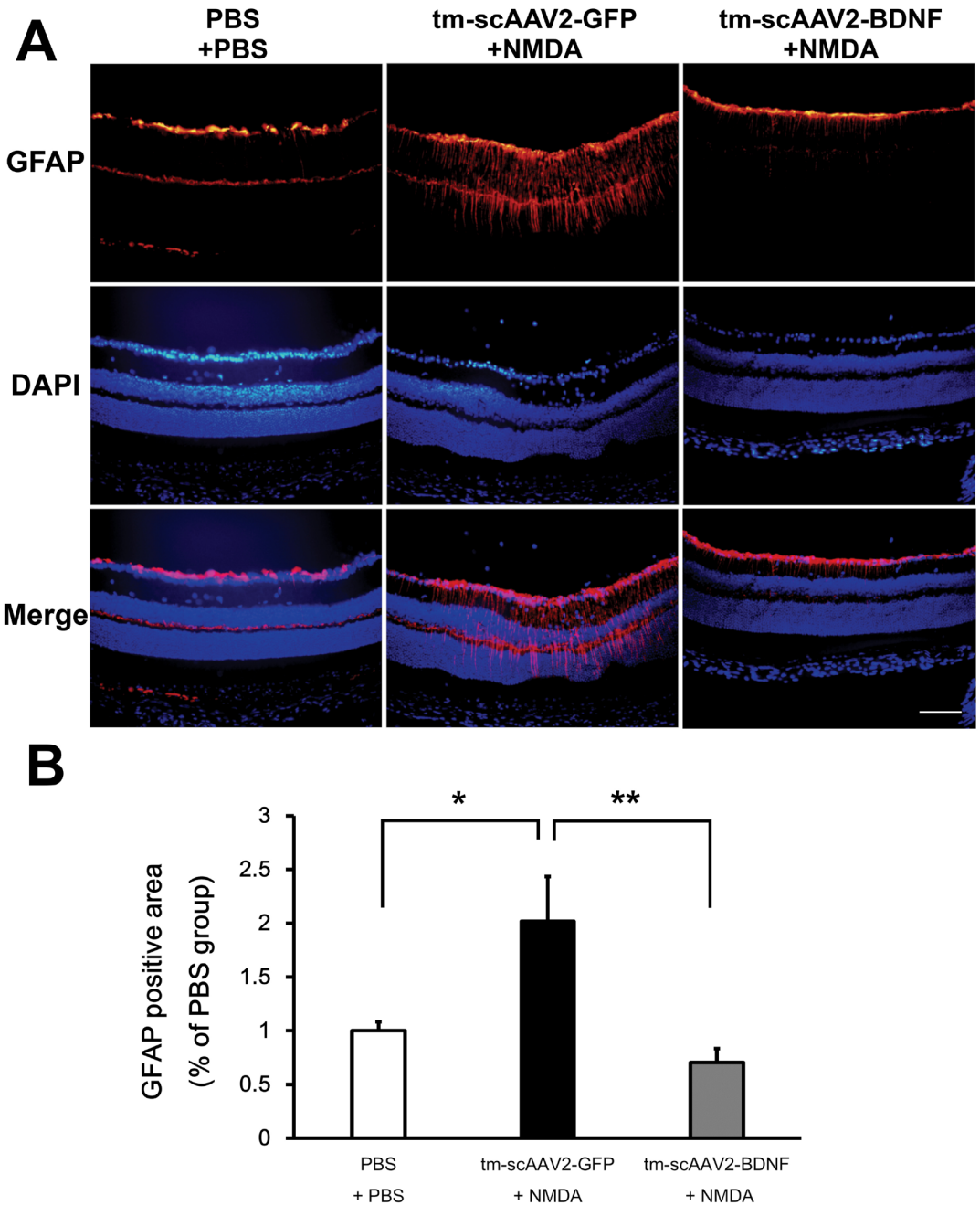

Figure 5. Immunofluorescence analysis of cross sections. **A**: Exemplary images of cross sections obtained from the buffer-treated (left), control vector-NMDA-treated (middle), and therapeutic vector-NMDA-treated (right) groups. Red: GFAP, Blue: DAPI. Images were captured at a distance of 1,000 µm from the ONH. Scale bar represents 100 µm. **B**: Quantification of GFAP-positive area in the three groups (buffer only, n = 4; control vector-NMDA, n = 3; therapeutic vector-NMDA, n = 3, *p<0.05 and **p<0.01 in SNK test).

referred to as glutamate/aspartate transporter, was remarkably reduced in glaucomatous retinas [45]. Thus, the elevation in glutamate levels in the retina may be associated with the glaucoma pathogenesis.

Glutamate is one of the major excitatory neurotransmitters found in retinas, and it activates glutamate receptors, including NMDA receptors [46]. NMDA receptors are ionotropic glutamate receptors and permit the influx of cations, such as calcium ions [46].

Excessive NMDA administration induces the excitotoxicity of retinal cells [47,48] via a calcium influx [49]. Subsequently, this leads to the activation of the apoptosis cascade retinal neurons, especially the RGCs [50]. Moreover, the pathologic increase of the calcium influx induces several harmful effects on neural cells, such as endoplasmic-reticulum (ER) stress [51], oxidative stress [52], and mitochondrial dysfunction [53].

The NMDA injection model is a useful inner retinal injury model that is based on the "glutamate hypothesis" of glaucoma. However, it should be clarified that the retinal injury induced by NMDA is highly acute, while the progression of glaucoma occurs over a much longer period. Thus, this model does not perfectly mimic the glaucoma pathogenesis. Nevertheless, while this model cannot be considered a complete model of glaucoma, it can be modified simply and it is an easily controllable inner retinal injury model based on the "glutamate hypothesis." In line with the findings of the Nakano et al.'s study [54], our current study was designed to use the fewest number of NMDA moles to cause retinal degeneration and provide the expected pathology level.

BDNF is a neurotrophic factor that contributes to the maintenance and survival of the nervous system. Other previous studies have reported that BDNF is important to the integrity of the central nervous system, and decreases in this protein can lead to several neurodegenerative diseases [55,56]. The relationship between BDNF and glaucoma has also been examined in the field of ophthalmology. Several researchers have reported finding decreases in serum BDNF levels in patients with early or moderate primary open-angle glaucoma [57-59]. Uzel et al. reported finding a strongly positive correlation between serum and aqueous BDNF concentrations [60]. Thus, it is possible that BDNF levels in RGCs might also be low in glaucoma patients.

In neural cells, BDNF prevents ER stress, oxidative stress, and mitochondrial dysfunction [61,62]. Binding of BDNF induces phosphorylation of tropomyosin-related kinase B (TrkB), while phosphorylated TrkB activates survival cascades, such as the mitogen-activated protein kinase and phosphoinositide 3-kinase/Akt cascade [63]. Therefore, it is assumed that transduced BDNF offers neuroprotective effects against the programmed cell deaths of RGCs induced by NMDA.

Although a previous study reported that a single administration of BDNF led to a neuroprotective effect against NMDA-induced toxicity, this effect proved limited [23]. On the other hand, Feng et al. showed that their Thy-1-CreERT(T^2) $BDNF^{stop}$ mice were resistant to chronic IOP elevations because of the sustained expression of BDNF in their retinas [64]. Therefore, these findings suggest that a continuous and stable expression of neurotropic factors could be beneficial for preventing retinal degeneration, including chronic moderate damage.

When using gene therapy with neurotropic factors to treat glaucoma, several challenges must be overcome. One of the important issues is that retinal must be protected before the RGCs completely die out. However, during the early stages, glaucoma is often asymptomatic; thus, RGC loss has already progressed by the time the visual field defect appears [14]. Recently, Cordeiro et al. invented a novel method for diagnosing RGC loss during the early stages of glaucoma by using annexin to detect apoptotic retinal cell death [17,19]. Furthermore, when novel diagnoses are combined with the use of gene therapy with neurotropic factors, this can lead to the development of more powerful tools for treating glaucoma.

The other factor that must be considered is how to preserve the expressions of the protein levels to maintain effective concentrations. While AAV2 is the best studied and most used vector in clinical trials, its transduction efficacy is limited [33]. Although recombinant AAV2 vectors that encode BDNF have been used to protect RGCs from inner retinal injuries, previous use resulted in only a mild effect [65,66]. It is presumed that the vectors failed to provide immediate and strong BDNF expression levels. To overcome this problem, several AAV mutants have been developed. McCarty et al. created a self-complementary AAV to bypass the genome replicating step from single-stranded DNA to double-stranded DNA in the host cell [32]. Moreover, AAV capsid modifications have been studied extensively. Petrs-Silva et al. reported that a site-directed mutation of tyrosine to phenylalanine (Y-F) modified the rAAVs to avoid ubiquitination, thereby providing an efficient transgene expression in the retinal cells [34].

Based on these mutant AAVs, we previously reported that our recombinant AAV encoding BDNF, tm-scAAV2-BDNF, successfully protected the retina in a transient IOP elevation rat model [35]. Although retinal ischemia and

reperfusion cause injury of the inner retinas and activation of glial fibrillary acidic protein positive cells, our treatment virus was able to prevent these retinal damages. In the current study, tm-scAAV2-BDNF also exhibited beneficial effects when using a different retinal injury model, the NMDA administration model. Therefore, the present findings suggest that this treatment virus might potentially be effective for use in other retinal disease models and, most importantly, in clinical situations.

APPENDIX 1. CELL LINE AUTHENTICATION

To access the data, click or select the words "Appendix 1."

APPENDIX 2. STATISTICAL ANALYSIS OF INNER RETINAL THICKNESS AMONG THE CONTROL AND THERAPEUTIC VECTOR TREATED GROUPS

To access the data, click or select the words "Appendix 2."

APPENDIX 3. COMPARISON OF B-WAVE AMPLITUDES AMONG THE CONTROL AND THERAPEUTIC VECTOR-TREATED GROUPS

To access the data, click or select the words "Appendix 3."

ACKNOWLEDGMENTS

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