Noninvasive Light Flicker Stimulation Promotes Optic Nerve Regeneration by Activating Microglia and Enhancing Neural Plasticity in Zebrafish

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Purpose. Forty-hertz light flicker stimulation has been proven to reduce neurodegeneration, but its effect on optic nerve regeneration is unclear. This study explores the effect of 40-Hz light flicker in promoting optic nerve regeneration in zebrafish and investigates the underlying mechanisms.

Methods. Wild-type and *mpeg1*:EGFP zebrafish were used to establish a model of optic nerve crush. Biocytin tracing and hematoxylin and eosin staining were employed to observe whether 40-Hz light flicker promotes regeneration of retinal ganglion cell axons and dendrites. Optomotor and optokinetic responses were evaluated to assess recovery of visual function. Immunofluorescence staining of *mpeg1*:EGFP zebrafish was performed to observe changes in microglia. Differentially expressed genes that promote optic nerve regeneration following 40-Hz light flicker stimulation were identified and validated through RNA-sequencing analysis and quantitative real-time PCR (qRT-PCR).

Results. Zebrafish exhibited spontaneous optic nerve regeneration after optic nerve injury and restored visual function. We observed that 40-Hz light flicker significantly activated microglia following optic nerve injury and promoted regeneration of retinal ganglion cell axons and dendrites, as well as recovery of visual function. Transcriptomics and qRT-PCR analyses revealed that 40-Hz light flicker increased the expression of genes associated with neuronal plasticity, including *bdnf*, *npas4a*, *fosab*, *fosb*, *egr4*, and *ier2a*.

Conclusions. To our knowledge, this study is the first to demonstrate that 40-Hz light flicker stimulation promotes regeneration of retinal ganglion cell axons and dendrites and recovery of visual function in zebrafish, which is associated with microglial activation and enhancement of neural plasticity.

Keywords: light flicker, optic nerve regeneration, microglia, neural plasticity, zebrafish

Optic neuropathies, such as glaucoma, traumatic, ischemic, or inflammatory optic neuropathy, can lead to loss of retinal ganglion cells (RGCs) and axonal damage. Among these diseases, glaucoma is the main cause of irreversible blindness worldwide and is characterized by progressive loss of RGCs, leading to progressive visual impairment.[1] RGCs are the only output neurons that transmit optical signals from the retina to the brain, and the optic nerve is a structure composed of RGCs axons, which are highly susceptible to damage and cannot regenerate in neurodegenerative diseases such as glaucoma. Unlike mammals, zebrafish have a high regenerative capacity after central nervous system injury, and the optic nerve can fully regenerate after optic nerve crush (ONC).[2,3] Microglia are involved in the process of zebrafish optic nerve regeneration,[4] and the depletion of microglia impairs optic nerve regeneration in adult zebrafish after ONC.[5,6] Microglia-mediated neuroinflammation is considered an important factor for optic nerve regeneration in adult zebrafish, and modulating microglia may contribute to optic nerve regeneration in zebrafish. The cytokines produced by microglia in response to transient 40-Hz light flicker stimulation are different from those produced in response to acute inflammation and have neuroprotective effects.[7]

Forty-hertz light stimulation, sound stimulation, or a combination of both reduces amyloid beta (Aβ) protein levels in Alzheimer's disease (AD) mouse models and improves cognitive function, and the underlying mechanism is related to morphological changes in microglia.[8–11] Clinical studies have also shown that 40-Hz light and sound stimulation safely and effectively induces gamma oscillations in patients with AD and improves recall ability and daily activity rhythmicity in patients with AD.[12] Also, 40-Hz light flicker stimulation promotes the activation of adult-born neurons, accelerates the learning of difficult odor discrimination, and improves memory.[13] Neurological activity has a positive impact on axonal regeneration, and light and electrical stimulation have been reported to have great potential for promoting axonal regeneration in RGCs.[14] In addition, adult mice exposed to high-contrast visual stimulation exhibited regeneration of RGC axons within a short distance from the site of optic nerve injury.[15] Transcorneal electrical stimulation was found to increase the survival of RGCs after optic nerve transection injury in rats,[16] and an in vitro study showed that this stimulation guides the direction of RGCs axon growth.[17] It has been shown that 40-Hz light flicker stimulation reduces neurodegeneration, but its role in optic nerve regeneration is unknown. We speculate that 40-Hz light flicker stimulation modulates microglia and promotes zebrafish optic nerve regeneration.

Given the enormous potential therapeutic significance of 40-Hz light flicker stimulation, this study used the classic ONC model to explore whether 40-Hz light flicker stimulation promotes optic nerve regeneration and improves visual function. We found that 40-Hz light flicker stimulation significantly increased the activation of microglia after zebrafish optic nerve injury, promoted optic nerve regeneration, and improved visual function. Both the transcriptomic and quantitative real-time PCR (qRT-PCR) results showed that 40-Hz light flicker stimulation increased the expression of genes related to neuronal plasticity.

Materials and Methods

Animals

This study involved 3- to 6-month-old male and female zebrafish, including wild-type AB and *mpeg1*:EGFP transgenic zebrafish, which were obtained from the China Zebrafish Resource Center (Wuhan, China). All adult zebrafish were raised in a zebrafish feeding system (Haisheng Biotechnology, Shanghai, China) on a light/dark cycle of 14 hours/10 hours at a temperature of 28.5°C and were fed twice a day with brine shrimp (Wudi Fengtai Aquaculture, Binzhou, China). The zebrafish maintenance and experimental procedures were approved by the Ethical Review Committee of the Affiliated Eye Hospital of Nanchang University (protocol number: YLP20220617) and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Light Flicker Stimulation

As shown in Figure 1A, zebrafish were kept in a transparent aquarium (10 cm × 10 cm × 10 cm), a light-emitting diode (LED) light was placed above an opaque LED light box, and the transparent aquarium was placed in the center of the light box (12 cm × 12 cm × 12 cm). White LEDs with a correlated color temperature of 6500 K were used to produce light flickers of different frequencies, with luminescence intensities ranging from 50 to 500 lux, as measured from the bottom and top of the aquarium. The electroluminescence spectra and CIE1931 chromaticity diagrams of the LEDs are shown in Figure 1B. The LEDs were automatically controlled to produce light flickers of different frequencies (Fig. 1C), such as 20 Hz (25 ms on/25 ms off), 40 Hz (12.5 ms on/12.5 ms off), and 80 Hz (6.25 ms on/6.25 ms off). The zebrafish were stimulated for 1 hour every morning at 8 AM for 7 days after optic nerve injury. During the 1-hour light flicker stimulation period, the zebrafish were allowed to move freely in the aquarium but had no opportunity to obtain food. After 1 hour, the zebrafish were placed in a normal breeding environment.

Optic Nerve Crush

ONC was induced in adult zebrafish as described previously.[18] Adult zebrafish were anesthetized in 0.02% tricaine solution (MS-222; Sigma Aldrich, St. Louis, MO, USA) and placed on damp tissue paper with their eyes facing upward. The zebrafish were placed under an anatomical microscope, and after the connective tissue around the eyes was removed the eyes were gently removed from the orbit, exposing the optic nerve and ophthalmic artery. Sterile forceps (Dumont #5; Fine Science Tools, Foster City, CA, USA) were placed around the optic nerve, and the optic nerve was crushed for 10 seconds at a distance of 0.5 mm from the optic nerve head to avoid damaging the ophthalmic artery. As shown in Figure 2A, zebrafish in which ONC was successful exhibited a clear gap within the translucent nerve sheath of the optic nerve. Subsequently, the eyes were placed back in their sockets, and the zebrafish were placed back in the fish tank for recovery.

Optomotor Response

As shown in Figure 2B, the apparatus used to measure the optomotor response (OMR) was composed of a round white light, a rotating grating, a transparent circular fish chamber, and an opaque column from the outside to the inside. Under the illumination of a round white light, the transparent plastic cylinder with black-and-white striped paper on the inside could be rotated to form a rolling black-and-white grating stimulus, and the speed and direction of rotation, as well as the spacing between the black-and-white gratings, could be adjusted. Adult zebrafish were allowed to freely swim in a transparent, circular fish chamber with an opaque column placed in the center to prevent the zebrafish from swimming directly through the central area. When the black-and-white grating rotated, the zebrafish followed the grating to swim in a regular pattern. Each fish underwent OMR testing in two consecutive 1-minute experiments. The rotation direction was clockwise in one of the experiments and counterclockwise in the other; the order was randomized to eliminate any directional deviation. The concordance rate was calculated as the proportion of time the zebrafish swam in the grating direction relative to the entire observation time.[4,19]

Optokinetic Response

As shown in Figure 2C, the device used to measure the optokinetic response (OKR) was similar to that used to measure the OMR; it consisted of a round white light, a rotat-

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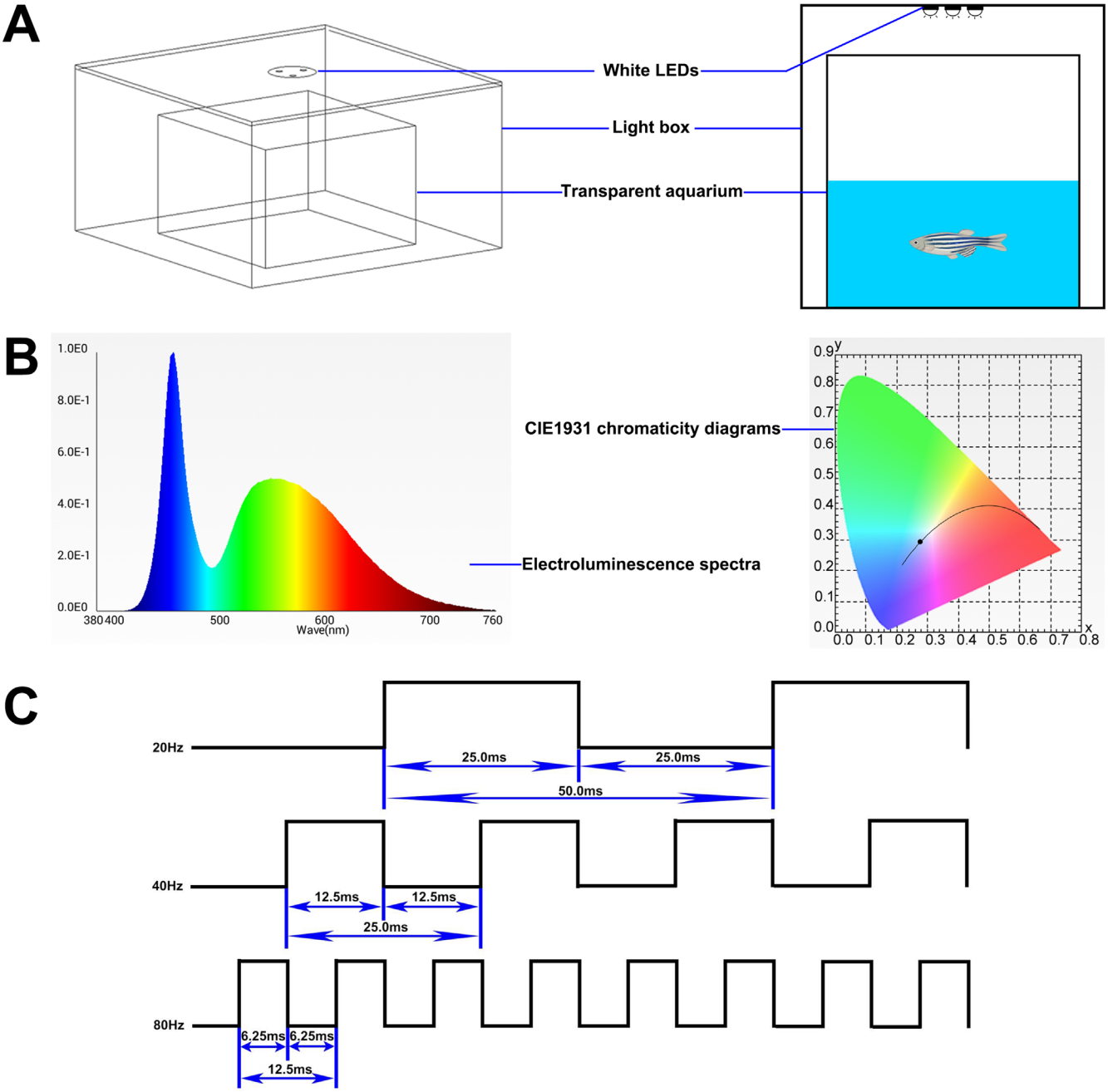

Figure 1. Light flicker stimulation. (**A**) Schematic diagram of the light flicker stimulation device, which included a transparent aquarium placed in the center of a lightbox and LEDs placed above the light box. (**B**) Electroluminescence spectra and CIE1931 chromaticity diagrams of the LEDs; LEDs with a correlated color temperature of 6500 K were used to produce light flickers of different frequencies. (**C**) Schematic diagram of light flicker stimulation at different frequencies. The LEDs were automatically controlled to produce light flickers of different frequencies, such as 20 Hz (25 ms on/25 ms off), 40 Hz (12.5 ms on/12.5 ms off), and 80 Hz (6.25 ms on/6.25 ms off).

ing grating, and a transparent circular fish chamber from the outside to the inside. The bodies of the zebrafish were fixed so that their eyes were located in the center of the circular fish chamber. The black-and-white grating was rotated, and the zebrafish eyes followed the grating until their eyes could no longer rotate; then, the eyes were returned to the normal position with a sharp eye tremor and began another cycle of rotation. To assess vision, the number of cycles per degree (cpd) was calculated using the following formula: cpd = 1/2[arctan(h/2a)], where a is the distance from the center of the zebrafish lens to the grating, and h is the length of one cycle of the smallest grating.[20,21]

Optic Tectum Reinnervation

Optic nerve axons were traced as described previously.[2] Briefly, the zebrafish were anesthetized, and the optic nerve was exposed. To trace the regenerated axons, the optic nerve was transected near the optic nerve head. A gel sponge soaked in 0.5% biocytin (B4261; Sigma Aldrich) was placed

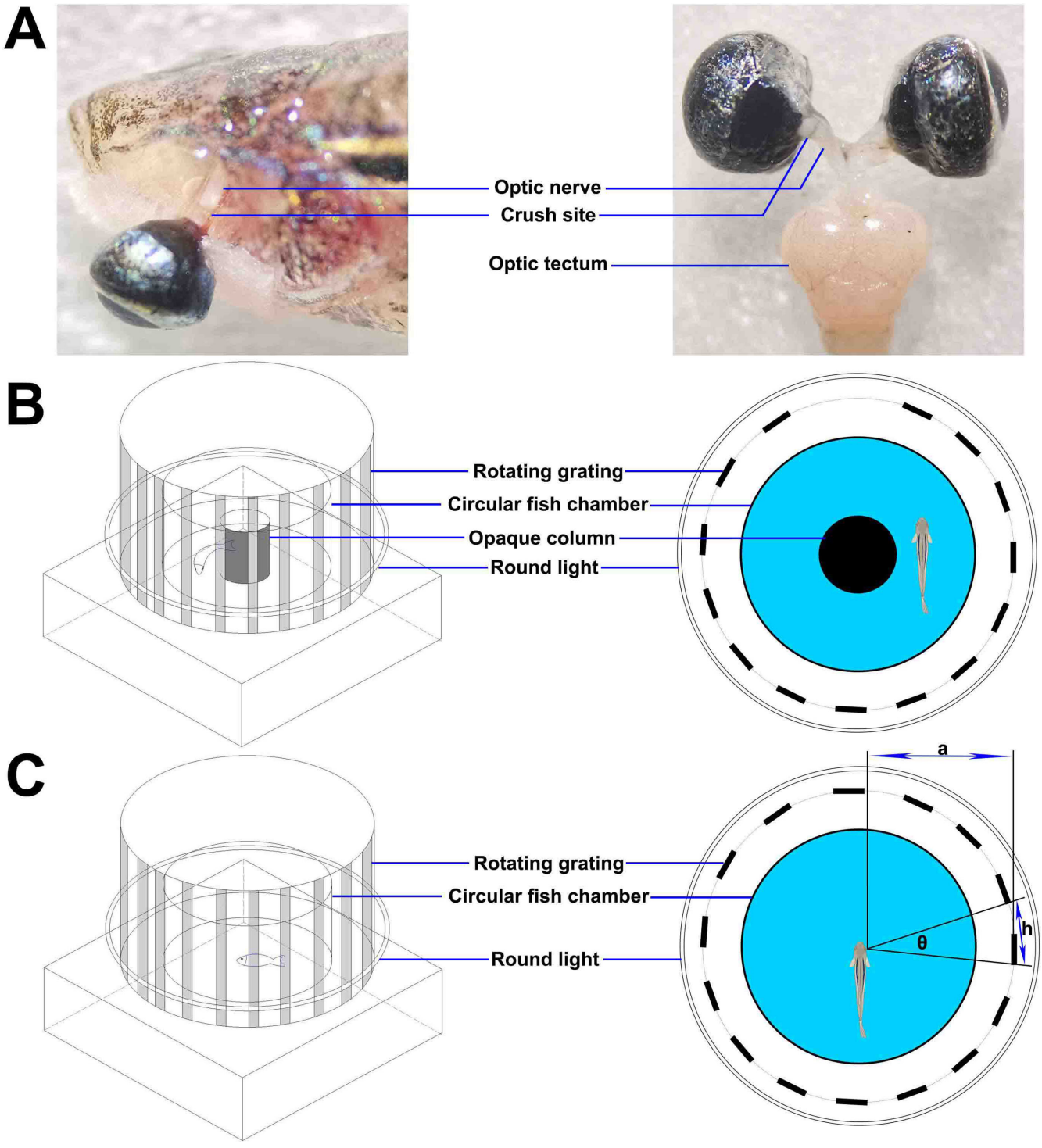

Figure 2. Schematic diagram of the method used to establish the zebrafish ONC model and the device used to assess visual function. (**A**, *left*) A clear gap could be observed inside the translucent nerve sheath of the zebrafish optic nerve after optic nerve injury. (**A**, *right*) The axons of retinal ganglion cells converge in the optic nerve and project to the contralateral optic tectum through the optic chiasm. (**B**) Schematic diagram of the device used to measure the OMR; the device was composed of a round white light, a rotating grating, a transparent circular fish chamber, and an opaque column. (**C**) Schematic diagram of the device used to measure the OKR; the device was composed of a round white light, a rotating grating, and a transparent circular fish chamber. The number of cpd was calculated using the following formula: cpd = 1/2[arctan (h/2a)], where a is the distance from the center of the zebrafish lens to the grating, and h is the length of one cycle of the smallest grating.

at the location of optic nerve transection. After the eyes were returned to their sockets, the zebrafish were returned to the fish tank and allowed to move freely for 3 hours, allowing for the anterograde transport of biocytin. Then, the

zebrafish were euthanized in a 0.1% tricaine solution, the brains were removed, and the tissues were fixed overnight with 4% paraformaldehyde (PFA). Frozen zebrafish brain sections (50 µm) were prepared and stained using a VECTASTAIN ABC Kit (PK-6100; Vector Laboratories, Neward, CA, USA) and DAB (AR1027-1; Boster, Wuhan, China) as a chromogen. All slices used in the experiment were stained simultaneously, taking care to avoid overstaining. We analyzed the optic tectum of normal and healthy adult zebrafish to determine the ratio of the biocytin-labeled area to the total analyzed area of the optic tectum, which was set to 100% as a reference value. After optic nerve injury, the percentage of optic nerve reinnervation relative to the reference value was used as an indicator of optic nerve regeneration.

Hematoxylin and Eosin Staining

Zebrafish eyes were fixed overnight in 4% PFA and gradually dehydrated in 30%, 50%, 70%, 90%, and 100% ethanol, with each step lasting 1 hour. Subsequently, the sections were treated with xylene for 1 hour, soaked in 70°C paraffin for 1 hour, and embedded in paraffin. A paraffin slicing machine (Leica RM2235 microtome; Leica Biosystems, Wetzlar, Germany) was used to cut the embedded eyes into 5-µm slices (horizontal to the optic nerve head). Each slice contained the entire retina from the superior and inferior hemispheres, with slices along the vertical meridian. Hematoxylin and eosin (H&E) staining was used to stain the slices. Finally, the retinal thicknesses were measured using Image-Pro Plus 6.0 in a region beginning 250 µm from the center of the optic nerve head.

Immunofluorescence

To visualize microglia in the retina, adult *mpeg1*:EGFP transgenic zebrafish were euthanized in a 0.1% tricaine solution. The eyeballs of the zebrafish were removed and fixed overnight with 4% PFA at 4°C. Then, the retinas were dissected and cut into four-leaf clover shapes. The retinas were blocked at room temperature for 1 hour using 5% donkey serum and then incubated overnight with anti-green fluorescent protein (GFP) primary antibodies (1:500, 600-101-215; Rockland Immunochemicals, Pottstown, PA, USA) at 4°C, followed by incubation with the corresponding fluorescently labeled secondary antibodies (1:200, ab150129; Abcam, Cambridge, UK) at room temperature for 1 hour. Finally, imaging of retinal GFP^+ cells was performed via confocal microscopy. The number and morphology of microglia were quantitatively analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

RNA-Sequencing

We conducted transcriptomic analysis of retina samples from the normal group and the optic nerve injury group. Zebrafish were randomly divided into the following five groups: the control (Con) group; a group of zebrafish analyzed on the fourth day after optic nerve injury (4-dpi group); a group of zebrafish subjected to optic nerve injury after 40-Hz light flicker stiulation for 4 days (4-dpi+40-Hz group); a group of zebrafish analyzed on the seventh day after optic nerve injury (7-dpi group); and a group of zebrafish subjected to optic nerve injury after 40-Hz light flicker stimulation for 7 days (7-dpi+40-Hz group). Four samples were obtained from each group, with each sample consisting of six retinas from different zebrafish. The RNA sequencing protocol involved RNA extraction, RNA detection, mRNA library construction, and sequencing. Briefly, total RNA was collected and isolated using Invitrogen TRIzol Reagent (R401-01; Vazyme, Nanjing, China), and mRNAs with poly(A) tails were enriched using Oligo(dT) magnetic beads (12629ES, Yeasen Biotechnology, Shanghai, China). Subsequently, first- and second-strand cDNA was synthesized. After the quality of the library was confirmed, the different libraries were pooled according to the effective concentration and the target sequencing output data volume before Illumina sequencing. The cDNA libraries were sequenced on the Illumina sequencing platform by MetWare Biotechnology Co., Ltd. (Wuhan, China). Feature counts were used to calculate gene alignment statistics, and the fragments per kilobase of transcript per million fragments mapped (FPKM) value of each gene was subsequently calculated based on its length. DESeq2 was used for differential expression analysis between two groups, and the Benjamin–Hochberg method was used to correct *P* values. The screening criteria for differentially expressed genes in this study were a fold change (FC) ≥ 1.2 and an false discovery rate (FDR) < 0.05. Enrichment analysis was performed based on the hypergeometric test, with pathway-based hypergeometric distribution analysis being used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Gene Ontology (GO) term-based analysis being used for GO enrichment analysis. The protein interactions of the differentially expressed genes were analyzed based on the STRING database of known and predicted protein–protein interactions.

Quantitative Real-Time PCR

RNAiso Plus reagent (9108; Takara Bio, Shiga, Japan) was used to extract total RNA from the retina. Each sample was a combination of four retinas from different zebrafish. HiScript Q RT SuperMix for qPCR (R323; Vazyme) was used for reverse transcription of total RNA. Using cDNA as a template, qRT-PCR was performed using Taq Pro Universal SYBR qPCR Master Mix (Q712; Vazyme). The specific primers used in the experiment were obtained from PrimerBank, and the primer sequences are shown in Supplementary Table S1. All the data were taken from the linear amplification stage of each gene. The comparative CT method ($2^{-\Delta\Delta CT}$) was used to calculate the relative changes in gene expression.

Western Blotting

Radioimmunoprecipitation (RIPA) lysis buffer (C1053; Applygen Technologies, Beijing, China) was used to extract proteins from the retinas of zebrafish. Each sample was a combination of four retinas from different zebrafish. A bicinchoninic acid (BCA) protein detection kit (ZJ102; Epizyme, Shanghai, China) was used to determine the protein concentration. For western blotting (WB) analysis, equal amounts of protein samples were electrophoresed on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred to polyvinylidene fluoride membranes (PVDF; Millipore, Burlington, MA, USA). After the membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBST) buffer containing 5% skim milk for 1 hour, they were incubated overnight with the appropriate primary antibodies at 4°C. The primary antibodies used were as follows: anti-BDNF (1:1000, ab108319; Abcam) and anti-

β-actin (1:200,000, AC026; ABclonal, Woburn, MA, USA). Then, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 hour. Finally, the proteins were detected using an enhanced chemiluminescence immunoblotting kit (Advansta, San Jose, CA, USA). The gray values of the bands were quantitatively analyzed with ImageJ software.

Statistical Analysis

All analyses were performed using Prism 9.0 (GraphPad, Boston, MA, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used for comparisons of three or more groups. The data are presented as the mean ± standard error of the mean (SEM), and $P < 0.05$ was considered to indicate statistical significance.

Results

Spontaneous Recovery of Retinal Structure and Visual Function in Zebrafish After Optic Nerve Injury

The dendrites of RGCs are located within the inner plexiform layer (IPL). To evaluate the dendritic remodeling of RGCs in zebrafish after ONC, we used H&E staining to compare the retinal IPL thickness/total retinal thickness (TRT) ratio between normal zebrafish and zebrafish subjected to optic nerve injury on days 1, 4, 7, 10, and 14. Figure 3A shows representative images of the retinal thickness at different times. At 4 and 7 days postinjury (dpi), the IPL thickness/TRT ratio in the central retina significantly decreased compared to the normal group of zebrafish. Notably, the IPL thickness/TRT ratio at 10 dpi increased compared to those at 4 dpi and 7 dpi and approached the baseline value again starting at 14 dpi, indicating dendritic remodeling of RGCs after ONC injury.

To evaluate the axonal regeneration of RGCs in zebrafish after ONC, we labeled the optic nerves of zebrafish with biocytin. Figure 3B shows representative images of optic nerve reinnervation in the optic tectum at different times. The results showed that, in normal zebrafish, 100% of the optic tectum was innervated by the optic nerve, whereas on the first day after ONC injury there was no biocytin labeling in the optic tectum of zebrafish. On the fifth day after ONC, regenerated RGC axons labeled with biocytin began to enter the optic tectum. At 7 dpi, approximately 50% to 70% of the optic tectum was labeled; at 10 dpi and 14 dpi, the biocytin-labeled area of the optic tectum was consistent with that observed in normal zebrafish, indicating optic nerve regeneration in adult zebrafish after ONC.

To evaluate changes in visual function in zebrafish after ONC, we assessed OMRs and OKRs to detect the recovery of visual function in zebrafish after ONC. Figure 3C shows the changes in visual function at different times. The results indicate that healthy adult zebrafish had a stable OMR and could continuously track a rotating grating while swimming. One day after optic nerve injury, the OMR was severely impaired, as the zebrafish no longer followed the grating movement but rather moved in a disorderly manner. Over time, the OMR gradually recovered, and at 10 dpi and 14 dpi the OMR basically returned to the preinjury level. Moreover, OKRs were evaluated, and no eye movement was observed on the first or fourth day after ONC. On the seventh day, an OKR was detected in a small number of zebrafish. At 10 dpi and 14 dpi, the OKR basically recovered to preinjury levels. The results of the statistical analysis of retinal thickness, optic tectum reinnervation, OMR, and OKR in zebrafish after optic nerve injury at different times are shown in Figure 3D. These results indicate that the optic nerve can regenerate and that visual function can be restored after ONC in adult zebrafish.

40-Hz Light Flicker Stimulation Accelerated the Recovery of Retinal Structure and Visual Function

Subsequently, we explored whether 40-Hz light flicker stimulation could promote the recovery of retinal structure and visual function after ONC in zebrafish. We found that the IPL thickness/TRT ratio decreased, the regenerated optic nerve innervated 50% to 70% of the optic tectum, and visual functions were impaired after 7 days of optic nerve injury in zebrafish; however, retinal structure and function were normalized after 10 and 14 days. To evaluate whether 40-Hz light flicker stimulation can promote optic nerve regeneration in zebrafish, zebrafish were subjected to 1 hour of 40-Hz light flicker stimulation daily for 7 consecutive days after ONC, and we assessed whether 40-Hz light flicker stimulation could promote the recovery of retinal structure and visual function in zebrafish. The results showed that 40-Hz light flicker stimulation promoted dendritic remodeling (Fig. 4A) and axonal regeneration of RGCs (Fig. 4B) in zebrafish after ONC, as well as visual function recovery (Fig. 4C). Figure 4D shows the statistical data for retinal thickness, optic nerve reinnervation, OMR, and OKR after 40-Hz light flicker stimulation.

40-Hz Light Flicker Stimulation Modulated Microglia in Zebrafish With Optic Nerve Injury

Previous studies have shown that exposing mice to 40-Hz light flicker stimulation can cause morphological changes in microglia.[8–10] To investigate whether microglia are involved in the process of zebrafish optic nerve regeneration, we compared changes in microglia in the retina between zebrafish subjected to ONC and normal zebrafish on days 1, 4, 7, 10, and 14. We found that the number of microglia significantly increased from day 1 to day 7 after ONC and decreased to normal levels after 14 days. Analysis of microglial morphology revealed an increase in cell size and a decrease in branching from day 1 to day 4, and microglial morphology returned to normal on the seventh day (Fig. 5A). Figure 5B shows the statistical analysis of the number and morphology of microglia at different times after optic nerve injury in zebrafish. These results confirmed that microglia are dynamically involved in the entire process of optic nerve regeneration and play an important role in this process. Modulating microglia may be a strategy for promoting optic nerve regeneration.

Furthermore, we investigated whether 40-Hz light flicker stimulation could modulate microglia in zebrafish after optic nerve injury. We found that on the seventh day after ONC, 40-Hz light flicker stimulation significantly activated microglia in the retina, as indicated by an increase in cell body size and a decrease in process length (Fig. 5C). Figure 5D shows the statistical analysis of the number and morphology of microglia in zebrafish with optic nerve injury after 40-Hz light flicker stimulation. These results indicate that 40-Hz light flicker stimulation may have a beneficial effect on optic nerve regeneration by modulating microglia in the zebrafish retina.

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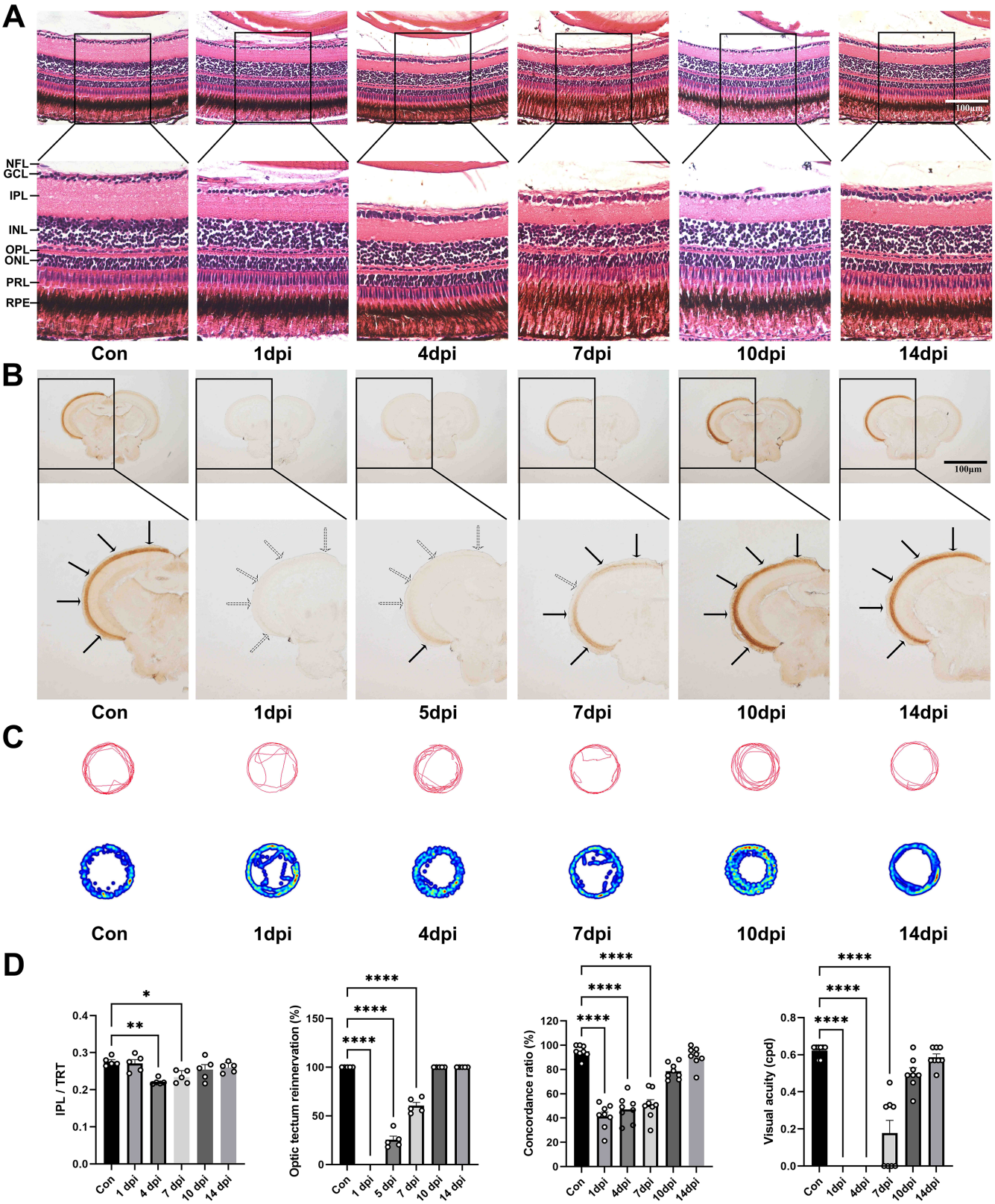

Figure 3. Recovery of retinal structure and visual function after optic nerve injury in zebrafish. Retinal thickness (**A**), optic tectum reinnervation (**B**), and OMRs and OKRs (**C**) in zebrafish after optic nerve injury. (**D**) Statistical analysis of retinal thickness, optic tectum reinnervation, OMRs, and OKRs in zebrafish after optic nerve injury. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PRL, photoreceptor layer; RPE, retinal pigment epithelium; TRT, total retinal thickness; Con, control; dpi, days postinjury. The data are shown as the mean ± SEM; $n = 5$ per group for **A** and **B** and $n = 8$ per group for **C**. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$.

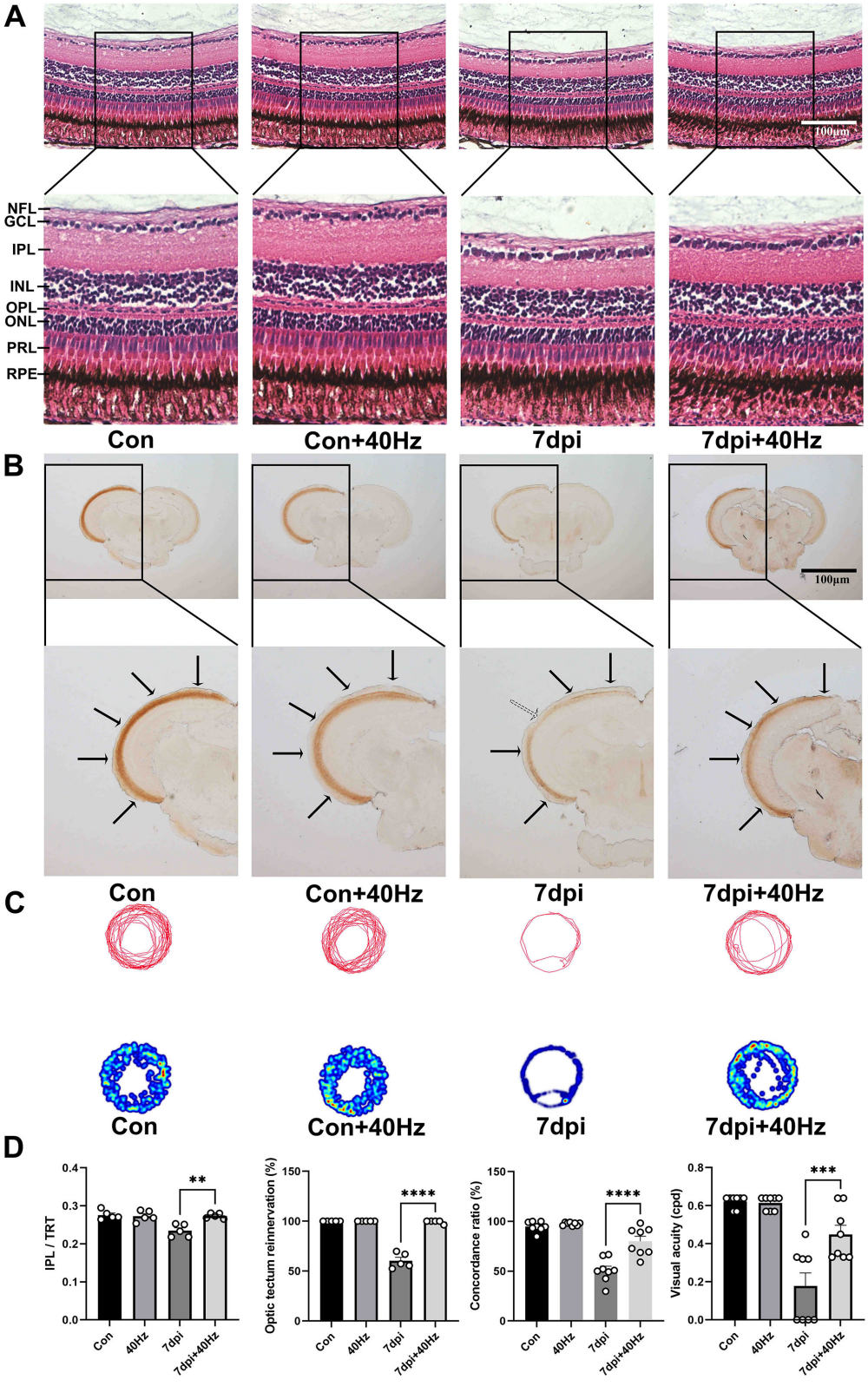

Figure 4. Forty-hertz light flicker stimulation promoted the recovery of retinal structure and visual function in zebrafish with optic nerve injury. Retinal thickness (**A**), optic tectum reinnervation (**B**), and OMRs and OKRs (**C**) in zebrafish with optic nerve injury after 40-Hz light flicker stimulation for 7 days. (**D**) Statistical analysis of retinal thickness, optic tectum reinnervation, OMRs and OKRs in zebrafish with optic nerve injury after 40-Hz light flicker stimulation for 7 days. The data are shown as the mean ± SEM; $n = 5$ per group for **A** and **B** and $n = 8$ per group for **C**. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

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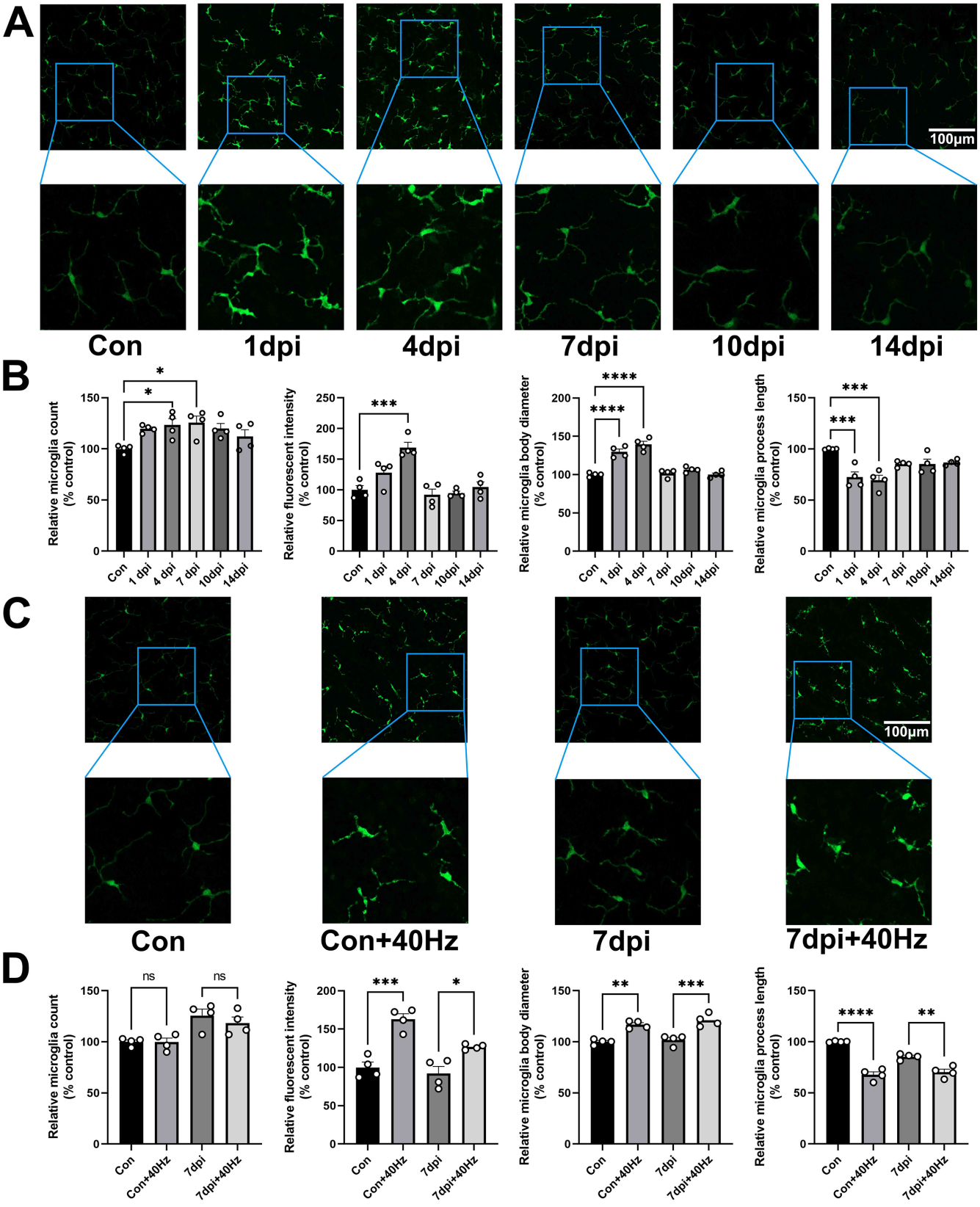

Figure 5. Forty-hertz light flicker stimulation modulated microglia in zebrafish with optic nerve injury. (**A**) Changes in retinal microglia at different times after optic nerve injury in zebrafish. (**B**) Statistical analysis of the number and morphology of microglia at different times after optic nerve injury in zebrafish. (**C**) Forty-hertz light flicker stimulation modulated microglia in zebrafish after optic nerve injury. (**D**) Statistical analysis of the number and morphology of microglia in zebrafish with optic nerve injury after 40-Hz light flicker stimulation. The data are shown as the mean ± SEM (four repeats from four zebrafish); ns, not significant. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

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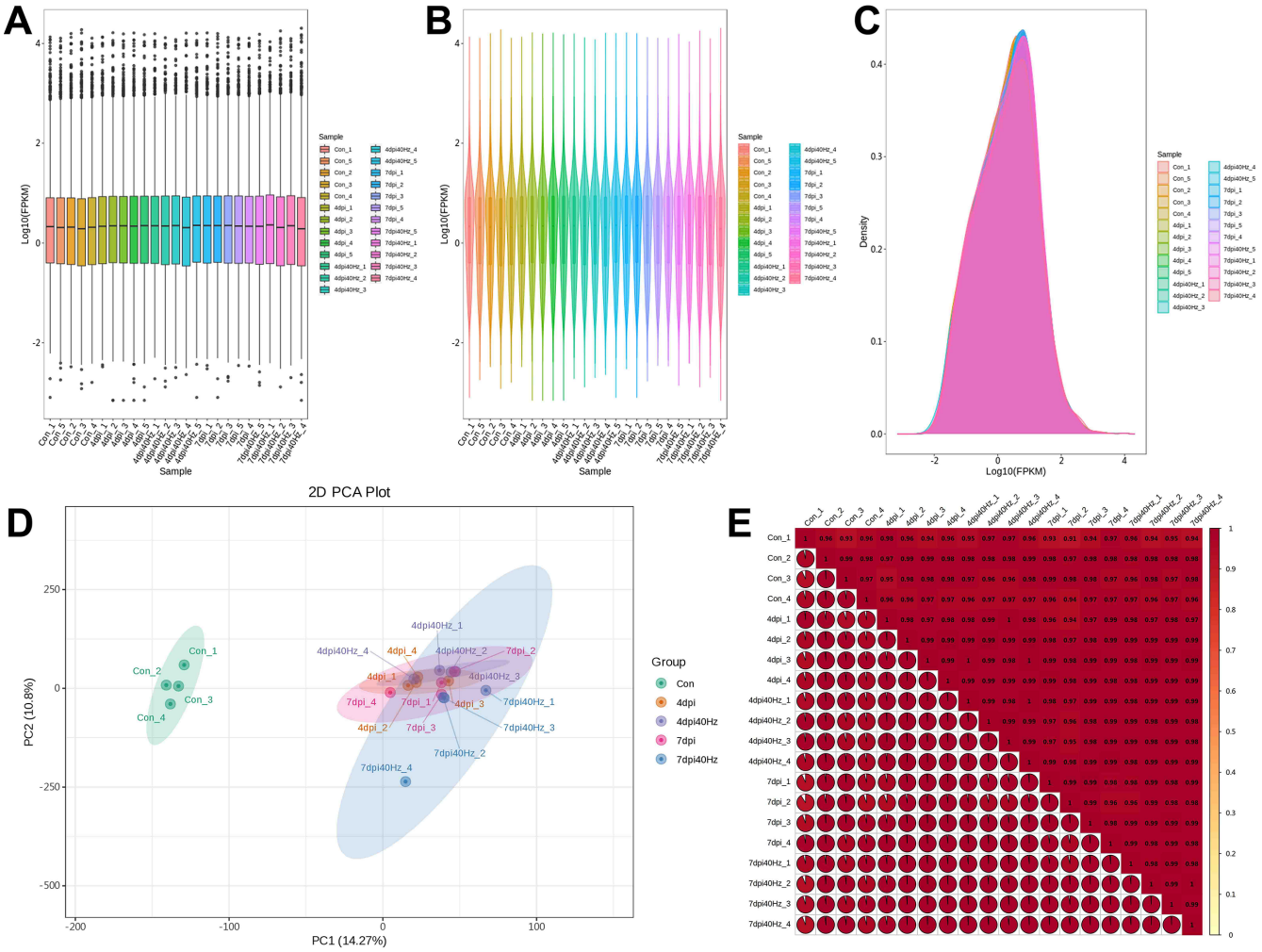

Figure 6. Quantitative analysis of gene expression. (**A**) Box plot. (**B**) Violin plot. (**C**) Density distribution plot. (**D**) PCA plots. (**E**) Sample correlation plots.

40-Hz Light Flicker Stimulation Enhanced Neural Plasticity in Zebrafish With Optic Nerve Injury

To investigate the mechanism by which 40-Hz light flicker stimulation promotes optic nerve regeneration in zebrafish after ONC, we conducted transcriptomic analysis of the zebrafish retina. FPKM was used as to evaluate gene or transcript expression levels. The box plot (Fig. 6A), violin plot (Fig. 6B), and density distribution plot (Fig. 6C) show the dispersion, probability density, and concentration interval of the gene expression level distribution for each sample, respectively. The results indicated that the overall distribution of FPKM values in each sample was similar, indicating the reproducibility of the RNA-sequencing data. Two-dimensional principal component analysis (PCA) showed that the retinal samples of zebrafish after ONC were separate from those of the normal group (Fig. 6D). Pearson's correlation coefficient R was used to evaluate the correlation between biological replicates and the reliability of the differentially expressed gene data. The closer the absolute value of R^2 is to 1, the stronger the correlation between duplicate samples. Our sample correlation plots showed that the R^2 values of the samples in this study were all greater than 0.9 (Fig. 6E).

Due to the spontaneous regeneration of the optic nerve in zebrafish after ONC, we conducted transcriptomic analysis of the zebrafish retina on the fourth and seventh days after ONC. There was a significant difference in gene expression between zebrafish subjected to ONC and normal zebrafish on the fourth and seventh days after ONC (Supplementary Figs. S1A–S1E, S2A–S2E). On the fourth day, a total of 4089 differentially expressed genes were identified, 2144 of which were upregulated and 1945 of which were downregulated (Supplementary Fig. S1B). On the seventh day, a total of 4211 differentially expressed genes were identified, of which 2391 were upregulated and 1820 were downregulated (Supplementary Fig. S2B). Supplementary Tables S2 and S3 list the 10 upregulated and downregulated genes with the greatest difference in gene expression on the fourth and seventh days after ONC in zebrafish. Compared with the normal zebrafish, there was a significant overlap of differentially expressed genes between zebrafish analyzed on the fourth day after ONC and those analyzed on the seventh day after ONC. The 10 overlapping upregulated and downregulated genes with the highest fold difference in expression included *flnca*, *myl6*, *pfdn5-3*, *txn-2*, *hbba2*, and *mtbl-2*. These findings indicate that zebrafish exhibit the same transcriptional changes at different times after ONC. KEGG enrichment

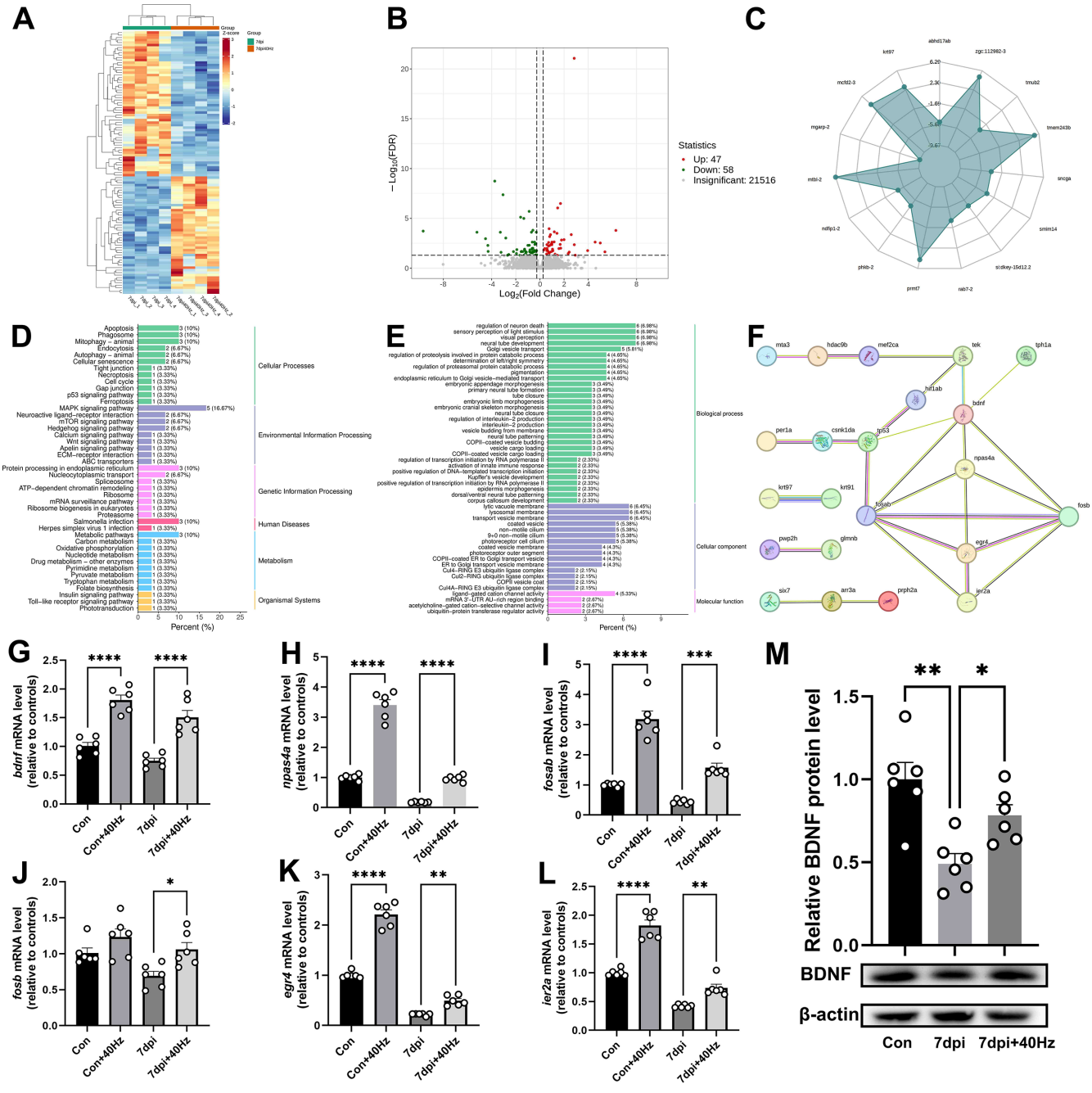

Figure 7. Forty-hertz light flicker stimulation enhanced neural plasticity in zebrafish with optic nerve injury. (**A**) Cluster heatmap of FPKM values. (**B**) Volcano map. (**C**) Radar charts. (**D**) Histogram of KEGG enrichment analysis results. (**E**) Histogram of the GO enrichment analysis results. (**F**) PPI network of the differentially expressed genes. (**G–L**) The gene expression of *bdnf*, *npas4a*, *fosab*, *egr4*, *fosb*, and *ier2a* determined via qRT-PCR. The data are shown as the mean ± SEM ($n = 6$ per group; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). (**M**) Representative western blotting showing BDNF protein levels. The data are shown as the mean ± SEM ($n = 6$ per group; *$P < 0.05$, **$P < 0.01$).

analysis revealed that the differentially expressed genes on the fourth and seventh days after ONC were enriched mainly in metabolism-related pathways, such as oxidative phosphorylation, carbon metabolism, and amino acid biosynthesis (Supplementary Figs. S1D, S2D). GO enrichment analysis of zebrafish at 4 and 7 days after ONC revealed that the differentially expressed genes were enriched mainly in the axon regeneration (GO: 0031103, GO: 0048679, GO: 0048680, GO: 0048681) and retinal ganglion cell axon guidance (GO: 0031290, GO: 0090259) pathways (Supplementary Figs. S1E, S2E).

To explore the potential mechanism by which 40-Hz light flicker stimulation promotes optic nerve regeneration in zebrafish after ONC, we conducted transcriptomic analysis of the retinas of zebrafish treated with 40-Hz light flicker stimulation on the fourth and seventh days after ONC. There was a significant difference in gene expression between the group subjected to 40-Hz light flicker stimulation and the untreated group on the fourth and seventh days after treatment (Supplementary Figs. S3A–S3E, Figs. 7A–7F). On the fourth day after 40-Hz light flicker stimulation, a total of 64 differentially expressed genes were

Table. Top 10 Upregulated and Top 10 Downregulated Genes in Zebrafish With Optic Nerve Injury After 40-Hz Light Flicker Stimulation for 7 Days

Symbol	Description	Gene Accession Number	Log_2FC	*P*, Adjusted
Upregulated				
mtbl-2	Metallothionein-B-like protein	NW_018394694.1	78.25	0.000162002
tmem243b	Transmembrane protein 243b	NC_007115.7	41.73	0.022899783
prmt7	Protein arginine *N*-methyltransferase 7	NC_007136.7	23.78	0.00240263
mcfd2-3	Multiple coagulation factor deficiency protein 2 precursor	NW_018395306.1	15.48	0.017088634
krt97	Keratin 97	NC_007133.7	7.59	0.049415937
ttll6	Tubulin polyglutamylase ttll6	NC_007123.7	7.19	0.004398617
sec13-2	Protein SEC13 homolog	NW_018395121.1	7.16	8.62625E-22
rpl12	60S Ribosomal protein L12	NC_007116.7	6.04	0.00042652
krt91	Keratin 91	NC_007130.7	5.07	0.034050054
npas4a	Neuronal PAS domain-containing protein 4A	NC_007125.7	3.99	0.001363795
Downregulated				
abhd17ab	Protein ABHD17A	NC_007119.7	0.03	0.000245446
ndfip1-2	NEDD4 family-interacting protein 1 isoform X1	NW_018394908.1	0.04	0.001089806
si:dkey-15d12.2	Mitotic-spindle organizing protein 1	NC_007112.7	0.05	0.005158375
phkb-2	Phosphorylase b kinase regulatory subunit beta isoform X5	NW_018394705.1	0.05	0.020696329
sncga	Synuclein, gamma a	NC_007124.7	0.07	1.81933E-09
tmub2	Transmembrane and ubiquitin-like domain-containing protein 2	NC_007114.7	0.10	0.000245446
smim14	Small integral membrane protein 14	NC_007112.7	0.11	0.025349463
rab7-2	Hypothetical protein HF521_008781	NW_018394809.1	0.12	4.25949E-08
uprt-2	Uracil phosphoribosyltransferase homolog	NW_018394574.1	0.14	0.000942109
mef2ca	Myocyte-specific enhancer factor 2C isoform X1	NC_007121.7	0.16	0.049952966

identified, 31 of which were upregulated and 33 of which were downregulated (Supplementary Fig. S3B). On the seventh day after 40-Hz light flicker stimulation, a total of 105 differentially expressed genes were identified, 47 of which were upregulated and 58 of which were downregulated (Fig. 7B). The Table and Supplementary Table S4 list the 10 upregulated and downregulated genes with the greatest change in gene expression on the fourth and seventh days after 40-Hz light flicker stimulation. According to the transcriptome sequencing results, the overlapping genes between zebrafish with optic nerve injury exposed to 40-Hz light flicker stimulation for 4 and 7 days were *egr4* and *rtn4rl2a*. As shown in Supplementary Figure S3D, KEGG enrichment analysis revealed that on the fourth day after 40-Hz light flicker stimulation, the differentially expressed genes were enriched mainly in metabolic pathways, purine metabolism, and *O*-glycan biosynthesis. On the seventh day after 40-Hz light flicker stimulation, the differentially expressed genes were enriched in molecular processes, such as apoptosis, phagosome, mitophagy, endocytosis, and autophagy, and were affecting metabolic pathways, carbon metabolism, oxidative phosphorylation, and nucleotide metabolism (Fig. 7D). As shown in Figure 7E, GO enrichment analysis revealed that 40-Hz light flicker stimulation affected biological processes such as the regulation of neuronal death (GO: 1901214), the sensory perception of light stimulus (GO: 050953), and visual perception (GO: 0007601).

As shown in Figure 7F, we used the STRING protein interaction database to construct a protein–protein interaction (PPI) network of the differentially expressed genes, and we used gene expression data and the PPI network to identify the regulatory differentially expressed genes. By constructing a PPI network of differentially expressed genes after 7 days of 40-Hz light flicker stimulation, the following hub genes related to neural plasticity were identified: *bdnf*, *npas4a*, *fosab* (*c-fos*), *fosb*, *egr4*, and *ier2a*. To validate the transcriptome sequencing results, we used qRT-PCR to validate the change in the expression of the aforementioned genes and found that 40-Hz light flicker stimulation promoted the expression of genes related to neuronal plasticity, such as *bdnf*, *npas4a*, *fosab* (*c-fos*), *fosb*, *egr4*, and *ier2a* (Figs. 7G–7L). Brain-derived neurotrophic factor (BDNF) is a key regulatory factor in the neuroimmune response. WB revealed that 40-Hz light flicker stimulation increased the expression of BDNF in the retinas of zebrafish after ONC (Fig. 7M).

Discussion

In the present study, we demonstrated that the optic nerve can spontaneously regenerate and that visual function can be restored after ONC in zebrafish. We found that 40-Hz light flicker stimulation modulated microglia in the zebrafish retina after ONC, promoted dendritic remodeling and axonal regeneration of RGCs, and restored visual function. Transcriptomic and qRT-PCR results both indicated that 40-Hz light flicker stimulation increased the expression of genes related to neural plasticity. Our study confirmed that 40-Hz light flicker stimulation promotes optic nerve regeneration in zebrafish.

Unlike in mammals, the optic nerve can regenerate in zebrafish after ONC. In the present study, on the fifth day after ONC, RGC axons begin to enter the optic tectum. At 7 dpi, approximately 50% to 70% of the optic tectum was innervated; at 10 and 14 dpi, the optic tectum area labeled by biocytin matched that in normal zebrafish, indicating optic nerve regeneration in adult zebrafish after ONC.[2,3] In addition, at 4 days after optic nerve injury in zebrafish, the dendrites of RGCs contracted, resulting in a significant decrease in the retinal IPL thickness, which gradually recovered. At 10 and 14 dpi, the retinal IPL thickness returned to normal.[3] A previous study showed that young zebrafish immediately lose complete visual function after ONC, but over time their vision gradually recovers. Visual function begins to recover at 3 dpi and returns to its initial level at

16 dpi.[22] Our study findings are similar to previous ones, and the retinal structure and visual function of zebrafish can serve as evaluation criteria for optic nerve regeneration.

Our study showed that 1 hour of 40-Hz light flicker stimulation for 7 consecutive days promoted dendritic remodeling and axonal regeneration of RGCs in zebrafish after ONC, as well as visual function recovery. These results are similar to those of previous studies confirming that 40-Hz light flicker stimulation reduces the number of β-amyloid plaques, tau phosphorylation, and neuronal and synaptic loss and has neuroprotective effects on AD mouse models.[10] In addition, 40-Hz light flicker stimulation reduces cerebral ischemic damage by increasing synaptic plasticity, thereby providing neuroprotection and improving cognitive function.[23] Alonso et al.[13] confirmed that 40-Hz light flicker stimulation activates adult-born neurons, accelerates the learning of difficult odors, and improves memory.

During the process of optic nerve regeneration, the number of microglia in the zebrafish retina gradually increases until 7 days after ONC and subsequently decreases to baseline levels.[4] The depletion of microglia disrupts optic nerve regeneration in zebrafish after ONC.[5,6] Our results also indicated that microglia are dynamically involved in the entire process of optic nerve regeneration. In addition, 40-Hz light flicker stimulation was found to activate microglia in the retina of zebrafish after optic nerve injury. This finding was similar to that of previous studies showing that 40-Hz light flicker stimulation activates microglia in AD model mice.[8–10] In a rat partial optic nerve transection model, the regulation of microglia alleviated the loss of RGCs.[24] Our results suggest that 40-Hz light flicker stimulation may have a beneficial effect on optic nerve regeneration by modulating microglia in the zebrafish retina.

Transcriptome sequencing and qRT-PCR revealed that 40-Hz light flicker stimulation increased the expression of genes related to neuronal plasticity, such as *bdnf*, *npas4a*, *fosab* (*c-fos*), *fosb*, *egr4*, and *ier2a*. Neuronal PAS domain protein 4 (Npas4) is a calcium-dependent transcription factor that regulates the activation of genes involved in the regulation of excitatory–inhibitory homeostasis and is crucial for the formation, function, and sustained plasticity of neural circuits.[25] Npas4 can induce visual cortex plasticity, and overexpression of Npas4 restores the plasticity of the visual system in adult rats after form deprivation; however, downregulation of Npas4 prevents the plasticity of the visual system.[26] Npas4 promotes the growth and functional maturation of neuronal processes, playing an important role in the structural and functional plasticity of neurons.[27] The target gene of Npas4 is *bdnf*, and the transcription of *bdnf* is significantly reduced in the hippocampus of Npas4 knockout mice.[28] Overexpression of *bdnf* delays the loss of RGCs and prolongs the survival of axons after ONC in mice.[29] The expression of transcription factors related to early gene neuronal activity, such as *c-fos* and *fosb*, is upregulated during nerve growth and regeneration.[30] *c-fos* can serve as a marker for neuronal activation, and 40-Hz light flicker stimulation increases the number of *c-fos*–positive cells in the visual cortex and other areas outside the visual cortex in mice.[10] Forty-hertz light flicker stimulation can also increase the expression levels of *c-fos* in the granule cell layer[13] and in brain regions such as the hippocampus and hypothalamus.[31] A zinc finger transcription factor, *egr4* belongs to the early growth response gene family and is necessary for the formation of brain primordia and head regeneration in planarians.[32]

A previous study revealed that 40-Hz light flicker stimulation regulates synaptic signaling pathways and synaptic plasticity-related proteins.[10] Also, 40-Hz light flicker stimulation has been shown to enhance synaptic plasticity and promote information transmission between neurons and the formation of learning and memory.[23] Our study also suggested that 40-Hz light flicker stimulation increases the expression of genes related to neural plasticity, such as *bdnf*, *npas4a*, *fosab* (*c-fos*), *fosb*, *egr4*, and *ier2a*, which may provide beneficial effects on optic nerve regeneration. Microglia play important roles in synaptogenesis, synaptic pruning, neurogenesis, and neural plasticity.[33–35] Microglia promote the formation of learning-related synapses through BDNF signaling and play an important role in brain plasticity.[36] The depletion of microglia significantly affects the density of *c*-fos–positive cells in the brain.[37]

In summary, our results confirmed for the first time that 40-Hz light flicker stimulation promotes dendritic remodeling and axonal regeneration of RGCs in zebrafish after ONC, as well as the recovery of visual function, which is related to the activation of microglia and enhancement of neural plasticity.

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