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Evaluation of intravitreal AAV-mediated MANF gene delivery for retinal ganglion cell protection in a mouse retinal ischemia-reperfusion pre-clinical model

Zhenya Gao
University of Xuchang
Yanming Huang
Xiamen eye center,Xiamen University
Fang Yu
University of Xuchang
Yuqing Wang
Xuchang Central Hospital
Hongge Hu
honggehu@126.com

Xuchang Central Hospital

Research Article

Keywords: MANF, Retinal ischemia-reperfusion, Retinal ganglion cell, Adeno-associated virus gene therapy

Posted Date: November 24th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11478864/v1

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Additional Declarations: No competing interests reported.

Abstract

Purpose

To investigate whether intravitreal AAV-7-mediated MANF gene delivery produces neuroprotective effects on retinal ganglion cells (RGCs) under retinal ischemia-reperfusion (RIR) injury in mice, and to compare the efficacy between sustained MANF gene overexpression and single-dose MANF protein injection.

Methods

This is a pre-clinical randomized laboratory animal experiment. Three-month-old male C57BL/6J mice were randomly assigned to multiple intervention and control groups (n = 5 per group). Intravitreal injections of AAV-MANF, AAV-GFP, MANF protein, PBS or dry-needle sham puncture were performed before establishing intraocular-pressure-induced RIR injury. At day 7 after reperfusion, retinal flat-mount immunohistochemistry for NeuN was used to quantify relative RGC survival rate; western blot was applied to detect retinal MANF protein abundance. One-way ANOVA was used for inter-group statistical comparison. Animal experiments complied with ARVO statement and ARRIVE 2.0 reporting guidelines. Pre-defined inclusion/exclusion criteria for experimental animals were applied.

Results

Seven days after RIR insult, the RGC survival rate was 25 ± 1% in untreated ischemic retinas and 21 ± 3% in AAV-GFP control group. AAV-MANF intervention achieved a RGC survival rate of 78 ± 8%, which was statistically higher than both control groups ($P<0.001$). Retinal MANF protein expression was markedly elevated in AAV-MANF-injected eyes. Single intravitreal bolus MANF protein injection offered limited protective effect against RGC loss.

Conclusions

Our findings demonstrate that intravitreal AAV-mediated MANF overexpression robustly mitigates ischemia-induced RGC degeneration and preserves inner-retinal and RGC-dependent visual pathway function. Accordingly, MANF could be considered as a clinical therapeutic neuroprotective agent for degenerative retinal and ischemic ocular disorders.

What’s known and what’s added

What’s known

Retinal ischemia-reperfusion (I/R) injury triggers progressive retinal ganglion cell (RGC) loss and contributes to irreversible visual impairment. MANF is a neuroprotective factor that alleviates endoplasmic reticulum stress and exerts protective effects on neurons. Intravitreal AAV delivery represents a promising strategy for sustained gene therapy in retinal diseases.

What's added

This study demonstrates that intravitreal AAV-mediated MANF delivery preserves RGCs in a mouse retinal I/R model. Our findings provide pre-clinical evidence supporting the therapeutic potential of MANF gene therapy for RGC protection following ischemic retinal injury.

Introduction

Mesencephalic astrocyte-derived neurotrophic factor (MANF), also termed arginine-rich mutated in early-stage tumors (ARMET), is an evolutionarily conserved protein with potent neurotrophic functions. MANF is endogenously expressed in a wide range of neuronal and non-neuronal tissues throughout the body[1–3]. In the central nervous system, MANF exhibits abundant expression in the cerebral cortex, hippocampus, and cerebellar Purkinje cells[1, 4, 5]. Accumulating studies have demonstrated that MANF expression is markedly upregulated in response to ischemic insults and excessive endoplasmic reticulum (ER) stress[1, 6–11]. Mounting functional evidence reveals that MANF effectively mitigates ER stress, exerts prominent neurotrophic activities, and facilitates the survival and functional recovery of midbrain dopaminergic neurons[3, 12–15].

Retinal ischemia-reperfusion (RIR) is a common pathological condition among multiple sight-threatening ocular disorders, including glaucoma, high myopia, retinal artery or vessel occlusion, diabetic retinopathy, retinal detachment, retinopathy of prematurity syndrome, anterior ischemic optic neuropathy and traumatic optic neuropathy[16–19]. Because of limited clinical management options, RIR frequently results in irreversible vision loss and blindness. RIR causes progressive retinal ganglion cell (RGC) apoptosis, which is characteristic of many retinal neurons degenerative conditions. RGC death following RIR proceeds mainly via apoptosis[20, 21], with involving other contributions from necrosis, necroptosis, and autophagy.Since injured RGCs cannot regenerate, RGC loss after ischemia is irreversible. Hence, protecting RGCs from RIR-induced damage is thus critical to preserve vision,which has driven extensive investigations into RIR pathogenic mechanisms and corresponding neuroprotective tactics[22–25].

We previously demonstrated that recombinant human MANF preserves photoreceptors in rat retinal degeneration models. In this study, we constructed an Adeno-associated viral (AAV) vector encoding human MANF, verified its expression in vivo, and explored the neuroprotective potency of intravitreally delivered AAV-MANF against RIR-triggered RGC injury in mice.

Materials and Methods

Construction, Packaging, Purification, and Titering of AAV Vectors

The eGFP fragment was cut from dsAAV-GFP[26] with KpnI/XbaI. Human MANF cDNA from PCR3.1-MANF[1] was inserted into linearized dsAAV backbone to construct pdsAAV-MANF, verified by sequencing and Western blot in HEK293 cells. AAV7 was packaged by triple-plasmid calcium chloride transfection[27, 28]. Harvested cells underwent freeze–thaw lysis; viruses were purified via CsCl gradient centrifugation, dialyzed, quantified by CMV qPCR, aliquoted and stored at − 80°C.

Animals

All animal experiments followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of Xuchang University. All animal manipulations complied with the ARRIVE 2.0 guidelines and 3-R principles to minimise animal suffering and animal quantity. Three-month-old male C57BL/6J mice were housed under specific-pathogen-free (SPF) conditions, with stable temperature-humidity and a 12-hour light-dark cycle, ad libitum access to food and water. Mice underwent one-week environmental acclimation prior to any intervention. The experimental unit was defined as an individual mouse. Mice were randomly allocated into experimental groups. Pre-specified exclusion criteria included intraoperative retinal haemorrhage, lens opacity/cataract, or death under anaesthesia. No a-priori sample-size calculation was performed; group sample size (n = 5) was referenced from previously published retinal neuroprotection pre-clinical studies. Investigators conducting retinal ganglion cell counting were blinded to experimental group assignments. Humane endpoints were predefined: animals with obvious weight loss>20%, severe ocular inflammation or behavioural abnormality would be humanely euthanised ahead of planned time-point. No animals met humane endpoint criteria in this study.

Intravitreal injections

Mice were randomized into three viral intervention groups (n = 5 per group): IR only, IR + AAV-GFP, and IR + AAV-MANF. Three microliters of AAV-GFP or AAV-MANF (2×10^{13} vg/mL) was intravitreally injected into the left eye three weeks before IR injury, with the syringe held for 15 s to facilitate diffusion. For protein intervention, additional mice were divided into four groups (n = 5 each): IR only, dry needle, PBS, and MANF treatment. Two microliters of MANF protein or PBS was injected 24 h pre-modeling, while the dry needle group received only puncture.

Transient retinal ischemia

Mice were anesthetized with isoflurane via a fitted nose cone for 45 min under spontaneous breathing, with body temperature stably maintained at 37 ± 0.5°C using a heating pad. Prior to modeling, pupil

dilation was performed with a mixed solution of 1% tropicamide and 2.5% phenylephrine hydrochloride(NutraMax Products Inc, Gloucester, MA, USA), and corneal surface anesthesia was achieved using 0.5% proparacaine hydrochloride(Bausch and Lomb Pharmaceuticals, Tampa, FL, USA).

RIR injury was induced in the left eye by raising IOP to 120 mmHg for 45 min via an anterior-chamber 33-gauge needle connected to a saline reservoir; the cannulated right eye served as internal control. Ischemia was confirmed microscopically. Erythromycin ophthalmic ointment was applied postoperatively. Mice were euthanized by CO_2 inhalation one week later for immunohistochemistry.

Immunohistochemistry

After euthanasia, mouse eyes were enucleated and immersion-fixed in 4% paraformaldehyde for 1 hour at room temperature. Cornea, iris, lens, ciliary body and vitreous were dissected out. Posterior eye cups were cryoprotected overnight in 30% sucrose. Isolated retinas underwent three freeze–thaw cycles and were rinsed with 0.1 M PBS. Tissues were blocked in 1% BSA with 0.1% Triton X-100 for 1 h, then incubated overnight with FITC-conjugated NeuN antibody (1:100, Millipore, USA). Following PBS washes, retinas were flat-mounted. Images were acquired using a Leica SP5 confocal microscope.

Counting of ganglion cell layer neurons

Confocal microscopy was used to visualize NeuN-positive neurons in the retinal ganglion cell layer. Z-stack images with five optical sections were captured and reconstructed into maximum-intensity projections via Leica LAS AF software to ensure focal consistency. Using a 20× objective, twenty random images per retina were acquired from four quadrants, covering central, middle, and peripheral regions. NeuN-positive neurons were quantified semi-automatically using ImageJ. Neuronal density in ischemic retinas was normalized to paired contralateral control eyes, and neuronal survival was presented as relative percentages.

Electrophysiological assessment of retinal function

To evaluate retinal inner-layer function, scotopic flash electroretinogram (F-ERG) was recorded at day 7 after ischemia-reperfusion injury. All mice underwent overnight dark-adaptation (> 12 h) under dim red light before electrophysiological testing. Mice were anesthetized with isoflurane and body temperature was maintained at 37 ± 0.5°C by a heating pad. Pupils were fully dilated using mixed eye drops of 1% tropicamide and 2.5% phenylephrine hydrochloride. Corneal surface anesthesia was performed with 0.5% proparacaine hydrochloride, and ophthalmic lubricant gel was applied to prevent corneal desiccation.

For scotopic F-ERG recording, gold-wire ring corneal electrodes were placed on the corneal surface; a subcutaneous reference needle electrode was inserted between bilateral auricles, and a ground electrode was placed subcutaneously at the tail base. Flash stimuli (1.0 cd·s/m²) were delivered, and

a-wave (photoreceptor-derived) and b-wave (inner retinal/bipolar-cell-derived) amplitudes as well as implicit times were recorded. Amplitude ratios of ischemic left eyes relative to contralateral normotensive control eyes were calculated for statistical analysis.

Western blot analysis

Three weeks after intravitreal AAV injection, retinal tissues from three groups were collected, frozen, and homogenized in pre-chilled lysis buffer. After centrifugation at 13,000 rpm for 20 min at 4°C, supernatant proteins were extracted. Equal 50 μg protein samples were separated by 4–12% Bis-Tris gel electrophoresis and transferred to PVDF membranes. The membranes were blocked with 5% nonfat milk diluted in TBST for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies, including a mouse monoclonal MANF antibody (1:1000; Cell Signaling Technology, USA) and a mouse monoclonal β-actin antibody (1:3000; Santa Cruz Biotechnology, USA). After three 10-minute washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:1000; Santa Cruz Biotechnology, USA) for 1 hour at room temperature. Protein signals were detected using an ECL chemiluminescence reagent (Millipore, USA) with a corresponding imaging system. All Western blot experiments were repeated at least three times, and protein expression levels were normalized to β-actin for quantitative analysis.

Statistical analysis

All statistical analyses were conducted using SPSS 18.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was applied for intergroup comparisons, and a P value ≤ 0.05 was considered statistically significant.

Results

Characterization of AAV-MANF in vitro and MANF expression in the retina.

We successfully constructed a dsAAV packaging plasmid carrying the human MANF cDNA. Western blot analysis confirmed that MANF protein expression was markedly upregulated in HEK293 cells at 24 hours after transfection with the AAV-MANF plasmid (Fig. 1A, B). To determine the location of the increased levels of MANF induced by AAV-MANF, mice were injected AAV-MANF and PBS as control. The eyeballs were harvested at 6 hours after intravitreal injection, and frozen sections of the posterior eyecup were maked for immunocytochemical staining technique using an anti-MANF antibody. The location and morphology of the retinal cells treated with AAV-MANF suggested that increased expression of MANF was detected in Müller cells, including the cell bodies, fibers, and endfeet, as well as in the ganglion cells (Fig. 2A, B).To verify in vivo MANF overexpression, retinal tissues were harvested for protein detection at three weeks after intravitreal injection of AAV-MANF or AAV-GFP. Western blot results demonstrated that retinal MANF protein levels were significantly elevated in the AAV-MANF group. Quantitative analysis further revealed that MANF expression in AAV-MANF-injected retinas was approximately three-fold higher than that in the AAV-GFP group ($p < 0.001$, Student's *t*-test). Consistently, retinal MANF protein

levels was significantly increased in AAV-MANF-treated eyes compared with all control groups ($p < 0.001$, Student's *t*-test)(Fig. 2C, D).

Intravitreal injection MANF promotes survival of retinal neurons following RIR injury

The high IOP-induced transient RIR model is a widely established experimental paradigm for exploring the molecular mechanisms underlying neuronal ischemic injury [29]. This model achieves retinal ischemia by blocking both retinal and uveal blood circulation, thereby triggering pathological changes in the retinal ganglion cell layer (GCL) that closely mimic the characteristics of human central retinal artery occlusion [16]. To quantify surviving retinal neurons, whole retinal flat-mounts were immunolabeled with the neuronal marker NeuN. Consistent with typical IR injury responses, obvious neuronal loss in the GCL was detected in all RIR-injured eyes. Previous studies have demonstrated that IR-induced neuronal degeneration occurs in a biphasic manner, including an acute degenerative phase within 24 hours after reperfusion and a secondary progressive degenerative phase over subsequent days [29]. The NeuN immunohistochemistry further revealed that IR-induced neuronal damage was uniformly distributed throughout the ischemic retina across all experimental groups, with no regional or zonal patterns of degeneration observed.

A total of 20 mice were used to evaluate RGC survival at one week following RIR injury. Specifically, five mice received intravitreal dry needle puncture without any reagent injection, five mice underwent intravitreal PBS injection, and five mice were treated with intravitreal MANF protein injection. Compared with untreated control eyes, all IR-injured eyes exhibited evident neuronal degeneration in the GCL.

Quantitative analysis of RGC survival revealed distinct differences across experimental groups (Fig. 3A, B). The dry needle group exhibited a RGC survival rate of 44 ± 9%, which was significantly higher than the rates of the MANF group (32 ± 8%, $p < 0.05$), the PBS group (27 ± 8%, $p < 0.01$), and the untreated control group (18 ± 2%, $p < 0.01$). No significant difference in RGC survival was observed between the MANF and PBS groups ($p > 0.05$), and the survival rates were also comparable between the PBS and control groups ($p > 0.05$). Collectively, these results indicated that intravitreal MANF injection exerted no superior protective effect against RGC loss induced by RIR injury compared with PBS treatment at seven days post-operation. Nevertheless, the MANF group retained significantly more surviving RGCs than the control group ($p < 0.05$). Notably, the dry needle group unexpectedly presented the highest RGC survival level among all four experimental groups.

AAV-MANF significantly protects against RGC loss after ischemia- reperfusion injury

Seven days after IR injury, quantitative assessment of RGC survival showed that the untreated ischemic control retinas retained a RGC survival rate of 25 ± 1%, corresponding to approximately 75% neuronal

loss (Fig. 4A, B). The AAV-GFP control group showed a comparable RGC survival rate of 21 ± 3%, with no significant difference relative to the untreated control group. In contrast, the AAV-MANF-treated group exhibited a markedly elevated RGC survival rate of 78 ± 8%, which was significantly higher than both control groups ($p < 0.001$ for all comparisons). Furthermore, AAV-MANF administration yielded a higher RGC survival level than direct intravitreal MANF injection. This superior protective effect can be attributed to the sustained retinal MANF expression mediated by AAV-MANF transduction, as confirmed by Western blot analysis showing stable high MANF protein levels in AAV-MANF-treated retinas. Additionally, IR injury induced endogenous MANF redistribution in AAV-MANF-transduced retinas, which was consistent with injury-induced MANF protein secretion. Taken together, these results demonstrate that AAV-mediated sustained MANF delivery effectively alleviates retinal neuronal damage and improves functional outcomes following RIR injury in mice.

AAV-MANF preserves retinal inner-layer function following RIR injury

To determine whether the structural RGC protection afforded by AAV-MANF translated into preserved visual function, we performed scotopic F-ERG recordings 7 days post-IR injury (Fig. 5).

For scotopic ERG analysis: The a-wave amplitude ratio (ischemic eye / contralateral control eye) showed mild reduction in all IR-injured groups, with no significant inter-group difference (IR: 0.74 ± 0.06; IR + AAV-GFP:0.71 ± 0.05; IR + AAV-MANF:0.77 ± 0.07; P > 0.05). In contrast, the b-wave amplitude ratio was markedly depressed in IR only group (0.27 ± 0.04) and IR + AAV-GFP group (0.24 ± 0.05), indicating severe inner-retinal dysfunction after ischemia-reperfusion. The b-wave amplitude ratio was significantly preserved in IR + AAV-MANF group (0.68 ± 0.06), compared with both IR and IR + AAV-GFP control groups (P < 0.001 for both comparisons). No obvious differences in a-wave and b-wave implicit times were observed among three groups (P > 0.05).

Discussion

RIR injury triggers complex and multifaceted pathophysiological cascades, including oxygen and glucose deprivation[30], excessive glutamate release and subsequent excitotoxicity[31], reactive oxygen species formation[32, 33], protein and lipid modifications[34], intracellular calcium overload[35, 36], mitochondrial dysfunction[37, 38], ER-stress and neuronal apoptosis[22–24]. These pathological cascades drive progressive neurodegeneration in the ischemic retina, disrupt retinal neuronal integrity, and ultimately trigger irreversible visual impairment. Effective therapeutic interventions for RIR injury should target two core pathological stages. The primary and prioritized strategy is to mitigate acute neuronal damage in the early pathological phase by suppressing cell death-related mechanisms, including apoptosis, necroptosis, mitochondrial dysfunction, excitotoxicity, ER stress, inflammatory responses, and oxidative stress. The secondary strategy is to facilitate the functional repair and recovery of damaged neurons, which relies on multiple endogenous compensatory mechanisms, such as neural precursor proliferation

and migration, axonal guidance, neuritic outgrowth, and synaptogenesis. Accumulating evidence has validated that neurotrophic factors possess dual therapeutic potential to target both acute neuronal injury and subsequent neural repair processes in RIR models. Importantly, our present study combined histological structural evaluation with visual electrophysiological measurements though F-ERG. IR injury caused prominent reduction of ERG b-wave amplitude, reflecting inner-retinal dysfunction and impaired RGC output to visual cortex. AAV-MANF treatment not only increased surviving RGC counts, but also largely preserved inner-retinal and RGC-mediated visual pathway function. These functional observations reinforce our structural findings and demonstrate that MANF-mediated neuroprotection is not merely cell-number preservation; surviving RGCs retain physiological signal-transmission capacity after ischemic insult. Such functional data are critical for pre-clinical evaluation of retinal neuroprotective gene therapy, as histological cell survival alone cannot guarantee maintenance of visual function.

MANF was initially identified as a trophic factor supporting the survival of cultured embryonic dopaminergic neuron[39]. Both MANF and its homologous protein, cerebral dopamine neurotrophic factor (CDNF), have been well documented to promote the survival and functional recovery of midbrain dopamine neurons[15, 39]. In vivo studies have confirmed that intracortical delivery of recombinant MANF protein exerts protective effects against cerebral ischemic damage[41]. Notably, MANF exhibits superior and more selective neuroprotection toward dopaminergic neurons compared with glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) at low (0.05–0.25 ng/mL) and moderate (0.5–2.5 ng/mL) concentrations, with a protective potency ranked as MANF > GDNF > BDNF[39]. Our previous study also demonstrated that recombinant human MANF effectively protects photoreceptors in a rat model of retinal degeneration[40]. Many studies report elevated MANF expression in ischemic brain and heart tissue with activated ER stress[1, 5]. MANF knockdown aggravates IR-related cell death, while recombinant MANF protects cardiomyocytes[11]. Ischemia-induced ER stress promotes MANF secretion, which relieves ER stress damage via autocrine/paracrine signaling to limit RGC loss. Beyond ER homeostasis regulation, MANF acts as a neurotrophic factor. It binds unidentified membrane receptors and activates downstream neuroprotective pathways independent of ER stress modulation.

Here, we first report the in vivo neuroprotective effect of AAV-mediated human MANF delivery in a mouse RIR model. Serotype 7 AAV achieves widespread transduction in the ischemic retina. Sustained MANF overexpression by AAV-MANF effectively attenuates RGC loss and retinal injury. Intravitreal AAV-MANF administration elevates retinal MANF protein levels. This gene delivery strategy provides a promising therapeutic approach for RIR-related eye diseases.

Existing mechanistic studies have illustrated that MANF functions as a key regulator of ER stress and the unfolded protein response (UPR) signaling pathway[14, 41, 42]. Lindahl et al. reported that endogenous MANF deficiency triggers chronic UPR activation in vivo in mice, and recombinant human MANF treatment significantly promotes pancreatic β-cell proliferation in a dose-dependent manner[14]. As a unique neurotrophic factor with distinct sequences and structural characteristics, MANF exerts biological effects through dual regulatory modes. Notably, MANF has no obvious impacts on intact and

healthy neurons both in vitro and in vivo, whereas it effectively protects damaged dopaminergic neurons and facilitates neuronal repair after injury. Mechanistically, MANF directly binds to GRP78 and indirectly modulates the activity of three canonical UPR receptors, IRE1, PERK, and ATF6, thereby participating in the homeostatic regulation of UPR signaling. The finding that MANF supplementation relieves ER stress and rescues the chronic overactivation of all three UPR pathways in MANF-deficient mice strongly supports this regulatory mechanism. Moreover, MANF is implicated in protein kinase C signaling[43] and confers anti-apoptotic protection on cardiomyocytes and cultured astrocytes[11] [21]. Nevertheless, the precise molecular mechanism linking MANF-mediated ER stress alleviation to apoptotic pathway inhibition remains unclear and warrants further investigation.

AAV vectors are widely used in clinical trials for neurodegenerative diseases owing to favorable biosafety and transduction efficiency over lentivirus[44]. AAV-mediated neurotrophic factors protect neurons in brain injury models, including AAV7-MANF against stroke[41]. However, such gene delivery has rarely been investigated in RIR injury. Since direct MANF protein injection cannot rescue RGC loss, we generated dsAAV7-MANF for sustained retinal MANF expression. AAV7-MANF achieves broad retinal transduction and stronger neuroprotection than acute protein treatment, as persistent MANF counteracts prolonged ischemic cascades. Further work is needed to assess its long-term protective and regenerative effects. Notably, acute intravitreal MANF protein supplementation could not achieve satisfactory functional improvement, whereas AAV-driven sustained MANF overexpression conferred robust preservation of ERG b-wave responses. This further underscores that persistent MANF expression is required to sustain retinal neuronal function against the progressive pathological cascades triggered by retinal ischemia-reperfusion.

Confocal immunohistochemical imaging revealed uniform neuronal damage across all quadrants of individual ischemic retinal samples. This distribution pattern differs from that observed in cerebral cortical and brain ischemic models, in which neuroprotective effects are typically uneven and restricted to regions proximal to the injection site rather than distant areas. Suboptimal delivery strategies have been partially responsible for the modest therapeutic outcomes of neurotrophic factor-based clinical trials. Compared with conventional systemic or intracranial administration, intravitreal delivery of either recombinant MANF protein or AAV-MANF vectors offers distinct advantages for retinal targeting. This local administration strategy bypasses the blood–retina barrier, while the vitreous humor serves as an ideal medium for stable substance diffusion and efficient retinal tissue penetration. In addition, local intravitreal AAV delivery minimizes systemic off-target side effects. Furthermore, undesirable ubiquitous over-expression of target proteins can be precisely controlled by utilizing cell-specific promoters or synthetic regulatory systems, such as small molecule-inducible promoters, thereby improving the safety and controllability of gene therapy.

Multiple issues must be resolved before translating MANF-based therapy for RIR injury. First, the optimal therapeutic time window needs clarification to compare prophylactic versus post-injury MANF delivery. Second, combination therapies deserve exploration; combining MANF-mediated ER stress inhibition with treatments targeting mitochondrial dysfunction or neuronal necrosis may produce synergistic effects to

improve RGC survival. After addressing these basic questions, the safety, tolerability and efficacy of AAV-MANF should be comprehensively evaluated, laying the foundation for subsequent small-scale clinical trials. A clinical trial is currently re-evaluating the efficacy and safety of AAV2-based viral vectors, including AAV2-neurturin (Cere-120), in Parkinson's disease patients with modified injection sites and elevated viral titers, providing valuable reference data for the clinical application of ocular AAV gene therapy[45].

In conclusion, the present study demonstrates that AAV-mediated sustained MANF expression confers robust neuroprotection against RGC loss following retinal IR injury, highlighting MANF as a promising neuroprotective candidate for the clinical treatment of retinal degenerative and ischemic disorders. However, multiple unresolved issues regarding retinal MANF biology still require further investigation. Future studies are warranted to identify the specific MANF receptor, characterize the precise subcellular and tissue localization of MANF in the retina, and elucidate the complete signaling networks underlying MANF-mediated neuroprotective mechanisms. Long-term electrophysiological monitoring (> 28 days) is also needed to verify whether the functional benefits mediated by AAV-MANF can be stably maintained over extended time-courses. Collectively, these investigations will further advance the translational application of MANF-based ocular therapy.

Declarations

Competing interests:

None declared. All authors report no financial or non-financial competing interests related to this work.

Author Contribution

Conceived and designed the experiments: Zhenya Gao, Hongge Hu, Yanming Huang. Performed the experiments: Zhenya Gao,Yanming Huang, Hongge Hu. Analyzed the data: Zhenya Gao, Yanming Huang. Contributed reagents/materials/analysis tools: Fang Yu, Yuqing Wang. Wrote the paper: Zhenya Gao, Yanming Huang, Fang Yu. Obtained permission for use of animals: Yuqing Wang.

Acknowledgement

The authors thank the staff of the Ron Wen Laboratory(Bascom Palmer Eye Institute, University of Miami,FL, USA) for their technical assistance during the experiment.

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Figures

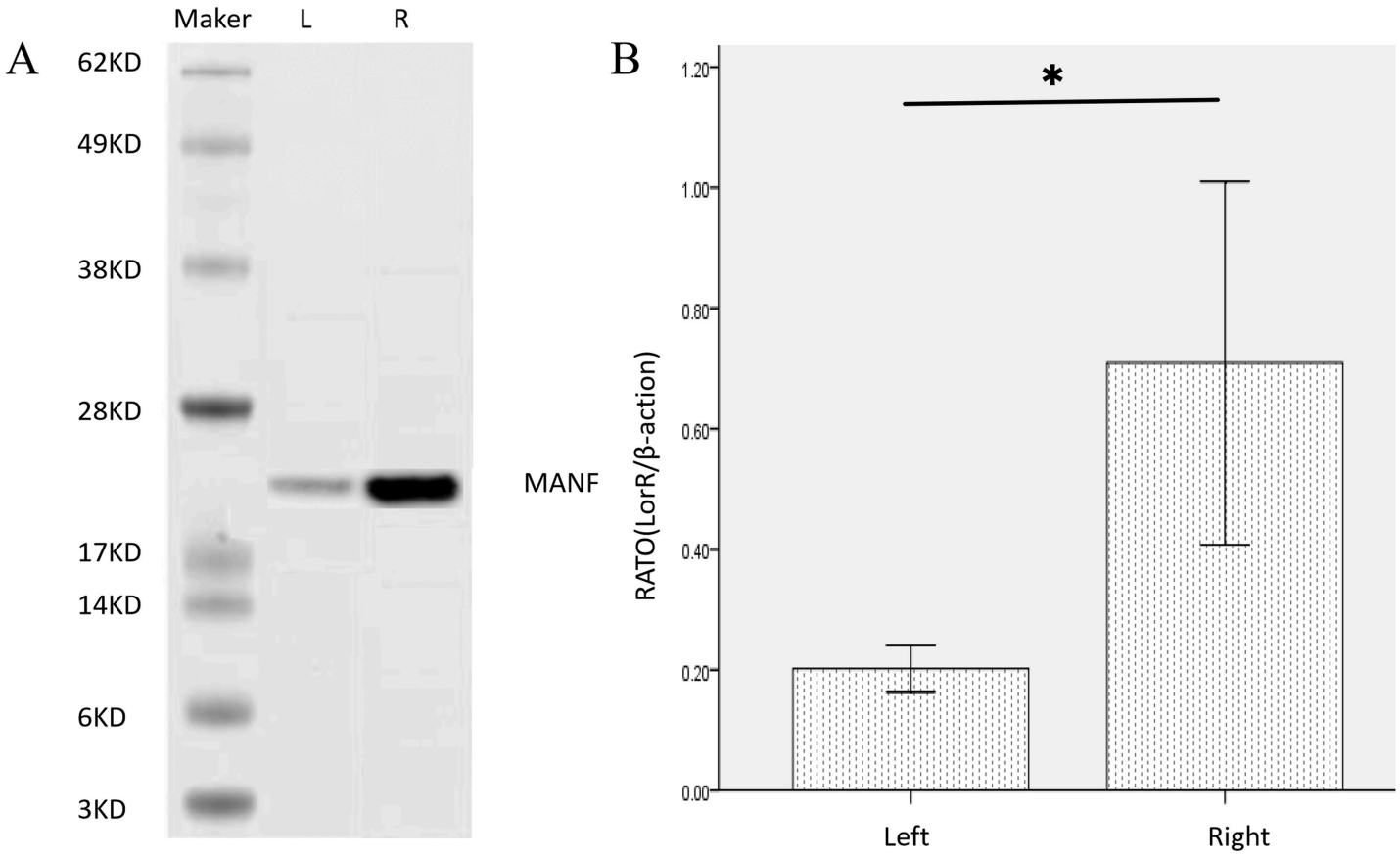

Figure 1

Expression of MANF. AAV-MANF was injected to the right eyes. The left eyes were untreated. The expression of MANF was examined by Western blots. Significant increase in MANF expression was evident in the right retinas.

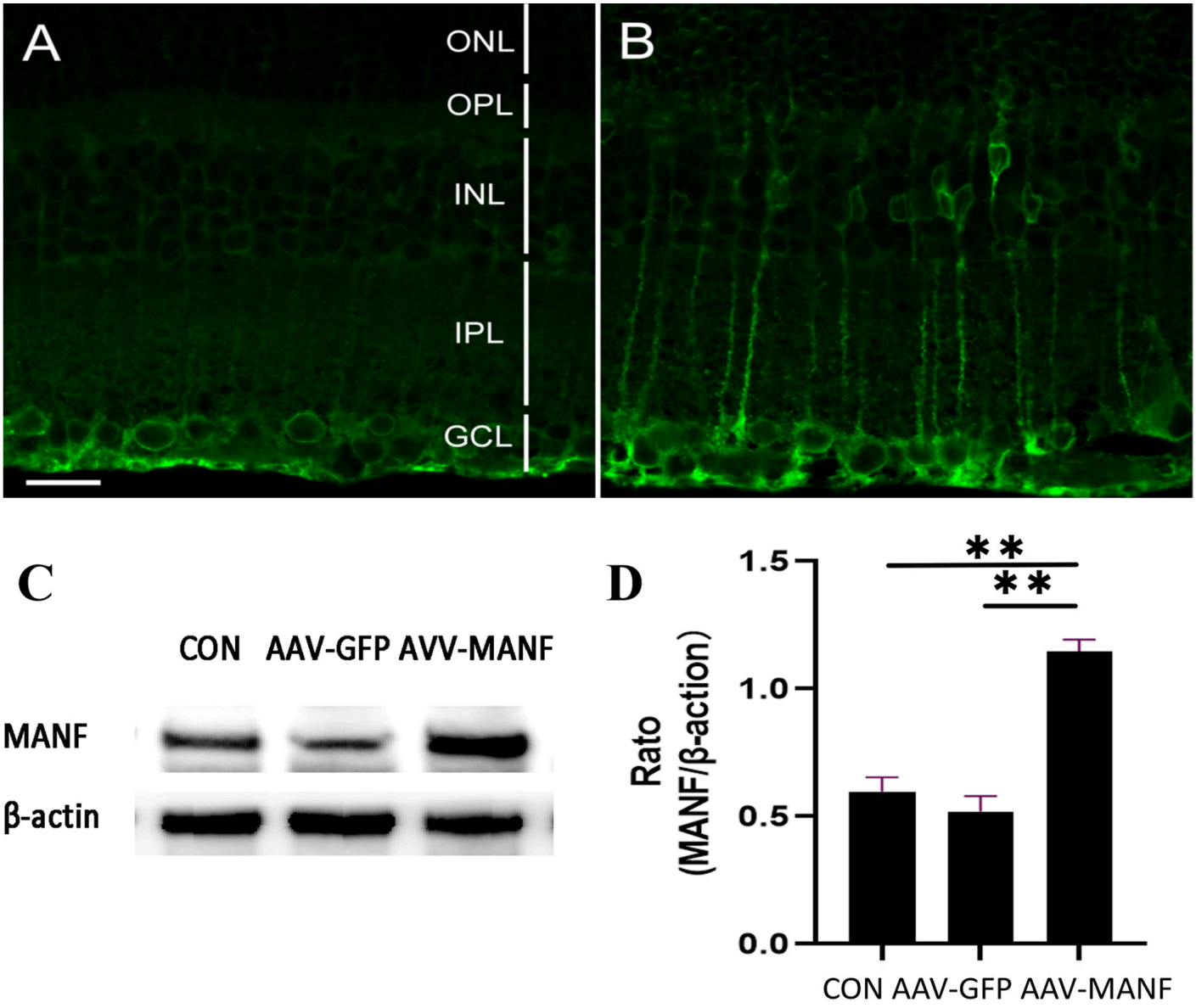

Figure 2

Localization of MANF expression. Low level expression is seen in the untreated retina in ganglion cells and at the inner limiting membrane, likely in the Muller cell endfeet (A). AAV-MANF induced MANF expression is mainly seen in Muller cells, including the cell bodies, fibers, and endfeet, as well as in the ganglion cells (B). Scale bar: 20 μm.

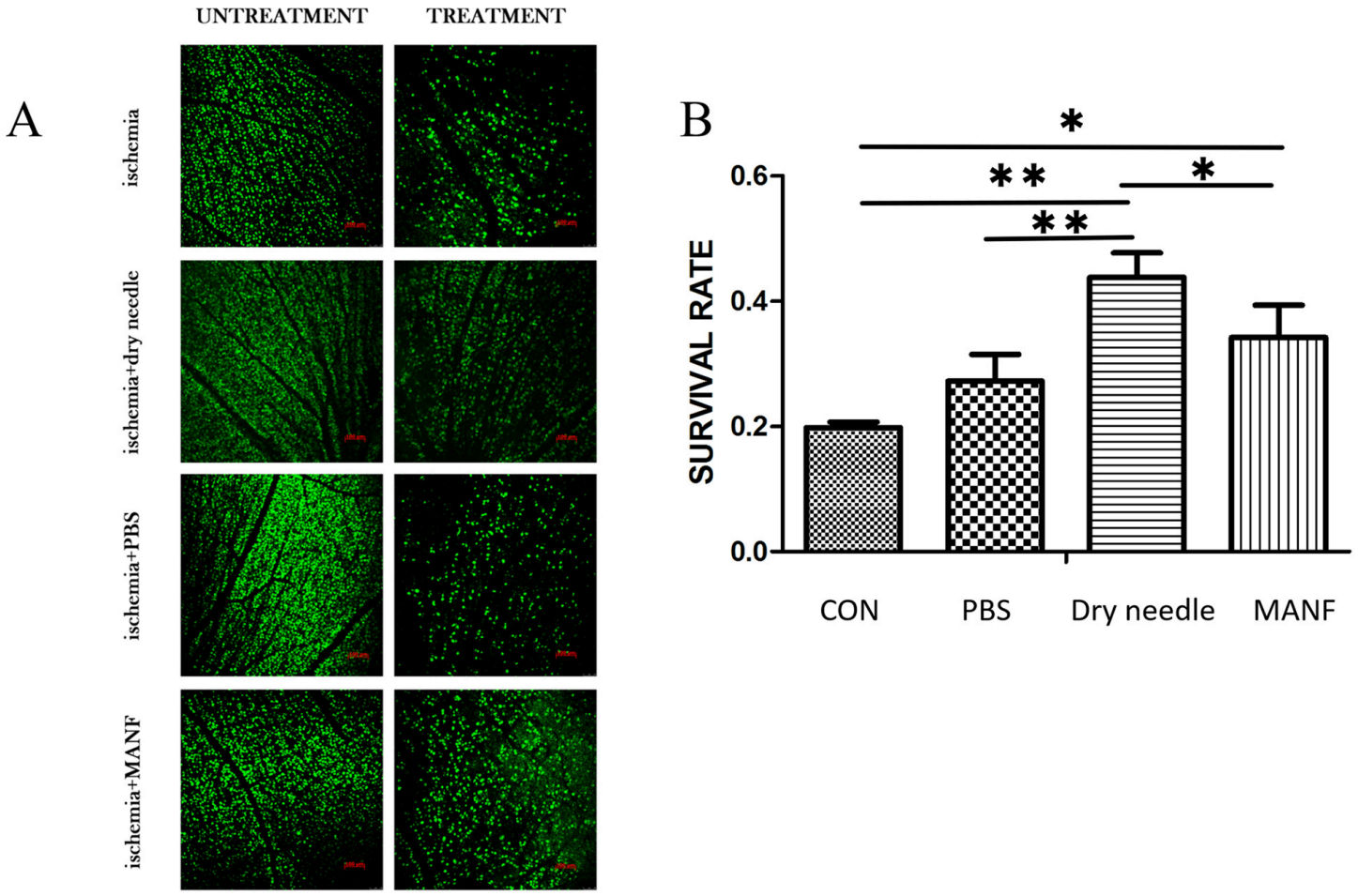

Figure 3

Intravitreal injection MANF results in neuroprotective effects in the GCL of retinas after IR. A: Representative confocal images of NeuN-labeled GCLs (green) in flat-mounted controls and ischemic retinas seven days after reperfusion. Scale bar equal to 100 μm. GCL, ganglion cell layer; IR, ischemia reperfusion; NeuN, neuronal nuclei; PBS, phosphate buffered saline.MANF, mesencephalic astrocyte-derived neurotrophic factor. **B**: The percent surviving of GCL neurons between 4 groups (IR, IR+Dry needle, IR+PBS, and IR+ MANF) in the IR retinas of C57BL/6J miceseven days after IR. (**p<0.01,*p<0.05,N=5).

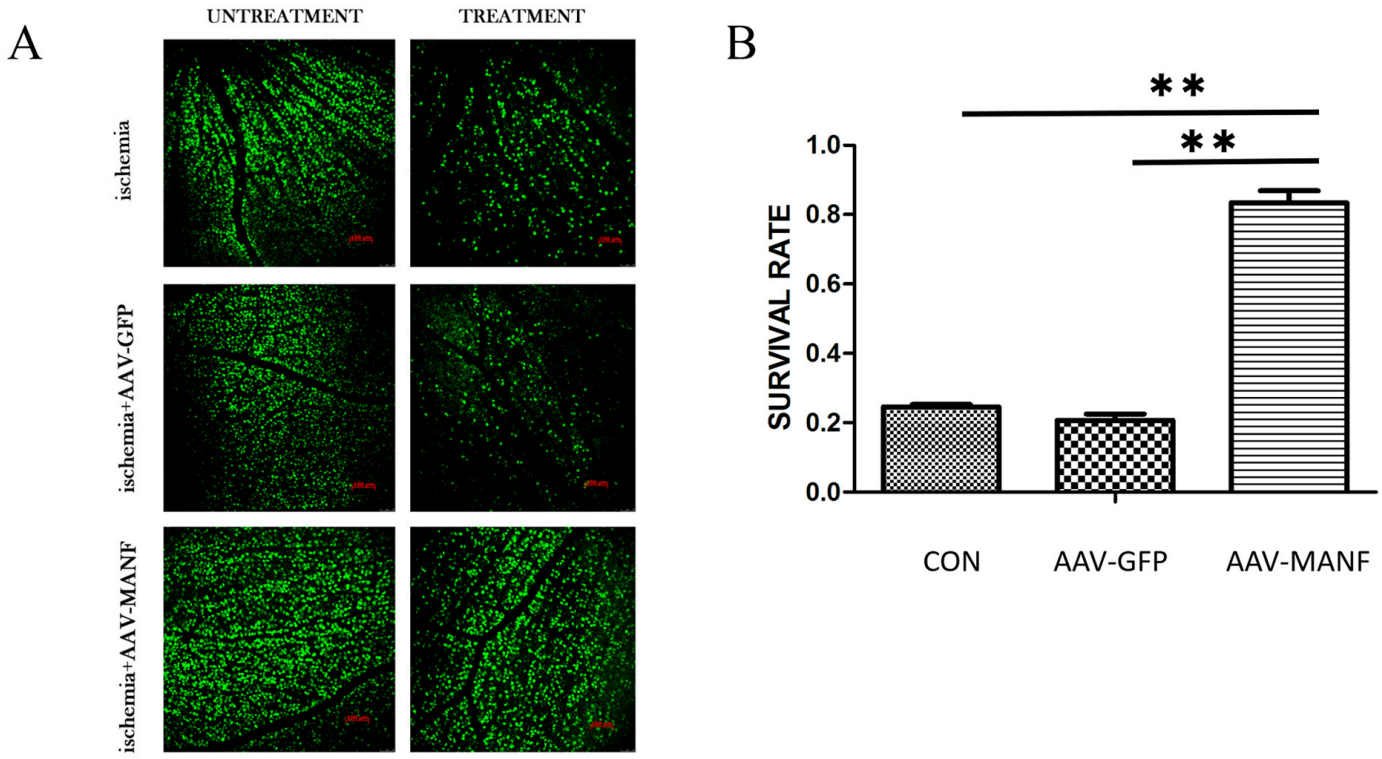

Figure 4

Intravitreal injection AAV-MANF results in neuroprotective effects in the GCL of retinas after IR.A: Representative confocal images of NeuN-labeled GCLs (green) in flat-mounted controls and ischemic retinas seven days after reperfusion. Scale bar equal to 100 μm. GCL, ganglion cell layer; IR, ischemia reperfusion; NeuN, neuronal nuclei; AAV-MANF, Adeno-associated viral MANF. **B**: The percent surviving of GCL neurons between 3 groups (IR, IR+AAV-GFP, and IR+AAV-MANF) in the IR retinas of C57BL/6J mice seven days after IR. (**p<0.01,N=5).

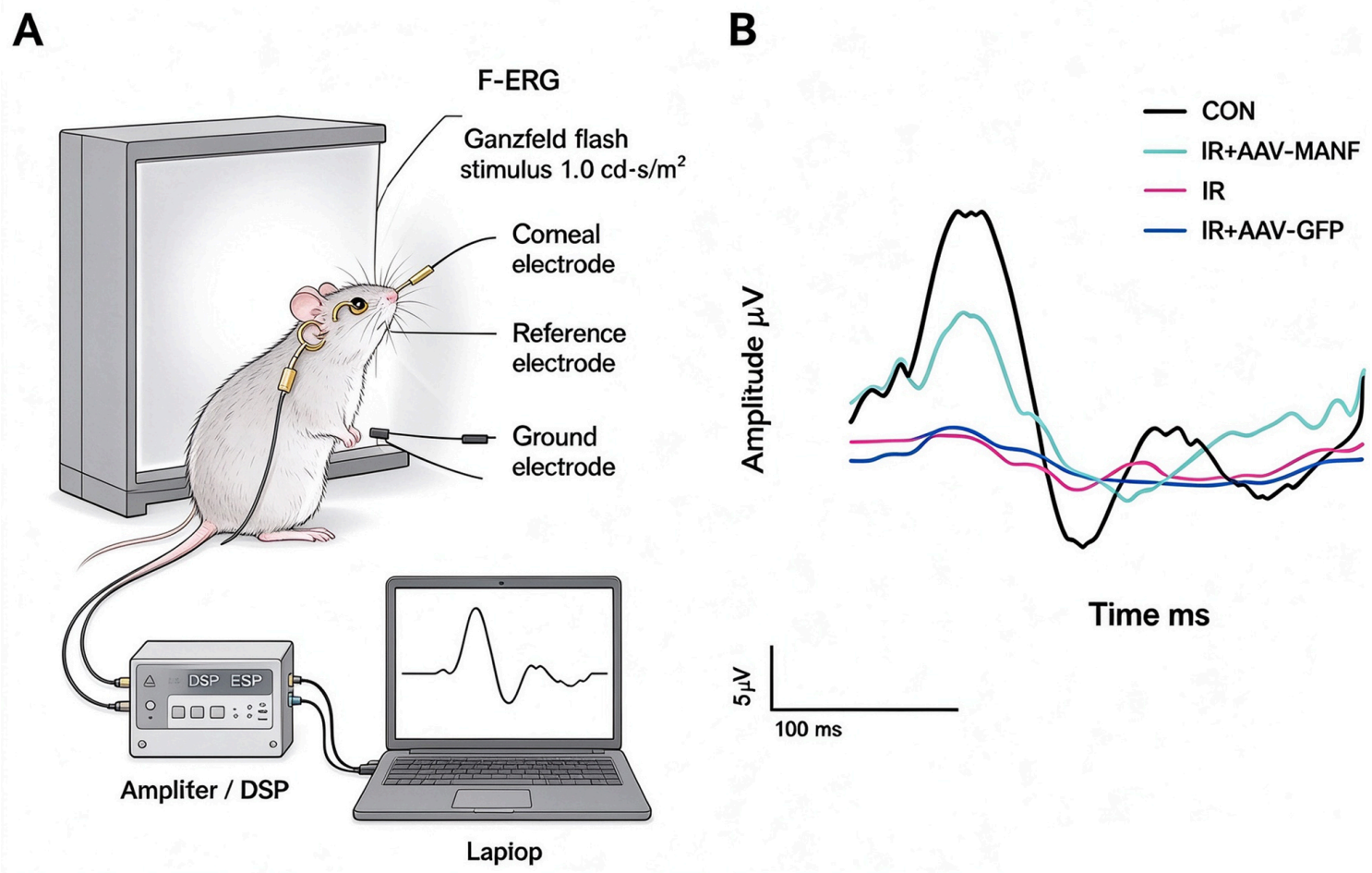

Figure 5

Scotopic flash electroretinogram (F-ERG) recording and representative waveforms after RIR injury. (A) Schematic setup for scotopic F-ERG recording in mice. Dark-adapted mice receive full-field flash stimuli; corneal recording electrode, subcutaneous reference and ground electrodes are connected to the electrophysiology acquisition system, and signals are displayed and saved on the computer.

(B) Representative scotopic F-ERG waveforms from three groups at day 7 post-IR injury: Control (black),IR group (pink), IR+AAV-GFP (blue), IR+AAV-MANF (light teal). Vertical scale bar = 5μV, horizontal scale bar =100 ms.

Supplementary Files

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