Cathepsin B promotes optic nerve axonal regeneration

Si Zhang[a], Hui Zhu[b], Guopei Li[a] and Min Zhu[a]

This study explored the role of cathepsin B (CTSB) in optic nerve regeneration. Sprague–Dawley rats were utilized for optic nerve crush and long-range crush injury model. Gene and protein expression changes were analyzed via reverse transcription quantitative polymerase chain reaction and western blot. Primary cortical neurons and BV2 cells were cultured to assess CTSB's effects on neuronal outgrowth and microglial activity. Local CTSB administration degraded chondroitin sulfate proteoglycans (CSPGs), promoting axonal growth in-vivo. In-vitro, CTSB neutralized CSPG-mediated inhibition of neuronal growth. Quantitative proteomics revealed elevated microglial marker proteins in the regenerative environment. Activation of signal transducer and activator of transcription 3 (STAT3) and signal transducer and activator of transcription 6 (STAT6) pathways in BV2 cells increased CTSB secretion. These findings suggest that postinjury regenerative microenvironment reconstruction is associated with upregulated CTSB, which degrades CSPGs to facilitate axonal growth. Microglia-derived CTSB, regulated by STAT3/STAT6 signaling, may play a key role in this process. Modulating CTSB expression could thus be a therapeutic strategy to enhance optic nerve regeneration by modifying the injury microenvironment. ***NeuroReport*** **36: 279–289 Copyright © 2025 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Introduction

In adult mammals, the regenerative capacity of the optic nerve following injury is generally extremely limited. Even when surrounding axons possess robust regenerative potential, they struggle to enter optic nerve grafts composed of glial cells from the central nervous system (CNS) [1]. The regenerative process of the optic nerve is a complex dynamic equilibrium involving the interplay between inhibitory and promoting factors for regeneration, with the ultimate growth length determined by the balance of these two aspects. Chondroitin sulfate proteoglycans (CSPGs), as crucial components of the CNS extracellular matrix (ECM), mediate inhibitory signals for axon growth through four signaling receptors: leukocyte common antigen-related tyrosine phosphatase, receptor protein tyrosine phosphatase sigma, nogo receptor 1, and nogo receptor 3 after nerve injury [2,3]. This subsequently inhibits the phosphorylation of protein kinase B and extracellular signal-regulated kinase, activates the Ras Homolog Family Member A/Rho-Associated Coiled-Coil Containing Protein Kinase pathway, and thus suppresses axon growth [2,3]. Experiments have shown that local application of arylsulfatase B (*ARSB*)/*ChABC* enzymes to enzymatically degrade CSPGs can significantly promote axon growth of retinal ganglion cells (RGCs) [4]. Therefore, alleviating the inhibitory effect of CSPGs on optic nerve axon growth is considered a key strategy to promote axon regeneration.

In previous studies [5,6], we successfully established a novel rat model of optic nerve crush (ONC) injury, which induces long-range ONC injury (LI, long-range injury) through consecutive clamping procedures. Experimental results indicate that the optic nerve environment after long-range clamping facilitates the growth of regenerating fibers, with decreased levels of various growth-inhibitory factors, including astrocytes, myelin, and members of the CSPGs family. Preliminary proteomic pathway enrichment analysis revealed significant alterations in the lysosomal pathway within the injury site. Specifically, cathepsin B (CTSB), a protein involved in the metabolism of CSPGs in the ECM, was one of the most significantly upregulated proteins. On the basis of these findings, we hypothesize that CTSB may play a crucial role in the repair process of optic nerve injury.

Microglia, which are rich in lysosome-associated proteases, are phagocytic cells in the CNS. In response to CNS injury stress, microglia undergo a range of phenotypic changes, and these different phenotypes may have dual roles in optic nerve regeneration, either promoting or inhibiting it [7–9]. This suggests a potential association between microglia and CTSB.

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In this study, we demonstrate for the first time that CTSB significantly promotes CNS axonal regeneration both *in vivo* and *in vitro*, and its increased expression may be associated with activated microglia. On the basis of these findings, we propose that modulation of CTSB represents a critical strategy for enhancing the local microenvironment at the site of optic nerve injury, thus promoting the repair of optic nerve damage.

Materials and methods

Animal models and groups

Animal models

All animal experiments were conducted according to the guidelines of ARRIVE and the National Institutes of Health (Bethesda, Maryland, USA). Sprague–Dawley (SD; 8-week-old) rats were used in this study and all procedures adhered to the standards set by the Animal Care and Use Committee of the Guangdong Medical Laboratory Animal Center.

Two animal models were used in this study:

(1) A modified ONC model, in a previous study, termed the long-range crush injury (LI) model, was used [5]. This model extends the injury range compared with the classic ONC model. Specifically, ophthalmic forceps (tip width: 0.5 mm; YAI020, Jinzhong, China) were used to crush the optic nerve 2 mm behind the eyeball, followed by a second crush 2–4 mm behind the eyeball, creating a 2-mm long crush injury. A previous study demonstrated that the LI model significantly increased the number of regenerating axons compared with the ONC model, suggesting a more regenerative environment. In addition, significant changes in the lysosomal pathway were observed in this regenerative microenvironment [5]. Therefore, the LI model is particularly useful for studying the injury microenvironment and intrinsic regeneration of retinal RGCs. In this study, we further investigated changes in lysosomal pathway proteins (e.g. CTSB) using this model.

(2) The second model was the traditional ONC model. Microsurgical forceps (tip width: 0.1 mm; WA3010, Jinzhong, China) were used to crush the optic nerve 2 mm behind the eyeball for 10 s, creating a 0.1-mm long crush injury without damaging the ophthalmic artery. This model, which induces pure optic nerve injury, was used to study the local effects of CTSB on optic nerve axon regeneration, reducing the interference of other regenerative factors.

Experimental groups

A total of 48 SD rats were randomly assigned to two groups, with 24 rats in each group: the LI group and the sham group. On days 3, 7, and 14, six rats from each group were randomly selected to extract optic nerve tissue for quantitative proteomics analysis. In addition, on day 7, six rats from each group were randomly chosen to harvest optic nerve tissue for PCR and western blot (WB) analysis.

A total of 24 SD rats underwent common optic nerve injury (crush group) and were subsequently injected with 5 μl of zymosan into the vitreous cavity on day 3 postinjury. The rats were then randomly divided into four groups of six each: (a) the control group; (b) the buffer group; (c) the leupeptin group (a CTSB inhibitor at 10 mM/ml); and (d) the CTSB group (at 200 μg/ml). The injured optic nerves were re-exposed, and a 31G insulin syringe needle was used to incise the dura mater surrounding the optic nerve. A gelatin sponge soaked in the corresponding solution was placed around the site of the optic nerve. On day 7, WB assays were conducted. On day 19, anterograde axonal labeling was performed by intravitreal injection of cholera toxin subunit B (CTB). On day 21, the rats underwent cardiac perfusion and tissue harvesting, followed by frozen sectioning and immunohistofluorescence to observe the regeneration of optic nerve axons.

Cholera toxin subunit B anterograde labeled axons

Anterograde axonal labeling was performed by intravitreal injection of CTB, following established protocols [5]. Briefly, CTB was dissolved in PBS to prepare a 1 mg/ml solution and stored at −20 °C for 1 month. Two days before optic nerve sampling, 5 μl of CTB was injected intravitreally using a 33G needle and a 10 μl Hamilton syringe, taking care to avoid puncturing the lens. The syringe was thoroughly rinsed with ethanol followed by PBS after each injection.

Cell culture and groups

Primary cortical neurons from rats culture and groups

The extraction method for primary brain neurons from rat fetuses was based on a previously published study [4]. Following the procedures of washing, digestion, mechanical pipetting, filtration, and centrifugation of fetal rat brain tissue, the cells were resuspended in a neurobasal medium and counted. These cells were then seeded into 48-well plates at a density of 5×10^4 cells per well and incubated at 37 °C with 5% CO_2. Once the cells had adhered to the substrate for 4 h, they were randomly divided into three groups: (a) normal culture medium group (neurobasal + B27 + 24 mM KCl; control group), (b) CSPGs-containing culture medium group (neurobasal + B27 + 24 mM KCl + 5 μg/ml CSPGs; CSPGs group), and (c) CTSB-treated CSPGs culture medium group (neurobasal + B27 + 24 mM KCl + 5 μg/ml CSPGs digested with CTSB enzyme; CTSB group). The cells were further cultured for 5 days before immunofluorescence experiments.

BV2 culture and group

BV2 cells (Procell, Wuhan, China) were plated in six-well plates 24 h before the experiment and cultured until they

reached a 70% confluence. Subsequently, following a previously published methodology [10], interleukin (IL)-4 (20 ng/ml; 214-14, Peprotech) and IL-13 (20 ng/ml; 210-13, Peprotech, Cranbury, New Jersey, USA) were added to the cell culture system. The cells were treated with these cytokines for 48 h, followed WB and immunofluorescence experiments.

Cell immunofluorescence

After fixation with 4% paraformaldehyde, the cells were washed with PBS, permeabilized with 0.5% Triton X-100, and blocked with 10% goat serum. Subsequently, the cells were incubated with primary antibodies overnight at 4 °C. The primary antibodies used were as follows: anti-βIII tubulin (1 : 100; MA1-118, Invitrogen, Carlsbad, California, USA), anti-signal transducer and activator of transcription 3 (STAT3) (1 : 100; 10253-2-AP, Proteintech, Rosemont, Illinois, USA), anti-STAT6 (1 : 100; 82630-1-RR, Proteintech), anti-IRE1α (1 : 100; 27528-1-AP, Proteintech), and anti-CTSB (1 : 100; 12216-1-AP, Proteintech). After washing with PBS, the cells were incubated with the corresponding secondary antibodies for 2 h at room temperature. Following incubation, the wells were washed again with PBS. Finally, fluorescent images were captured using an EVOS inverted fluorescence microscope at a magnification of ×200. ImageJ software was used to measure neurite outgrowth length and analyze immunofluorescence intensity.

Frozen sections of optic nerve tissue and fluorescence imaging

Rats were anesthetized with 1–2% isoflurane and euthanized by cervical dislocation. They were then perfused with cold PBS followed by 4% paraformaldehyde. The eyeballs and optic nerves were isolated and fixed overnight in 4% paraformaldehyde. Subsequently, the tissues were processed using standard frozen sectioning protocols. Imaging was conducted using a Leica DM4B upright fluorescence microscope. The appropriate fluorescence channels were selected to capture images at a magnification of ×200. The acquired images were analyzed for fluorescence intensity using ImageJ software. The method for counting optic nerve axons was adopted from previously published literature.

Counting of regenerated axons

ImageJ was used to count CTB-labeled regenerated axons at distances of 200, 500, 1000, and 1500 μm from the lesion site. The counts were averaged from three optic nerve sections. The width of the nerve's cross-section ($2r$) was measured to estimate the axon count per millimeter of nerve width. The$\sum a_d$ extending over a distance d within a nerve of radius r was estimated by summing data across all segments of thickness t (12 mm) [11]:

$$\sum a_d = \pi r^2 \times (\text{average axons/mm})/t.$$

RT-qPCR

Total RNA was extracted from the rat optic nerve using an Animal Tissue Total RNA Extraction Kit (DP431; Tiangen Biotech, Beijing, China). The extracted RNA was then reverse-transcribed into cDNA using a reverse transcription kit (R323-011; Vazyme, Nanjing, Jiangsu province, China). qPCR reactions were conducted using the ChamQ Universal SYBR qPCR Master Mix Kit (Q711; Vazyme). An initial denaturation step was performed at 95 °C for 30 s, followed by temperature cycling, which consisted of denaturation at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension for 30 s per cycle. A total of 40 cycles were performed. The primer sequences used are listed in Supplementary Table S1, Supplemental digital content 1, *http://links.lww.com/WNR/A808*. Normalization was conducted using an endogenous control [glyceraldehyde-3-phosphate dehydrogenase (GAPDH)], and relative units were calculated by comparing the C_t values. The experiments were repeated three times, and the results are presented as mean ± SD.

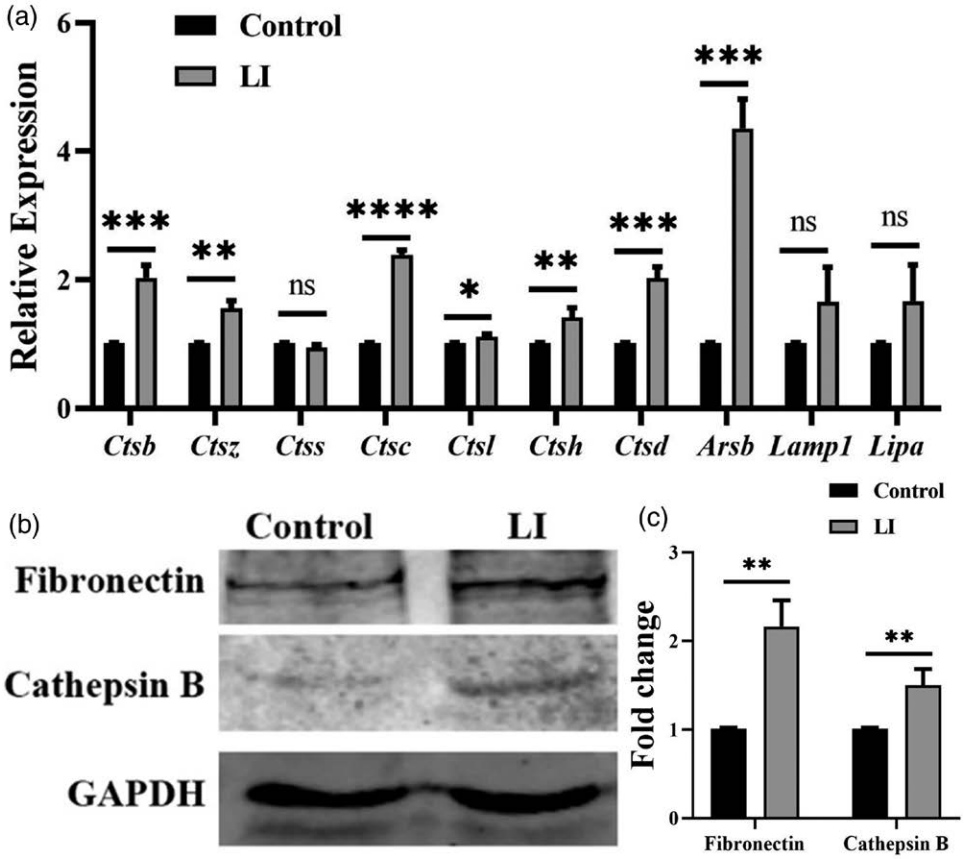
Western blot

Tissue protein extraction

Animals were anesthetized with 1–2% isoflurane and euthanized by cervical dislocation. Optic nerve connective tissue was dissected. For long-range injury models, a 3 mm nerve segment (including a 2-mm lesion) was excised 0.5 mm from each end of the injury. Other groups' samples were taken from corresponding optic nerve regions. Three nerve segments were pooled, transferred to sterile 1.5 ml tubes with 100 μl cold lysis buffer (100 mM phenylmethylSulfonyl fluoride and radioimmunoprecipitation assay buffer, 1 : 100; Beyotime, Shanghai, China), homogenized, and centrifuged to obtain tissue protein supernatant. Cellular protein extraction: cultured cells were washed with cold PBS, lysed with buffer, scraped, and centrifuged to obtain protein supernatant.

Following protein quantification using the BCA protein concentration assay kit, SDS-PAGE gel electrophoresis was conducted. The subsequent steps included membrane transfer and blocking. The membranes were incubated overnight at 4 °C with primary antibodies specific to the target bands. Afterward, the membranes were washed three times with phosphate-buffered saline with tween 20 (PBST) and incubated with the appropriate secondary antibodies. Following additional PBST washes, the membranes were scanned and exposed using the Odyssey WB fluorescence imaging system. The intensity of the WB bands was analyzed and statistically evaluated using the ImageJ software. All results were normalized to GAPDH as an internal reference. The primar antibodies utilized were: anti-fibronectin (15613-1-AP; Proteintech), antineurocan (1 : 1000; 27087-1-AP, Proteintech), anti-STAT3 (1 : 1000; 10253-2-AP, Proteintech), anti-STAT6 (1 : 1000; 82630-1-RR, Proteintech), anti-IRE1α (1 : 1000; 27528-1-AP,

Fig. 1



Analysis of lysosomal pathway proteins and fibronectin expression in the optic nerve following LI injury. (a) RT-qPCR analysis targeting the mRNA corresponding to differentially expressed proteins within the lysosomal pathway in the optic nerve of the LI group on the seventh postoperative day, compared with the corresponding region in the unoperated control group. These results suggest a significant upregulation of genes such as CTSB within the LI group. (b) Western blot bands for cathepsin B and fibronectin in the injured optic nerve segment of the LI group on the seventh postoperative day, alongside the corresponding region in the control group. (c) The grayscale analysis of the corresponding proteins, demonstrating a notable increase in the expression levels of both fibronectin and cathepsin B in the LI group compared with the control group. $N = 3$, $P < 0.05$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NS, no significance; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

Proteintech), and anti-CTSB (1 : 1000; 12216-1-AP, Proteintech).

Quantitative proteomics

The same method for optic nerve tissue harvesting and protein extraction, as used in WB analysis, was applied for proteomics analysis. The proteomic analysis was conducted by the Beijing Genomics Institute (Shenzhen, Guangdong, China).

Statistical analysis

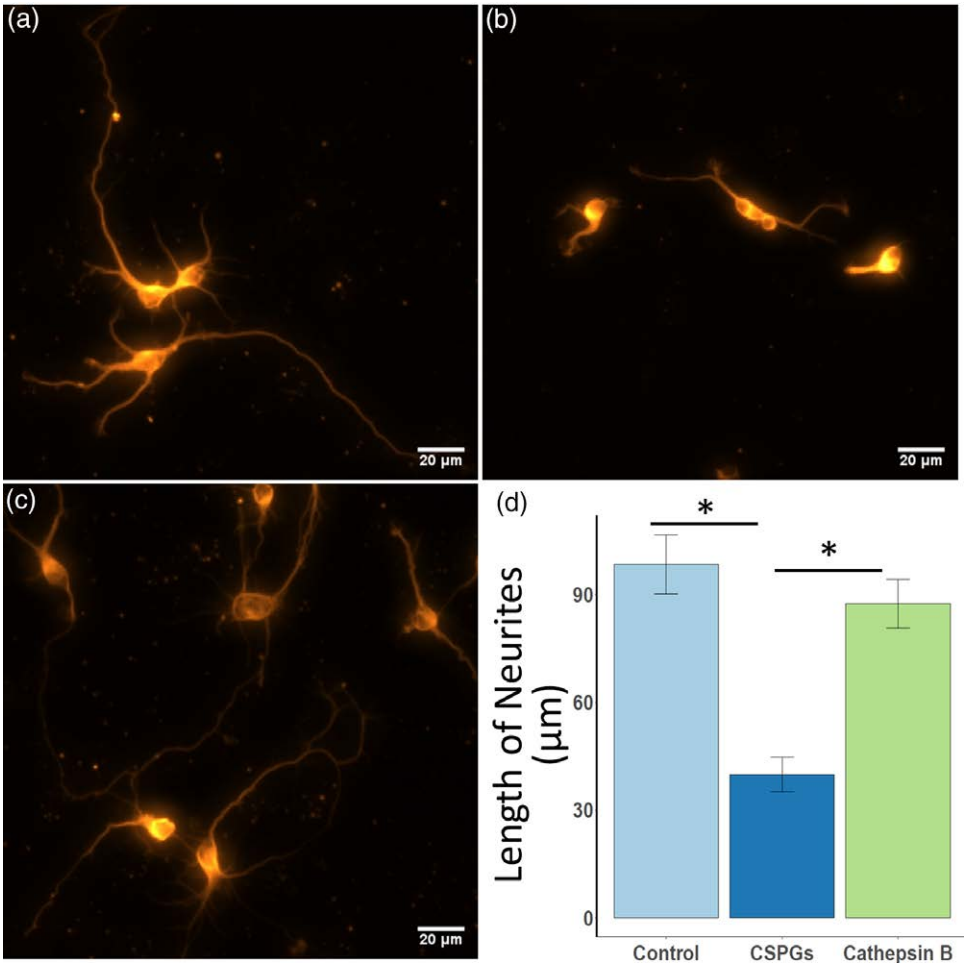
All statistical analyses were conducted using R software (version 4.0.2, available at http://www.r-project.org) [12]. The statistical significance was determined using two-tailed tests with an α level of 0.05. Differences between the two groups were assessed using a two-sample t-test, and multiple comparisons were adjusted using the Bonferroni correction.

Results

Elevated expression of cathepsin B in long-range crush injury group

In previous studies [5], we demonstrated that the microenvironment of the optic nerve following long-range crush injury (LI group) favors the growth of regenerating nerve fibers. In addition, the quantitative proteomic analysis indicated that the lysosome pathway was the most markedly affected, with significant increases in the expression levels of differentially expressed proteins, including CTSB, CTSD, CTSZ, and ARSB. On the basis of these findings, we further employed PCR and WB to validate the expression of lysosome-derived enzymes. Using reverse transcription quantitative polymerase chain reaction (RT-qPCR), we compared the mRNA expression levels of lysosome-derived enzymes between the LI group and the control group at day 7 (Fig. 1a). The results revealed that the transcription levels of genes encoding CTSB, CTSC, CTSD, and CTSZ were

Fig. 2



Effects of CSPGs and CTSB on cortical neuron growth. (a) The growth of cortical neurons in a normal neurobasal medium (control group). (b) The growth of cortical neurons in neurobasal medium supplemented with CSPGs at a final concentration of 5 μg/ml (CSPGs group). (c) The growth of cortical neurons in neurobasal medium containing CSPGs that have been treated with CTSB (CTSB group). (d) Statistics of axonal length. $N = 30, P < 0.05$,scale bar = 20 μm. CTSB, cathepsin B; CSPG, chondroitin sulfate proteoglycan.

significantly increased after optic nerve injury. Consistent with the proteomic and PCR findings, the WB results also showed a significant elevation in CTSB levels in the LI group (Fig. 1b and c). Furthermore, fibronectin, a marker secreted by microglia that promotes axon growth [13], showed significantly increased expression levels in the LI group compared with the control group.

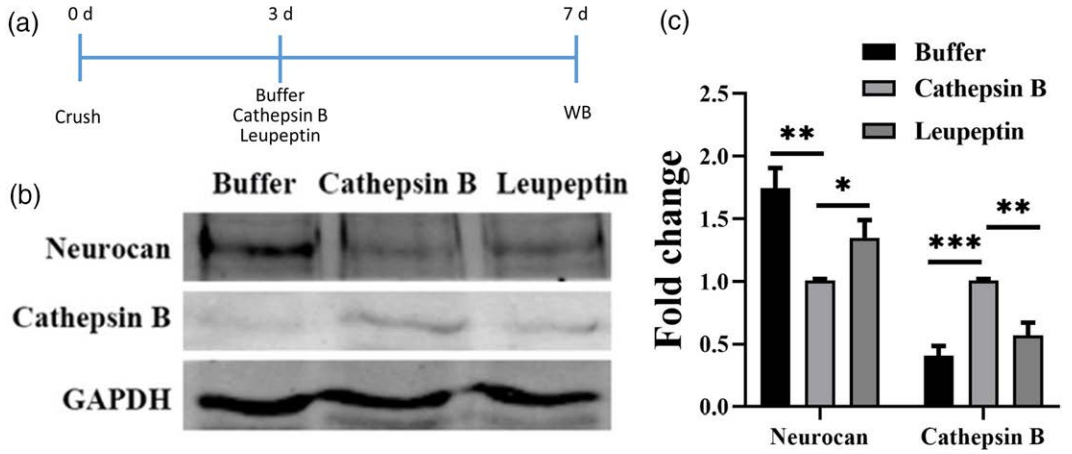
Cathepsin B alleviates chondroitin sulfate proteoglycans–mediated inhibition of axonal growth *in vitro*

To investigate the impact of CTSB on the growth of CNS axonal cells *in vitro*, we extracted and cultured primary brain neurons from fetal rats. Subsequently, these neurons were compared under three distinct culture conditions: a normal culture medium group serving as the control, CSPGs group, and a group treated with CTSB in the presence of CSPGs (CTSB group). The primary measure for the experiment was the length of neuronal outgrowth. Immunofluorescence staining was performed using anti-βIII tubulin. The results demonstrated that the average length of neuronal outgrowth in the control group was 98.47 ± 8.20 μm, while in the CSPGs group, it was 39.87 ± 4.80 μm. In contrast, the average length in the CTSB group was 87.46 ± 6.78 μm. These data indicate that CTSB can effectively counteract the inhibitory effect of CSPGs on neuronal outgrowth (Fig. 2).

Local application of cathepsin B *in vivo* enhances axonal regeneration

To investigate whether local application of CTSB can break down CSPGs, which inhibit axonal growth within the optic nerve, we administered CTSB buffer (buffer group), CTSB (CTSB group), and the CTSB inhibitor leupeptin (leupeptin group) locally to the site of ONC injury on the third-day postinjury, as illustrated

Fig. 3



Analysis of cathepsin B and neurocan protein levels following optic nerve crush injury. (a) Schematic representation of operational time points: On the third day following optic nerve crush, buffer, cathepsin B, or the cathepsin B inhibitor leupeptin was locally administered to the site of optic nerve injury. Subsequently, on the seventh-day postcrush, the optic nerve was harvested for western blot analysis to assess alterations in the protein levels of cathepsin B and its substrate, neurocan. (b) Western Blot band analysis of cathepsin B and neurocan in the optic nerve following various treatments. (c) Grayscale analysis of the corresponding bands. $N = 3$, $P < 0.05$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

in Figure 3a. On the seventh-day postcrush, the optic nerves were harvested for WB analysis to assess changes in protein levels of CTSB and neurocan, a member of the CSPGs family. Compared with the buffer and leupeptin groups, local application of CTSB led to a significant decrease in neurocan levels, indicating that CTSB effectively digested CSPGs. This finding suggests that CTSB may play a crucial role in facilitating the regeneration of optic nerve axons (Fig. 3b and c).

We further compared the number of axons that traversed the injury site 3 weeks postcrush among the control, buffer, leupeptin, and CTSB group (Fig. 4). The results showed that, at a distance of 200 μm from the initial site of ONC injury, the number of axons in the CTSB group was 695.2 ± 59.9, significantly higher than that in the buffer group (471.3 ± 50.3) and the leupeptin group (459.1 ± 47.9). This finding suggests that local application of CTSB can significantly increase the number of regenerating axons that traverse the injury site. These results imply that increased expression of CTSB reduces axonal growth inhibitors at the injury site, thus enhancing the local microenvironment and facilitating axonal regeneration.

Increased expression of cathepsin B is associated with microglial activation

In this study, we performed quantitative proteomics analysis on optic nerve tissues extracted from the LI and control group on days 3, 7, and 14. Through pathway enrichment and differential analysis, we observed a significant upregulation of microglial cell marker expressions (Iba-1, HexB, CD14, ferritin, and Arg-1) in the LI group (Fig. 5).

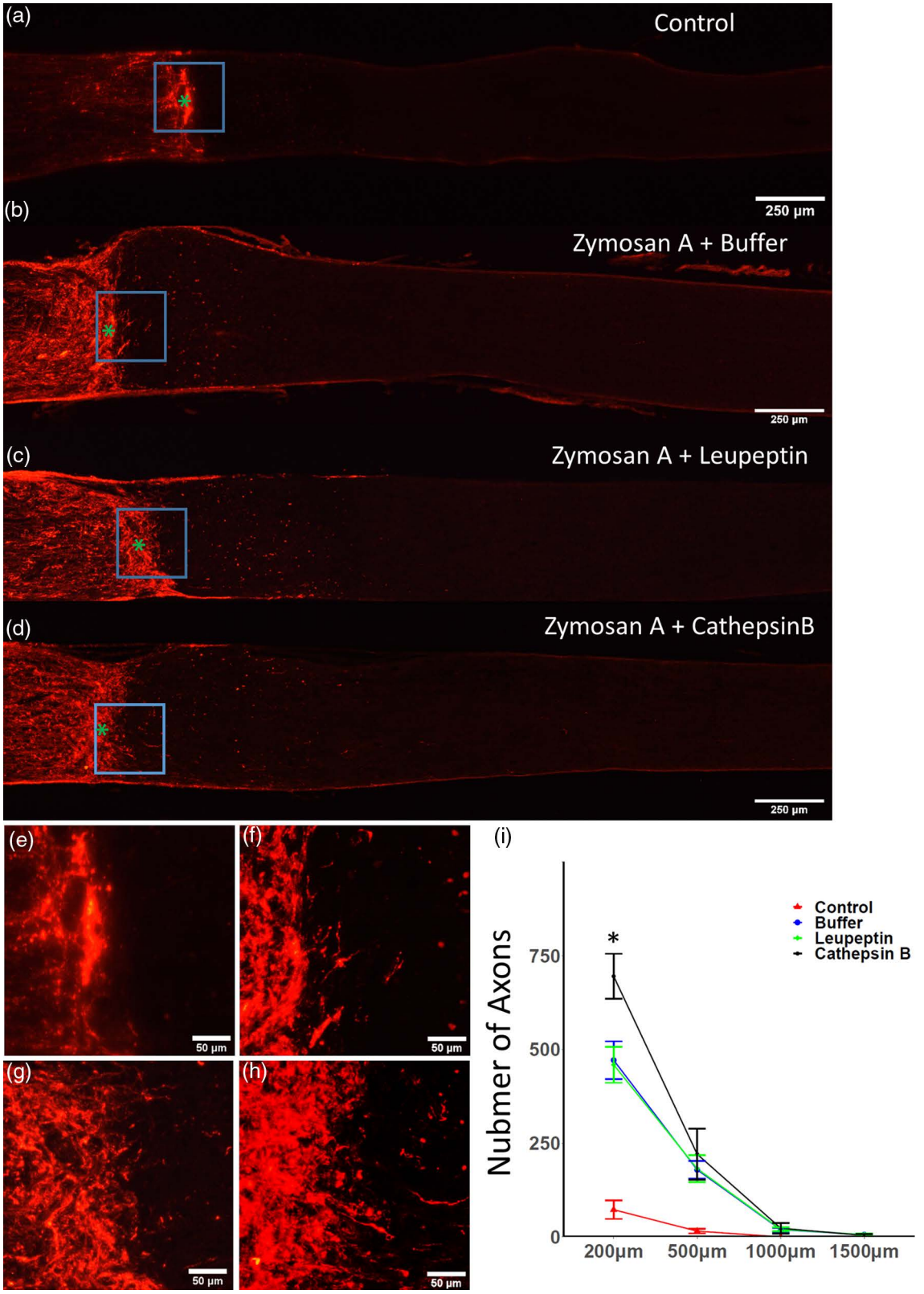
We hypothesized that microglia activation might promote increased CTSB expression. To test this, we cultured the BV2 microglial cell line *in vitro* and treated the cells with 20 ng/ml IL-4 and IL-6 for 48 h. Subsequently, we used WB to assess the expression levels of STAT3, STAT6, IRE1α, and CTSB in microglia. The results showed that, compared with the control group, the IL-4 + IL-6 group exhibited significant higher protein expression levels of STAT3, STAT6, IRE1α, and CTSB in microglia (Fig. 6).

Immunofluorescence analysis further supported these findings, demonstrating an upward trend in the expression of STAT3, STAT6, IRE1α, and CTSB in the IL-4 + IL-6 group (Fig. 7). These results confirm that activation of the STAT3 and STAT6 pathways promotes increased CTSB expression in microglia.

Discussion

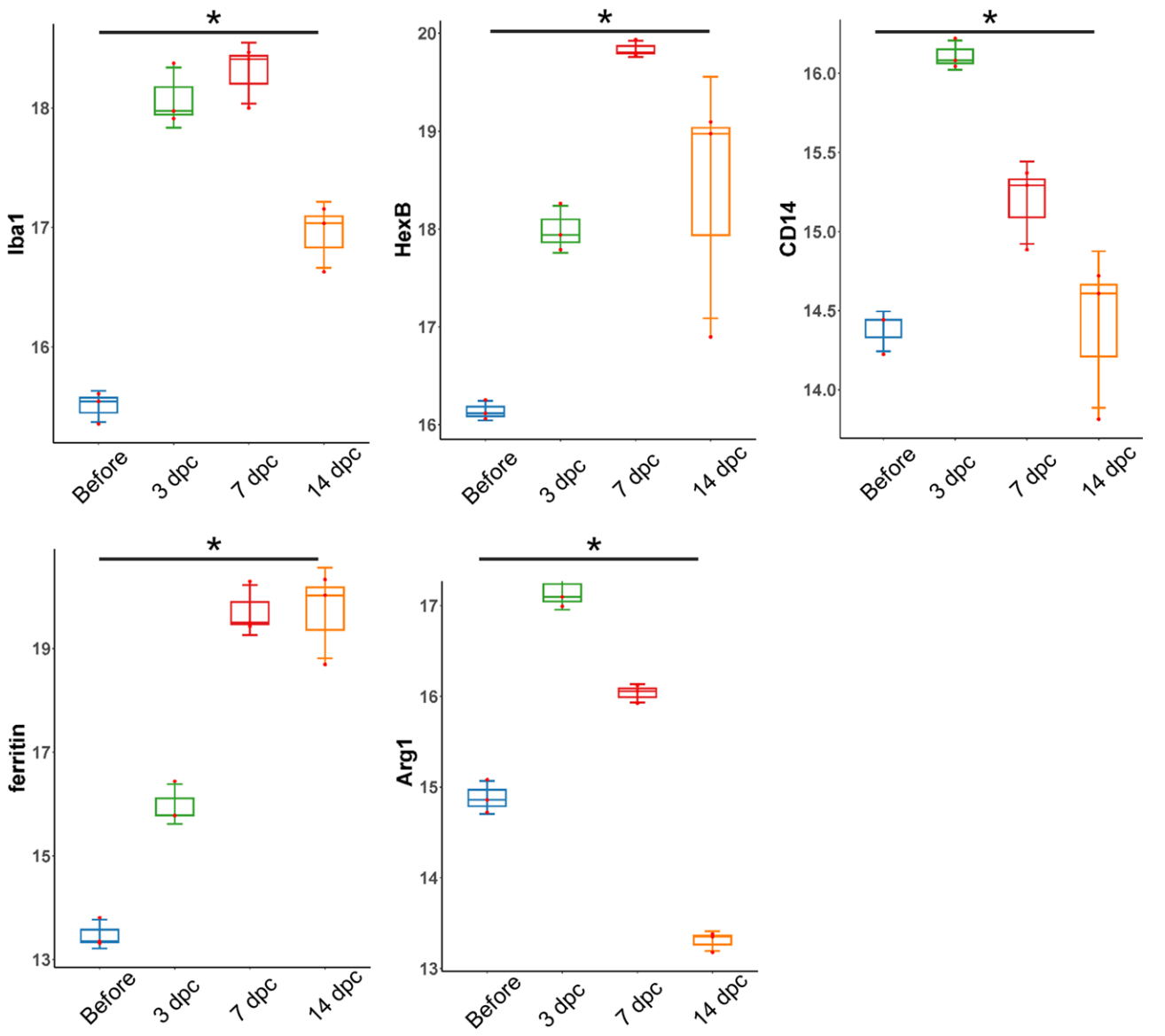
In the present study, we revealed a significant upregulation of CTSB expression in a long-range optic nerve injury model conducive to axonal regeneration. Further, *in vitro* models demonstrated that CTSB effectively mitigated the inhibitory impact of CSPGs on the growth of cerebral neuronal axons. In addition, when CTSB was locally applied to the site of ONC injury, it efficiently degraded CSPGs components (exemplified by neurocan) within the optic nerve, resulting in a substantial increase in the number of regenerating axonal fibers traversing the injury site. These results indicate that CTSB promotes neuronal outgrowth. Moreover, local application of CTSB to the site of optic nerve injury achieves similar effectiveness to enzymes such as *ARSB* and *ChABC*, enhancing the ability of regenerating axons to cross the injury site.

Fig. 4



Assessment of axonal regeneration following optic nerve crush injury. (a–d) Optical nerve sections of anterogradely labeled axons with CTB at 3 weeks post-crush injury for four groups, namely, the control group, the buffer group, the leupeptin group, and the cathepsin B group, respectively (scale bar = 250 μm) (e–h) The partial enlargements of the blue-boxed sections labeled as a–d, respectively (scale bar = 50 μm). (i) The statistical analysis of the number of axonal regenerations in groups located at varying distances (200, 500, 1000, and 1500 μm) from the initial site of optic nerve crush injury. CTB, cholera toxin subunit B.

Fig. 5

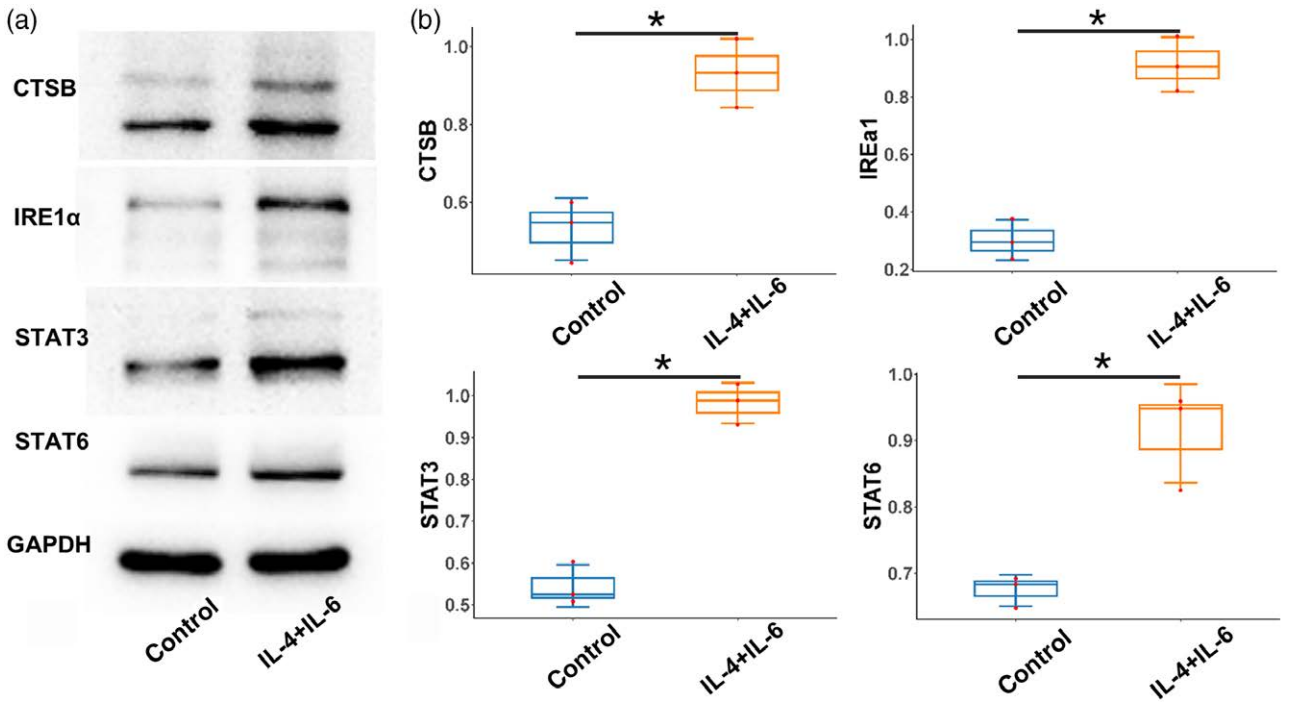


Proteomic analysis of microglial marker expression profiles.The proteomic analysis results depict the expression profiles of microglial markers (Iba-1, HexB, CD14, ferritin, and Arg-1) on days 3, 7, and 14 in both the LI group and the control group. Notably, compared with the control group, the expression levels of all microglial markers were significantly elevated in the LI group. dpc, days post-crush.

In various other literature sources, it has been observed that following diverse forms of neuronal injury, there is an increased transcription and secretion of cathepsin [14]. According to documented evidence, the expression level of mRNA encoding CTSB increases within just 2 days following spinal cord injury [15]. WB analysis of tissue lysates from the injured spinal cord regions revealed that cathepsin expression peaks on the seventh-day postinjury in rats [16]. Furthermore, Tran *et al.* [17] demonstrated in their experiments with cultured peripheral dorsal root ganglion cells that the secretion of CTSB is essential for neurons to traverse CSPGs matrices, which inhibit axon regeneration. A novel peptide, ISP, effectively upregulates the secretion of neuronal CTSB, thereby specifically degrading CSPGs components in the ECM that hinder axon growth. *In vivo* studies on spinal cord injury in mice showed that intravenous administration of ISP significantly promotes the secretion of CTSB in peripheral neurons, accelerates neural growth postspinal cord injury, and facilitates the recovery of partial motor functions [17].

In vitro experiments were conducted to activate the microglial cell line BV2 using IL-4 and IL-6. We confirmed that this process led to an upregulation in the expression levels of STAT3, STAT6, IRE1α, and CTSB. On the basis of

Fig. 6



Western blot analysis of microglial protein expression following IL-4 and IL-6 activation. (a) Grayscale images depicting the expression levels of STAT3, STAT6, IRE1α, and CTSB in microglia following activation induced by IL-4 and IL-6, as assessed by western blot analysis. (b) The statistical analysis results indicate that, in comparison with the control group, the expression of STAT3, STAT6, IRE1α, and CTSB proteins in microglia is significantly upregulated in the IL-4 + IL-6 group. $N = 3$, $P < 0.05$. CTSB, cathepsin B; IL, interleukin; IRE1α, inositol-requiring enzyme 1 alpha; STAT3, signal transducer and activator of transcription 3; STAT6, signal transducer and activator of transcription 6.

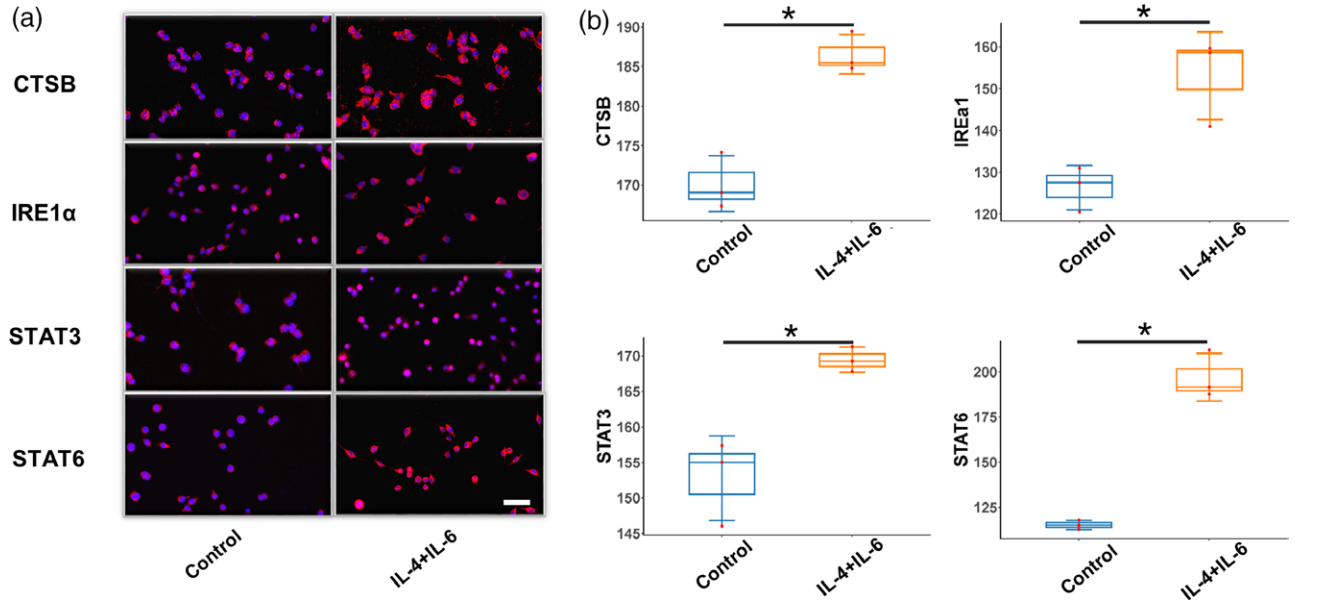
these findings, we initially hypothesized that the activation of STAT3 and STAT6 pathways may promote the secretion of CTSB by inflammatory microglia, potentially serving as one of the critical factors accelerating the ECM remodeling process. Literature documentation [15] indicates that the enzymatic activity of CTSB peaks between 5 and 7 days postinjury. Immune cells such as macrophages and microglia play a critical role in the infiltration at the lesion center. Following neural injury, glial cells at or near the lesion site release inflammatory cytokines and chemokines, thereby initiating an inflammatory response [18,19]. This process activates resident microglia and recruits immune cells, including blood-derived macrophages, to the site of injury [20,21]. In synergy with inflammatory cytokines such as IL-4, IL-6, or IL-10, macrophages can activate the STAT3 and STAT6 pathways, subsequently activating IRE1α and promoting the secretion of substances like CTSB [10]. These enzymatic substances are capable of degrading axon growth-inhibitory factors within the ECM components [22,23].

The regeneration of the optic nerve constitutes a complex process, encompassing the interplay between inhibitory and proregenerative factors. Studies have demonstrated that enhancing the intrinsic growth capacity of RGCs can, to a certain extent, facilitate the regeneration of optic nerve axons [24–26]. However, in parallel with augmenting the intrinsic regenerative capacity of RGCs, optimizing the microenvironment of optic nerve glial cells and the ECM, while mitigating inhibitory influences from the external environment, may further promote axonal growth. This comprehensive strategy exhibits more pronounced advantages compared with monotherapy [4]. This finding suggests that by focusing research efforts on alleviating the inhibitory effects of the injured microenvironment on axonal growth, manipulating this microenvironment through technological means, and combining therapeutic strategies that enhance RGC survival and their intrinsic regenerative capacity, effective repair of optic nerve injuries may be achievable.

Conclusion

This study investigates the effector protein CTSB within the ECM, aiming to validate its role in promoting optic nerve axon regeneration. The research has two key implications: first, it demonstrates that CTSB can mitigate the inhibitory effects of ECM components on axon growth during the repair process following optic nerve injury. Second, it established a foundation for future research by exploring the potential to enhance optic nerve axon regeneration through modulating CTSB secretion by inflammatory microglia, thereby identifying a promising

Fig. 7



Immunofluorescence analysis of microglial protein expression following IL-4 and IL-6 activation. (a) The expression levels of STAT3, STAT6, IRE1α, and CTSB in microglia following activation induced by IL-4 and IL-6 were assessed using immunofluorescence methods. (b) The statistical analysis results indicate that, in comparison with the control group, the expression of STAT3, STAT6, IRE1α, and CTSB proteins in microglia is significantly upregulated in the IL-4 + IL-6 group. $N = 3$, $P < 0.05$, scale bar = 50 μm. CTSB, cathepsin B; IL, interleukin; IRE1α, inositol-requiring enzyme 1 alpha; STAT3, signal transducer and activator of transcription 3; STAT6, signal transducer and activator of transcription 6.

therapeutic target. Overall, this study advances the field of optic nerve injury repair by providing a robust research framework and offers potential clinical insights for treating other CNS disorders.

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

There are no conflicts of interest.

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