Stimulation of c-Kit⁺ Retinal Progenitor Cells by Stem Cell Factor Confers Protection Against Retinal Degeneration

Xi Chen (✉ xichen@ccmu.edu.cn)

Capital Medical University Affiliated Beijing Friendship Hospital <https://orcid.org/0000-0002-8066-3394>

Shanshan Li

Capital Medical University Affiliated Beijing Friendship Hospital

Xiaoli Liu

Brigham and Women's Hospital

Jingjie Zhao

Capital Medical University Affiliated Beijing Friendship Hospital

Xiufen Yang

Capital Medical University Affiliated Beijing Friendship Hospital

Ran You

Capital Medical University Affiliated Beijing Friendship Hospital

Yanling Wang

Capital Medical University Affiliated Beijing Friendship Hospital

Research

Keywords: Retinal degeneration, c-Kit, Stem cell factor, Retinal ganglion cell; Crystallins

Posted Date: August 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-800933/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Frontiers in Pharmacology on March 31st, 2022. See the published version at <https://doi.org/10.3389/fphar.2022.796380>.

Abstract

Background

c-Kit/CD117, expressed in a series of tissue-specific progenitor cells, plays an important role in tissue regeneration and tissue homeostasis. We previously demonstrated that organoid-derived c-Kit⁺ retinal progenitor cells can facilitate the restoration of degenerated retina. Meanwhile, we have identified a population of endogenous c-Kit⁺ cells in retinas of adult mouse. However, the exact role of these cells in retinal degeneration remains unclear.

Methods

Retinal degeneration was induced by intravitreal injection of N-methyl-D-aspartate (NMDA). Two days post NMDA challenge, intravitreal injection of stem cell factor (SCF) was performed. Distribution and abundance of c-Kit⁺ cells and other retinal cells were evaluated by immunochemistry. Retinal function of treated mice was tested via flash electroretinogram (fERG) and the light/dark transition test. Possible regulatory pathways were evaluated by RNA sequencing.

Results

NMDA challenge increased the total number of c-Kit⁺ cells in the retinal ganglion cell layer (GCL), while slightly deregulated the protein level of SCF, which is mainly expressed in Müller cells. Both fERG and light/dark transition tests showed that intravitreal injection of SCF effectively improve the visual function of NMDA-treated mice. Consistently, the activation of microglia in injured retina has also been inhibited after SCF treatment. Mechanistically, SCF administration not only prevent the loss of retinal ganglion cells (RGCs), but also maintained the function of RGCs as quantified by fERG. Further, we performed transcriptome sequencing analysis of the retinal cells isolated from SCF-treated mice and the parallel control. Gene Ontology analysis showed that SCF-induced transcriptome changes were closely correlated with eye development-related pathways. Crystallins and several protective factors such as *Pitx3* were significantly upregulated by SCF treatment.

Conclusions

Our results revealed the role of c-Kit⁺ cells in the protection of RGCs in NMDA-treated mice, via inhibiting the loss of RGCs. Administration of SCF can act as a potent strategy for treating retinal degeneration-related diseases.

Background

The death of neurons is the leading cause of blindness in retinal degeneration [1, 2]. For example, in patients with glaucoma, retinal ganglion cells (RGCs), the neurons in the mammalian retina, undergo progressive degeneration, which leads to an irreversible vision loss [3]. Rescue of retinal neurons has been considered as an effective strategy for the retina regeneration therapy [4, 5]. Stem/progenitor cell transplantation can be differentiated into retinal neurons, while the preparation of transplantable cells is inevitably complicated [6–8]. Reprogramming Müller cells can also regenerate retinal neurons *via* virus-mediated genome editing, while it can yet be applied due to safety issues [9–13]. Neuroprotective factors, such as brain-derived neurotrophic factor (BDNF), can promote cell survival and prevent retinal neuron death, with some drawbacks such as short half-lives of these factors and the inability to cross the blood-retina barrier. Therefore, to develop a simple, safe and effective strategy to facilitate the repair of injured retina remains a key challenge.

In the previous study, we have demonstrated that c-Kit⁺ retinal progenitor cells (RPCs) would be a promising cell source for repairing the injured retina. By enriching c-Kit⁺ RPCs from human embryonic stem cell-derived retinal organoids, we found that subretinal transplantation of c-Kit⁺ RPCs into retinal degeneration models could significantly improve vision and delaying retinal degeneration [14]. Moreover, we have previously identified a population of c-Kit⁺ cells in retinas of both postnatal and adult mouse, especially containing regenerative potential during adulthood [15–17]. However, the exact role of these c-Kit⁺ cells in the retina remains unclear.

Here, we reported that activation of endogenous c-Kit⁺ cells can protect against retinal degeneration. In pathological conditions, such as N-methyl-D-aspartate (NMDA) challenge, the total number of c-Kit⁺ cells in the retina was increased, while the expression level of SCF was downregulated. Supplementation of exogenous SCF can effectively facilitate the preservation of the retinal function, by inhibiting the loss of RGCs. Transcriptome analysis showed that several eye development-related factors, such as Crystallins, were significantly upregulated by SCF treatment. In summary, our study demonstrated the protective role of SCF/c-Kit signaling on the retinal injury, and indicated exogenous SCF as a potent candidate for the treatment of retinal degeneration-related diseases.

Methods

Mice

C57BL/6J mice were provided by the Institutional Animal Care of Beijing Friendship Hospital Affiliated to Capital Medical University. Four-week-old mice (males and females) were randomly assigned to groups, and maintained under a standard 12-hour light/dark cycle at 24.5°C. All experimental procedures were approved by the Office of Research Ethics Committee at Beijing Friendship Hospital Affiliated to Capital Medical University (ethics approval number: 18-2020).

Intravitreal Injections

Animals were anaesthetized with 1.5%–2% isoflurane. Intravitreal injection of NMDA (100 mM in PBS) was performed using a sharp 32-gauge needle (micro-syringe equipped of Hamilton Storage, United States). Two days post NMDA challenge, intravitreal injection of recombinant SCF (Novus Biologicals, United States, 50 ng/ml in PBS) and anti-c-Kit neutralizing antibody (Tocris Bioscience, United Kingdom, 50 ng/ml in PBS) were performed. Mice injected with an equal volume of PBS (2 µl per eye) were served as control.

Tissue Preparation and Immunofluorescence

Immunohistochemistry was performed as described previously [16, 17]. Briefly, mouse eyeballs were prefixed in prefixation buffer (5% acetic acid, 0.4% paraformaldehyde, 0.315% saline, and 37.5% ethanol), then incubated in 4% paraformaldehyde overnight at 4°C, followed by embedded in paraffin. Eyecups were sectioned at 5 µm on a microtome (Leica, Germany). Slides were then deparaffinized, rehydrated, and boiled in 10 mM citrate buffer, followed by incubation in 5% donkey serum for 30 minutes at room temperature. Slides were then incubated with indicated primary antibodies at 4°C overnight, rinsed with PBS, and then incubated in species-matched fluorophore-conjugated secondary antibodies for 1 hour at 37°C. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were obtained using confocal microscopy of Fluo View FV1000 (Olympus, Japan). To perform the whole retina quantification, at least 6 sections across the optic disc were analyzed.

The primary antibodies used were as follows: anti-c-Kit at 10 µg/ml (AF1356, R&D Systems, United States), anti-glutamine synthetase (GS) at 1:200 (ab73593, ab64613, Abcam, United Kingdom), anti-SCF at 1:200 (ab64677, Abcam), anti-Connexin 43 (Cx43) at 1:100 (ab78055, Abcam), anti-Iba1 at 1:200 (ab178847, Abcam), anti-NeuN at 1:200 (ab209898, Abcam), and anti-Calretinin at 1:400 (MAB1568, Millipore). The secondary antibodies used were as follows: donkey anti-goat IgG Alexa Fluor 488 at 1:500 (ab150129, Abcam), donkey anti-rabbit Alexa Fluor 555 at 1:500 (ab150074, Abcam), donkey anti-mouse Alexa Fluor 555 at 1:500 (ab150106, Abcam), donkey anti-mouse Alexa Fluor 647 at 1:500 (ab150107, Abcam), donkey anti-rabbit IgG Alexa Fluor 488 at 1:500 (ab150073, Abcam), goat anti-chicken IgG Alexa Fluor 555 at 1:500 (ab150170, Abcam).

Analysis of Microglia

The method was performed as described previously [14]. Briefly, five 40× field views were captured from three 15 µm-thick retinal sections per eye using the Olympus confocal imaging system with 1-µm z-steps. By using a grid system, the number of grid-crossing points per individual microglia cell was counted ($n > 3$ eyes per group). The number of Iba1⁺ cells was counted in 5 eyes per group.

Electroretinogram Recording

Corneal scotopic flash electroretinogram (fERG) of mice was performed at corresponding time point after intravitreal injection of NMDA (at least 5 mice in each time point) as described previously [17]. Briefly, after adaption darkness overnight, mice were anesthetized with 1.5%–2% isoflurane. The animal

body temperature was maintained at 37°C by using a heating pad. The pupils of mice were dilated with tropicamide and phenylephrine eye drops (Santen Pharmaceutical, Japan). The recording electrodes of gold loops were placed on the cornea. The reference electrodes and grounding electrodes of gold needles were inserted subcutaneously into angulus oculi and tail respectively. We obtained flash recordings at the light intensities of -2.5 , -0.5 , -0.02 , and $0.5 \log(\text{cd} \cdot \text{s}/\text{m}^2)$ using Reti-scan system (Roland Consult, Germany). Waves measured at $0.5 \log_{10}(\text{cd} \cdot \text{s}/\text{m}^2)$ were presented. The fERG procedures were performed under the environment of dim red light. The amplitudes of a-wave and b-wave were analyzed among groups.

Scotopic threshold responses (STRs) were elicited using a $-4.5 \log_{10}(\text{cd} \cdot \text{s}/\text{m}^2)$ stimulus using Reti-scan system (Roland Consult) as described previously [18]. Thirty flashes with an interstimulus interval of 2s were averaged. Amplitudes of the positive STR (pSTR) and negative STR (nSTR) were measured for about 140 and 220 ms after the stimulus flash, respectively.

For photopic negative response (PhNR) analysis, flash strength was $10 \log_{10}(\text{cd} \cdot \text{s}/\text{m}^2)$, and 50 responses were averaged for each eye as described previously [19]. The PhNR was measured from baseline to the trough immediately following the b-wave.

Light/dark Transition Test

Light/dark transition test was performed as described previously [17]. The light/dark box consists of one light chamber ($45 \times 30 \times 40 \text{ cm}$) and one dark chamber ($15 \times 30 \times 40 \text{ cm}$), and these two compartments were connected with a door ($10 \times 10 \text{ cm}$). Mice were maintained in dark environment overnight, and adapted in the dark chamber for 2 minutes. The door was then opened, and mice were allowed to freely move into the light chamber for 5 minutes with 300 lux of tungsten filament bulb over the center of the compartment. All of the mice were tested naïve (only one test per mouse). Four paws completely through the door were defined as entering the light chamber. The time of exploratory behavior in the light compartment was analyzed.

Western Blotting

Eye samples were prepared after mice with euthanized. Retinas were then isolated and homogenized in an ice-cold mixture of RIPA buffer (Beyotime, China) containing protease inhibitor cocktail (Beyotime). Extracts were separated using 12% sodium dodecyl sulfate poly-acrylamide gels and transferred onto polyvinylidene fluoride membranes. Membranes were incubated in TBST (12.5 mM Tris-HCl, pH 7.6, 75 mM NaCl, 0.1% Tween 20) containing 5% fat-free milk for 1 hour at room temperature, then transferred into solution containing primary antibodies at 4°C overnight, and probed with indicated secondary antibodies in TBST for 2 hours at room temperature. Membranes were exposed on an Odyssey infrared imaging system with the Odyssey Application software V1.2.15 (LI-COR Biosciences, United States). All blots were analyzed by ImageJ (National Institutes of Health, United States). The relative levels of SCF were determined by normalizing against β -actin. The primary antibodies used were as follows: anti-SCF

at 1:1000 (ab64677, Abcam), anti- β -actin at 1:1000 (ab179467, Abcam). The secondary antibody used was peroxidase-conjugated goat anti-rabbit IgG at 1:2000 (Beyotime).

Gene Functional Annotation Analysis

For transcriptome analysis, total retinal cells were incubated in RNAiso Plus (Takara, Japan) at a concentration of 2×10^6 cells/ml and stored at -80°C . All samples were transported to the Genomics Institute on dry ice for the transcriptome study. The mRNA sample was enriched using oligo (dT) magnetic beads and fragmented into short fragments using fragmentation buffer. The corresponding cDNA libraries were produced and qualified using an Agilent 2100 Bioanalyzer and an ABI StepOnePlus Real-Time PCR System. Primary raw reads produced by HiSeq 4000 (Illumina, United States) were qualified and filtered to obtain clean reads. The Pearson correlation coefficients were based on all gene expression levels. A heatmap analysis of gene expression levels were created based on the averaged fragments per kilobase of exon per million fragments mapped (FPKM) values of genes. Genes with fold change ≥ 2 and adjusted P values ≤ 0.001 were considered as the differentially expressed genes (DEGs). Annotation analysis of Gene Ontology (GO) was performed to determine the on-going biological process. The KEGG database was used to perform pathway analysis of DEGs.

Statistical Analysis

All statistical differences were performed on SPSS 23.0 by one-way ANOVA test among comparisons groups. Data are presented as mean $\pm$ standard deviation (SD). Differences were considered as significant at $P < 0.05$.

Results

Increased Number of c-Kit⁺ Cells in NMDA-treated Mice

c-Kit, also known as CD117, is a type III receptor tyrosine kinase expressed in various types of stem cells, such as hematopoietic stem cells [20, 21]. c-Kit⁺ RPCs transplantation has been demonstrated as a potential strategy to improve vision and delay retinal degeneration. We have previously identified a population of c-Kit⁺ RPCs in the retinas of both postnatal and adult mice [16, 17]. Further, in c-Kit-Cre LacZ mice, the expression of β -galactosidase was restricted to RGCs and amacrine cells in the retina, suggesting these c-Kit⁺ cells may differentiate into retinal neurons [22]. However, the biological significance of these endogenous c-Kit⁺ RPCs in the degenerative retina remains unclear. To elucidate this issue, we examined the distribution and abundance of c-Kit⁺ cells in retina of the retinal degeneration mice model generated by NMDA injection. Consistent with previous reports, administration of NMDA led to a loss of RGCs and a decrease in the thickness of the inner plexiform layer (IPL), both of which are typical symptoms of retinal degeneration (Fig. 1A-C).

We next examined the distribution and abundance of c-Kit⁺ cells in retina of these retinal degeneration mice by immunohistochemistry. The morphology and distribution of c-Kit⁺ cells were not markedly affected by NMDA treatment (Fig. 1A-C). However, an increase in the number of c-Kit⁺ cells in both retinal ganglion cell layer (GCL) and inner nuclear layer (INL) were observed in retinas after 1 week post NMDA challenge, compared with that in the wild-type (WT) retinas, and the effect was sustained for at least 2 weeks ($P < 0.05$; Fig. 1D-E). These data confirmed that c-Kit⁺ cells indeed existed in both GCL and INL in the retina, and showed increased number of c-Kit⁺ cells in the retina of NMDA-treated mice, suggesting these cells might function in regulating the retinal degeneration.

The Expression of SCF was Slightly Downregulated after NMDA Treatment

c-Kit can be activated by its ligand stem cell factor (SCF), a growth factor that exists as a soluble or membrane-bound form [23]. The functional SCF/c-Kit signaling is critical for the survival and development of stem cells in hematopoiesis, pigmentation and reproduction [24]. We thus examined the expression of SCF in mouse retinas. In the retina of WT mice, SCF-expressing cells were mainly localized in INL as well as inner limiting membrane, adjacent to c-Kit⁺ cells (Fig. 2A). These SCF-positive cells can also express glutamine synthetase (GS), a marker of Müller cells, suggesting that in the INL, Müller cells act as an endogenous source of SCF. Moreover, the gap junction protein Connexin 43 (Cx43) was distributed between c-Kit⁺ cells and SCF-expressing Müller cells (**Additional file 1: Fig. S1**), suggesting the paracrine role of SCF/c-Kit signaling in the retina.

As NMDA challenge influenced the abundance of c-Kit⁺ cells, we then examined the expression of SCF in retinas with NDMA stimulation. Comparing to WT mice, the expression of SCF in retinas was slightly downregulated after NMDA exposure (Fig. 2B-D). Together with the previous observation showing the increased number of c-Kit⁺ cells (Fig. 1D-E), these data indicated that the insufficient activation of SCF/c-Kit signaling may be involved in the progression of NMDA-induced retinal degeneration.

Exogenous Scf Treatment Improved Visual Function Of Nmda-treated Mice

In view of the decreased SCF expression and increased number of c-Kit⁺ cells in the NMDA-induced degenerative retinas, we wondered whether exogenous SCF supplementation can compromise the retinal degeneration and improve the vision function. To do so, recombinant SCF (50 ng/ml) was intravitreally administrated at 2 days post NMDA injection, while control group received same amount of PBS or c-Kit neutralizing antibody (ac-Kit, 50 ng/ml) at the same timepoint after NMDA damage, respectively (Fig. 3A).

To detect the retinal function of indicated mice, we performed flash electroretinogram (fERG) and the light/dark transition tests at 1- and 2-week post SCF administration (Fig. 3). Mice receiving SCF showed markedly increased amplitudes of both the a-wave (Fig. 3B-C) and b-wave (Fig. 3B and 3D) at 0.5 log₁₀ (cd*s/m²; the data for the other light intensity not shown), compared with PBS and ac-Kit groups.

Moreover, in the light/dark transition test (Fig. 3E), mice receiving SCF injection for 1 and 2 weeks showed a behavioral aversion to light, and spent less time in the light chamber (Fig. 3F), indicating that exogenous SCF improved retinal function of NMDA-treated mice.

Microglia are considered as the major source of pro-inflammatory factors that contribute to retinal degeneration. Here we found that the activation of microglia has also been downregulated by SCF supplementation (Fig. 4). Based on the results of Iba1 staining, reactive microglia were mainly distributed in the GCL, IPL and outer plexiform layer (OPL) in NMDA-treated retina (Fig. 4A). However, in SCF-treated group, the number of activated microglia were significantly decreased, compared with control groups ($P < 0.05$; Fig. 4B). The morphology change is another key feature for microglia activation, we thus quantified the microglia morphology using a grid cross-counting system as reported previously [25–27]. By counting the grid-crossed points of Iba1⁺ cells, we found that SCF treatment markedly compromise the activation of the microglia. Moreover, the histogram data demonstrated that the microglia in the SCF-treated group mainly showed ramified shapes, while most microglia in the control group adopt an amoeboid morphology (Fig. 4C). Taken together, these data demonstrated that exogenous SCF treatment can inhibit the hyperactivation of microglia in the NMDA-treated retina, and thus improve the visual function.

SCF Supplementation Compensated for the Loss of RGCs in NMDA-treated Mice

The loss of RGCs, which can trigger the activation of microglia, is the leading cause of visual impairment in NMDA-induced retinal degeneration [28]. Since SCF supplementation can improve the NMDA-induced retinal degeneration, we then examined the protective role of SCF on RGCs. Both morphologic and functional assessments for RGCs were performed. As shown in Fig. 5A-B **and Additional file 2: Fig. S2**, the number of RGCs (indicated as Calretinin⁺ cells and NeuN⁺ cells) was markedly decreased after NMDA exposure. In line with previously observation (Fig. 3), SCF treatment indeed attenuated the loss of RGCs. Further, we found that the administration of SCF increased the proportion of c-Kit⁺ cells in GCL (**Additional file 3: Fig. S3**). The majority of the increased RGCs also expressed c-Kit (Fig. 5C), indicating that after SCF treatment, c-Kit⁺ cells may compensate for the loss of RGCs in NMDA mice.

We next examined the function of RGCs using specialized fERG. By comparing the ERG amplitudes recorded obtained from indicated groups, we found a significant increase in the pSTR of SCF-treated mice at 1- and 2-week post treatment ($P < 0.05$; Fig. 6A-C). Compared to the control eyes, the PhNR amplitudes in the SCF-injected eyes were consistently increased, and stayed negative for at least 2 weeks ($P < 0.05$; Fig. 6D-E). Taken together, these data demonstrated that exogenous SCF supplementation protected RGCs from NMDA-induced cell death, therefore delayed the progression of the retinal degeneration.

RNA-seq Reveals the Involvement of Key Genes for SCF Treatment

We next investigated the mechanism underlying the protective role of SCF/c-Kit signaling on retinal degeneration. To do so, we employed a parallel transcriptome analysis by RNA sequencing. Retinal cells from SCF-treated mice after 1 week were isolated as described previously [16]. The cells derived from

time-matched control retinas (NMDA plus PBS) were served as control. A total of 361 genes were found to be differentially expressed in retinal cells of SCF-treated mice, including 287 upregulated genes and 72 downregulated genes (Fig. 7A).

We then utilized the GO classification to analyzed the enriched pathways that were induced by SCF treatment. Accordingly, 361 differentially expressed genes (DEGs) identified in the present study were categorized into 14 functional groups ($P < 0.00001$). In the molecular function and biological process of GO classification categories, 1 and 13 functional groups were identified, respectively (**Additional file 4: Table S1**). SCF-treated retinas showed an enrichment in eye development-related pathways, including lens development, camera-type eye development, *etc.* (Fig. 7B), suggesting SCF stimulated c-Kit⁺ cells might contribute to RGC survival and retinal structure reconstruction.

To further analyze the DEGs, we found that the expression of multiple members from α , β and γ Crystallins family (*Cryaa*, *Cryab*, *Cryba1*, *Cryba2*, *Crygb*, *etc.*) were significantly up-regulated after SCF stimulation (Fig. 7C-D). Both α A- and α B-crystallin has been reported to protect retinal neurons from cell death [29]. Our previous research also demonstrated that the Crystallins members plays an important role in promoting the survival of RGCs [30], indicating that the upregulation of Crystallins may be a critical downstream pathway in SCF-mediated retinal protection. In addition, a series of factors that promote neuron survival were also significantly up-regulated with SCF exposure. The top10 up-regulated DEGs included *Pitx3*, *Foxe3*, *Gja3*, *Gja8*, *Wnt7a*, *Wnt7b*, *Rspo1*, *etc.* (Fig. 7E). Among them, *Pitx3*, *Foxe3* and *Gja3* are involved in neuronal degeneration, cell survival and immune modulation, and represent important candidate gene sets that regulate the retinal homeostasis [31–33]. Taken together, these RNA-seq data revealed a change in the molecular signature of the retina, and suggested that the protective effect of SCF/c-Kit pathway on NDMA-induced retinal degeneration may be mediated by both Crystallins and a series of protective factors.

Discussion

Degenerative retinal disease is one of the leading causes of vision loss, while there are currently a limited number of effective treatments available [34]. Here we found that stimulation of the endogenous c-Kit⁺ cells by SCF compromised the NMDA-induced experimental retinal injury. Mechanistically, this effect was mediated by the protection of RGCs, probably *via* the upregulation of Crystallins and neuron-protective genes such as *Pitx3*, *Foxe3* and *Gja3*. These finding suggested that applying SCF to stimulate endogenous c-Kit⁺ cells would be an effective strategy for retina therapy.

Despite the controversy regarding the role of c-Kit⁺ cardiac stem cells in the heart, various tissue-specific progenitor cells do express c-Kit, and can facilitate the tissue regeneration in response to SCF stimulation. Activation of SCF/c-Kit signaling in the progenitor cell niche stimulates several pathways mediating proliferation, survival, and migration [35]. We have previously identified a population of c-Kit⁺ RPCs in retinas of postnatal mice. Both photoreceptors in the outer nuclear layer, and retinal neurons and Müller cells in the INL are the progeny of c-Kit⁺ cells *in vivo* [16]. Further, we have demonstrated that subretinal

transplantation of c-Kit⁺ cells, either isolated from newborn mice retinas or human embryonic stem cell-derived retinal organoids, can improve the visual function in retinal degeneration mice [14, 17]. Both of these findings suggest the protective role of c-Kit⁺ cells in the degenerative retinal diseases. Intriguingly, we also found that c-Kit expression persisted at low levels in retinas of adult mice, up to 57 weeks of age [16]. However, whether SCF/c-Kit signaling in the retina of adult mice can also facilitate the restoration of the retinal function remains unclear. In the present study, we found that in degenerative retinas, the proportion of c-Kit⁺ cells increased, while the expression of c-Kit ligand SCF decreased. After supplementing SCF exogenously, the NMDA-induced RGC loss was alleviated, and reductions in visual function after NMDA treatment were ameliorated, suggesting that the SCF/c-Kit signaling contributes to the tissue homeostasis in the mice retinas. Of note, the proportion of c-Kit⁺ cells in the GCL increased significantly after SCF treatment, and the majority of c-Kit⁺ cells also express the RGC markers such as Calretinin, further confirming the protective role of SCF/c-Kit signaling against NMDA-induced RGC death.

Neuro-protective strategies could be promising to promote cell survival and prevent retinal neuron death [36]. Neuroprotective factors, including BDNF, ciliary neurotrophic factor, etc., have considerable potential to act as a powerful neuroprotective agent [37, 38]. However, significant challenges remain due to short half-lives of these factors and the inability to easily cross the blood brain or blood retina barrier [36]. Local delivery to the eye might avoid some of these limitations, while still causing increasing risk of infection and tissue injury. Here, we reported a long-lasting neuroprotective effect of SCF in the retinal degeneration mice. After a single administration of SCF, the protective effect sustained for at least two weeks, according to the reduction of RGC loss, the protection of RGC function and the restoration of visual function detected by light/dark transition test and fERG. The long-lasting effect of SCF has also been reported in the central nervous system [39]. In the traumatic brain injury (TBI) model, exogenous supplementation of SCF significantly showed superior efficacy in improving long-term functional outcome, enhancing neural plasticity, rebalancing neural structure networks disturbed by severe TBI, and promoting remyelination, as long as lasting for 21 weeks after treatment [39]. In addition, SCF has been covalently immobilized on polymeric substrate materials, such as hyaluronic acid/gelatin double network hydrogel, for the sustained release [40]. We will investigate the protective effect of these modifications of SCF in our further study.

During our study, Li et al. overexpressed SCF in photoreceptors by AAV8 virus, in attempts to treating photoreceptor degeneration [41]. In support of our findings, this study indicated the role of SCF in preventing retinal degeneration. However, we and three independent groups all suggested that in the retina, the expression of c-Kit is restricted to RGCs and amacrine cells, but not photoreceptors [22, 42, 43]. Here our data reported the pro-survival role of SCF/c-Kit signaling in NMDA-induced cytotoxicity in RGCs.

Müller cells can interact with neurons, and are responsible for the maintenance of the homeostasis of the retina. In the present study, we found that the endogenous SCF was mainly derived from Müller cells. Intriguingly, indicated by the expression of Cx43 (Gap junction alpha-1 protein), we found that c-Kit⁺ cells can interact with SCF⁺ Müller cells in the retina microenvironment, suggesting that the SCF/c-Kit

signaling may be required for the functional interactions between these cells, such as material exchange. Müller cell gliosis is another pathological hallmark in degenerative retinas, and gliotic Müller cells display an aberrant phenotype and lose the neuron-supportive functions [44]. Consistently, upon NMDA challenge, the expression level of SCF decreased in Müller cells, leading to the variation in the abundance of c-Kit⁺ cells in the GCLs. However, how these c-Kit⁺ cells and SCF⁺ Müller cells interact in the aftermath of retinal injury, and how they shape an adaptive or maladaptive overall response have not been fully explored. Further investigation is needed to demonstrate the mechanisms and functional significances underlying these interactions.

It has been documented that Wnt pathway is involved in processes of neurogenesis, dendritic development and axon guidance during development, and can inhibit neuronal damage by up-regulating anti-apoptotic proteins such as Survivin [45, 46]. *In vitro*, exogenous administration of Wnt3a can bind to the frizzled receptor and LRP5/6 on the surface of RGCs, and promote the survival and axon regeneration of RGCs by down-regulating Ripk1 and Ripk3, *etc.* [47]. With NMDA-induced RGCs injury, Wnt pathway was significantly suppressed, suggesting that Wnt-related pathway may be critical for the survival of RGCs [48]. In the present study, we found the upon SCF stimulation, Wnt7 pathway related genes such as *wnt7a*, *wnt7b* and *rspo1* were all significantly up-regulated, suggesting that this pathway may be critical for the protective effect of c-Kit⁺ cells on RGCs. Additional investigations, however, are needed to support this speculation, and both the origin and the targets in retina of Wnt7 should be addressed.

Conclusions

In summary, our study demonstrates that in the NMDA-induced retinal degeneration mice model, endogenous c-Kit⁺ cells can be stimulated by the treatment of SCF. The activation of c-Kit⁺ cells confer protection against retinal degeneration, *via* inhibiting the loss of RGCs. Administration of SCF can act as a potent strategy for treating retinal degeneration-related diseases.

List Of Abbreviations

SCF

stem cell factor; GCL, ganglion cell layer; RGC:retinal ganglion cell; BDNF:brain-derived neurotrophic factor; RPC:retinal progenitor cell; NMDA:N-methyl-D-aspartate; DAPI:4',6-diamidino-2-phenylindole; GS:glutamine synthetase; Cx43:Connexin 43; fERG:flash electroretinogram; STR:scotopic threshold response; PhNR:photopic negative response; FPKM:per million fragments mapped; DEG:differentially expressed gene; GO:Gene Ontology; SD:standard deviation; WT:wild-type; TBI:traumatic brain injury; OPL:outer plexiform layer; IPL:inner plexiform layer; INL:inner nuclear layer; ONL:outer nuclear layer

Declarations

Acknowledgements

We thank Prof. Haiwei Xu from Southwest Hospital for valuable advises on project design, and Dr. Nan Song from Beijing Friendship Hospital for generous help on data representation and manuscript revision.

Funding

This work was supported by the National Natural Science Foundation of China (No.81870686); Beijing Municipal Natural Science Foundation (No.7184201). The sponsor or funding organization had no role in the design or conduct of this research.

Availability of data and materials

The datasets from the current study are available from the corresponding author on reasonable request.

Authors' contributions

XC, SL, JZ, XY and RY contributed to the injections, immunofluorescence staining, ERG, western blotting, etc. XC, XL, and YW analyzed the data. XC and YW designed the project. XC and XL prepared the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The study protocol was approved by the Office of Research Ethics Committee at Beijing Friendship Hospital Affiliated to Capital Medical University (ethics approval number: 18-2020).

References

1. Pascolini D, Mariotti SP. Global estimates of visual impairment: 2010. *Br J Ophthalmol*. 2012;96(5):614–8. doi:10.1136/bjophthalmol-2011-300539.
2. Wong WL, Su X, Li X, Cheung CM, Klein R, Cheng CY, et al. Global prevalence of age-related macular degeneration and disease burden projection for 2020 and 2040: a systematic review and meta-analysis. *Lancet Glob Health*. 2014;2(2):e106-16. doi:10.1016/S2214-109X(13)70145-1.
3. Cuenca N, Fernandez-Sanchez L, Campello L, Maneu V, De la Villa P, Lax P, et al. Cellular responses following retinal injuries and therapeutic approaches for neurodegenerative diseases. *Prog Retin Eye Res*. 2014;43:17–75. doi:10.1016/j.preteyeres.2014.07.001.
4. Jin ZB, Gao ML, Deng WL, Wu KC, Sugita S, Mandai M, et al. Stemming retinal regeneration with pluripotent stem cells. *Prog Retin Eye Res*. 2019;69:38–56. doi:10.1016/j.preteyeres.2018.11.003.
5. Singh MS, Park SS, Albin TA, Canto-Soler MV, Klassen H, MacLaren RE, et al. Retinal stem cell transplantation: Balancing safety and potential. *Prog Retin Eye Res*. 2020;75:100779. doi:10.1016/j.preteyeres.2019.100779.

6. Mandai M, Watanabe A, Kurimoto Y, Hiramami Y, Morinaga C, Daimon T, et al. Autologous Induced Stem-Cell-Derived Retinal Cells for Macular Degeneration. *N Engl J Med*. 2017;376(11):1038–46. doi:10.1056/NEJMoa1608368.
7. Schwartz SD, Tan G, Hosseini H, Nagiel A. Subretinal Transplantation of Embryonic Stem Cell-Derived Retinal Pigment Epithelium for the Treatment of Macular Degeneration: An Assessment at 4 Years. *Invest Ophthalmol Vis Sci*. 2016;57(5):ORSFc1–9. doi:10.1167/iovs.15-18681.
8. Schwartz SD, Regillo CD, Lam BL, Elliott D, Rosenfeld PJ, Gregori NZ, et al. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *Lancet*. 2015;385(9967):509–16. doi:10.1016/S0140-6736(14)61376-3.
9. Jorstad NL, Wilken MS, Grimes WN, Wohl SG, VandenBosch LS, Yoshimatsu T, et al. Stimulation of functional neuronal regeneration from Muller glia in adult mice. *Nature*. 2017;548(7665):103–7. doi:10.1038/nature23283.
10. Jorstad NL, Wilken MS, Todd L, Finkbeiner C, Nakamura P, Radulovich N, et al. STAT Signaling Modifies Ascl1 Chromatin Binding and Limits Neural Regeneration from Muller Glia in Adult Mouse Retina. *Cell Rep*. 2020;30(7):2195 – 208 e5. doi:10.1016/j.celrep.2020.01.075.
11. Yao K, Qiu S, Wang YV, Park SJH, Mohns EJ, Mehta B, et al. Restoration of vision after de novo genesis of rod photoreceptors in mammalian retinas. *Nature*. 2018;560(7719):484–8. doi:10.1038/s41586-018-0425-3.
12. Zhou H, Su J, Hu X, Zhou C, Li H, Chen Z, et al. Glia-to-Neuron Conversion by CRISPR-CasRx Alleviates Symptoms of Neurological Disease in Mice. *Cell*. 2020;181(3):590–603 e16. doi:10.1016/j.cell.2020.03.024.
13. Hoang T, Wang J, Boyd P, Wang F, Santiago C, Jiang L, et al. Gene regulatory networks controlling vertebrate retinal regeneration. *Science*. 2020;370(6519):eabb8598. doi:10.1126/science.abb8598.
14. Zou T, Gao L, Zeng Y, Li Q, Li Y, Chen S, et al. Organoid-derived C-Kit(+)/SSEA4(-) human retinal progenitor cells promote a protective retinal microenvironment during transplantation in rodents. *Nat Commun*. 2019;10(1):1205. doi:10.1038/s41467-019-08961-0.
15. Zhou PY, Peng GH, Xu H, Yin ZQ. c-Kit(+) cells isolated from human fetal retinas represent a new population of retinal progenitor cells. *J Cell Sci*. 2015;128(11):2169–78. doi:10.1242/jcs.169086.
16. Chen X, Wang S, Xu H, Pereira JD, Hatzistergos KE, Saur D, et al. Evidence for a retinal progenitor cell in the postnatal and adult mouse. *Stem Cell Res*. 2017;23:20–32. doi:10.1016/j.scr.2017.06.010.
17. Chen X, Chen Z, Li Z, Zhao C, Zeng Y, Zou T, et al. Grafted c-kit(+)/SSEA1(-) eye-wall progenitor cells delay retinal degeneration in mice by regulating neural plasticity and forming new graft-to-host synapses. *Stem Cell Res Ther*. 2016;7(1):191. doi:10.1186/s13287-016-0451-8.
18. Holcombe DJ, Lengefeld N, Gole GA, Barnett NL. Selective inner retinal dysfunction precedes ganglion cell loss in a mouse glaucoma model. *Br J Ophthalmol*. 2008;92(5):683–8. doi:10.1136/bjo.2007.133223.

19. Jnawali A, Lin X, Patel NB, Frishman LJ, Ostrin LA. Retinal ganglion cell ablation in guinea pigs. *Exp Eye Res.* 2020;108339. doi:10.1016/j.exer.2020.108339.
20. Edling CE, Hallberg B. c-Kit—a hematopoietic cell essential receptor tyrosine kinase. *Int J Biochem Cell Biol.* 2007;39(11):1995–8. doi:10.1016/j.biocel.2006.12.005.
21. Lennartsson J, Ronnstrand L. Stem cell factor receptor/c-Kit: from basic science to clinical implications. *Physiological reviews.* 2012;92(4):1619–49. doi:10.1152/physrev.00046.2011.
22. Harada C, Guo X, Namekata K, Kimura A, Nakamura K, Tanaka K, et al. Glia- and neuron-specific functions of TrkB signalling during retinal degeneration and regeneration. *Nat Commun.* 2011;2:189. doi:10.1038/ncomms1190.
23. Cardoso HJ, Figueira MI, Socorro S. The stem cell factor (SCF)/c-KIT signalling in testis and prostate cancer. *J Cell Commun Signal.* 2017;11(4):297–307. doi:10.1007/s12079-017-0399-1.
24. Lennartsson J, Jelacic T, Linnekin D, Shivakrupa R. Normal and oncogenic forms of the receptor tyrosine kinase kit. *Stem Cells.* 2005;23(1):16–43. doi:10.1634/stemcells.2004-0117.
25. Luckoff A, Caramoy A, Scholz R, Prinz M, Kalinke U, Langmann T. Interferon-beta signaling in retinal mononuclear phagocytes attenuates pathological neovascularization. *EMBO Mol Med.* 2016;8(6):670–8. doi:10.15252/emmm.201505994.
26. Luckoff A, Scholz R, Sennlaub F, Xu H, Langmann T. Comprehensive analysis of mouse retinal mononuclear phagocytes. *Nat Protoc.* 2017;12(6):1136–50. doi:10.1038/nprot.2017.032.
27. Bian B, Zhao C, He X, Gong Y, Ren C, Ge L, et al. Exosomes derived from neural progenitor cells preserve photoreceptors during retinal degeneration by inactivating microglia. *J Extracell Vesicles.* 2020;9(1):1748931. doi:10.1080/20013078.2020.1748931.
28. Niwa M, Aoki H, Hirata A, Tomita H, Green PG, Hara A. Retinal Cell Degeneration in Animal Models. *Int J Mol Sci.* 2016;17(1):110. doi:10.3390/ijms17010110.
29. Piri N, Kwong JM, Gu L, Caprioli J. Heat shock proteins in the retina: Focus on HSP70 and alpha crystallins in ganglion cell survival. *Prog Retin Eye Res.* 2016;52:22–46. doi:10.1016/j.preteyeres.2016.03.001.
30. Zou AL, He T, Chen J, Sun X, Fan D. X, et al. Rescue of Retinal Degeneration in rd1 Mice by Intravitreally Injected Metformin. *Front Mol Neurosci.* 2019;12:102. doi:10.3389/fnmol.2019.00102.
31. Akula M, Park JW, West-Mays JA. Relationship between neural crest cell specification and rare ocular diseases. *J Neurosci Res.* 2019;97(1):7–15. doi:10.1002/jnr.24245.
32. Anand D, Agrawal SA, Slavotinek A, Lachke SA. Mutation update of transcription factor genes FOXE3, HSF4, MAF, and PITX3 causing cataracts and other developmental ocular defects. *Hum Mutat.* 2018;39(4):471–94. doi:10.1002/humu.23395.
33. Li H, Jiang H, Rong R, Jiang J, Ji D, Song W, et al. Identification of GJA3 p.S50P Mutation in a Chinese Family with Autosomal Dominant Congenital Cataract and Its Underlying Pathogenesis. *DNA Cell Biol.* 2020;39(10):1760–6. doi:10.1089/dna.2020.5605.

34. Ahmad I, Teotia P, Erickson H, Xia X. Recapitulating developmental mechanisms for retinal regeneration. *Prog Retin Eye Res.* 2020;76:100824. doi:10.1016/j.preteyeres.2019.100824.
35. Stankov K, Popovic S, Mikov M. C-KIT signaling in cancer treatment. *Curr Pharm Des.* 2014;20(17):2849–80. doi:10.2174/13816128113199990593.
36. Pardue MT, Allen RS. Neuroprotective strategies for retinal disease. *Prog Retin Eye Res.* 2018;65:50–76. doi:10.1016/j.preteyeres.2018.02.002.
37. Du R, Wang X, He S. BDNF improves axon transportation and rescues visual function in a rodent model of acute elevation of intraocular pressure. *Sci China Life Sci.* 2020;63(9):1337–46. doi:10.1007/s11427-019-1567-0.
38. Dulz S, Bassal M, Flachsbarth K, Riecken K, Fehse B, Schlichting S, et al. Intravitreal Co-Administration of GDNF and CNTF Confers Synergistic and Long-Lasting Protection against Injury-Induced Cell Death of Retinal Ganglion Cells in Mice. *Cells.* 2020;9(9):2082. doi:10.3390/cells9092082.
39. Qiu X, Ping S, Kyle M, Chin L, Zhao LR. Long-term beneficial effects of hematopoietic growth factors on brain repair in the chronic phase of severe traumatic brain injury. *Exp Neurol.* 2020;330:113335. doi:10.1016/j.expneurol.2020.113335.
40. Zhang Y, Pan X, Shi Z, Cai H, Gao Y, Zhang W. Sustained release of stem cell factor in a double network hydrogel for ex vivo culture of cord blood-derived CD34(+) cells. *Cell Prolif.* 2018;51(2):e12407. doi:10.1111/cpr.12407.
41. Li H, Lian L, Liu B, Chen Y, Yang J, Jian S, et al. KIT ligand protects against both light-induced and genetic photoreceptor degeneration. *Elife.* 2020;9:e51698. doi:10.7554/eLife.51698.
42. Koso H, Satoh S, Watanabe S. c-kit marks late retinal progenitor cells and regulates their differentiation in developing mouse retina. *Dev Biol.* 2007;301(1):141–54. doi:10.1016/j.ydbio.2006.09.027.
43. Morii E, Kosaka J, Nomura S, Fukuda Y, Kitamura Y. Demonstration of retinal cells expressing messenger RNAs of the c-kit receptor and its ligand. *Neurosci Lett.* 1994;166(2):168–70.
44. Pfeiffer RL, Marc RE, Jones BW. Persistent remodeling and neurodegeneration in late-stage retinal degeneration. *Prog Retin Eye Res.* 2020;74:100771. doi:10.1016/j.preteyeres.2019.07.004.
45. Kassumeh S, Weber GR, Nobl M, Priglinger SG, Ohlmann A. The neuroprotective role of Wnt signaling in the retina. *Neural Regen Res.* 2021;16(8):1524–8. doi:10.4103/1673-5374.303010.
46. Shi W, Tang Y, Zhi Y, Li Z, Yu S, Jiang J, et al. Akt inhibition-dependent downregulation of the Wnt/beta-Catenin Signaling pathway contributes to antimony-induced neurotoxicity. *Sci Total Environ.* 2020;737:140252. doi:10.1016/j.scitotenv.2020.140252.
47. Udeh A, Dvorianchikova G, Carmy T, Ivanov D, Hackam AS. Wnt signaling induces neurite outgrowth in mouse retinal ganglion cells. *Exp Eye Res.* 2019;182:39–43. doi:10.1016/j.exer.2019.03.004.
48. Boesl F, Drexler K, Muller B, Seitz R, Weber GR, Priglinger SG, et al. Endogenous Wnt/beta-catenin signaling in Muller cells protects retinal ganglion cells from excitotoxic damage. *Mol Vis.* 2020;26:135–49.

Figures

Figure 1

Increased Number of c-Kit+ Cells in NMDA-treated Mice. (A-C) Representative images of c-Kit+ cell staining (green) in retinas of indicated mice with NMDA exposure. Area in the white boxes (A-C) is shown at higher magnification and displayed to the right. White arrow, the c-Kit+ cell body in the retinal ganglion

cell layer (GCL); white arrowhead, the c-Kit⁺ cell body in the inner nuclear layer (INL). WT, wild-type; IPL, inner plexiform layer, OPL, outer plexiform layer; ONL, outer nuclear layer; DAPI, 4', 6-Diamino-2-Phenylendole. Scale bars represent 200 μ m of left panel, 20 μ m of right panel. (D-E) The total number of c-Kit⁺ cells in the GCL (D) and INL (E) were then quantitated and plotted as the mean $\pm$ standard deviation (SD) per slice ($n \geq 5$ for each time point). * $P < 0.05$ vs. WT, ** $P < 0.01$, *** $P < 0.001$.

Figure 2

The Expression of SCF was Slightly Downregulated after NMDA Treatment. (A) c-Kit-positive cells (green), glutamine synthetase (GS)-positive cells (red) and SCF-positive cells (white) located in the INL. The white box in left panel is shown at higher magnification in right panels. (B) Immunofluorescence staining of SCF (white) and GS (red) in the retinas of WT and NMDA-treated mice. Scale bars represent 20 μ m. (C-D) The expression of SCF were analyzed by western blotting analysis (C) and quantitated by ImageJ and plotted as the mean expression $\pm$ SD ($n = 3$ eyes/group, ** $P < 0.01$ vs. WT) (D). The level of SCF was corrected for β -actin loading control and normalized to the levels of control cells.

Figure 3

Exogenous SCF Treatment Improved Visual Function of NMDA-treated Mice. (A) Scheme of time points for intravitreal injections, function test and histology analysis. (B) Representative waves of three groups of mice measured through flash electroretinogram (fERG) tests at 0.5 log(cd*s/m²). (C-D) Statistical analysis of the amplitudes of fERG a-wave (C) and b-wave (D) in the indicated groups. (E) Diagram showing the setup of the light/dark transition test. (F) Time spent in the light compartment was recorded.

Data are shown as the mean $\pm$ SD ($n \geq 5$ for each time point). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with NMDA + PBS controls.

Figure 4

Activation of Microglia was Suppressed by SCF Supplementation. (A) Immunofluorescence detection of the morphology and distribution proportion of Iba1+ cells (red) in indicated mice. Scale bars represent 20 μ m. (B) Number of Iba1+ cells were quantitated in each group ($n \geq 5$ for each time point). (C) Representative images of ramified (left panel) or ameboid-like (right panel) Iba1+ microglia in grid crossing of each grid with a side length of 3 μ m. Statistical analysis of the grid-crossing points per microglia ($n \geq 21$ per group). The data are shown as mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$, compared with NMDA + PBS controls.

Figure 5

SCF Treatment Compensated for the Loss of RGCs in NMDA-treated Mice. (A) Immunofluorescence detection of Calretinin+ RGCs (red) in the GCL of indicated mice. White arrow, the Calretinin+/c-Kit+ cell in the GCL; white arrowhead, the Calretinin+/c-Kit- cell in the GCL. Scale bars represent 20 μ m. (B) Statistical analysis of the number of Calretinin+ RGCs in indicated groups. (C) Statistical analysis of the ratio of c-Kit+ cells in Calretinin+ RGCs in indicated groups. Data are shown as mean $\pm$ SD ($n \geq 5$ for each time point). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with NMDA + PBS controls.

Figure 6

SCF Treatment Maintained the Function of RGCs in NMDA-treated Mice. (A) Specialized fERG tests for detecting the function of RGCs were performed at designed timepoints. Representative waves of positive scotopic threshold responses (pSTR) and negative STR (nSTR) in these groups. (B-C) Amplitudes of pSTR (B) and nSTR (C) were compared among groups. (D) Representative waves of photopic negative response (PhNR). (E) Statistical analysis of PhNR amplitudes among groups. Data are shown as mean $\pm$ SD ($n \geq 5$ for each time point). * $P < 0.05$, ** $P < 0.01$, compared with the control group (NMDA + PBS).

Figure 7

RNA-seq Reveals the Involvement of Key Genes for SCF Treatment. (A) Volcano plot of differentially expressed genes (DEGs) in SCF group compared with controls. (B) Gene Ontology (GO) analysis showing the enriched gene functions of SCF group versus control group. (C) Heatmap analysis showing DEGs in indicated groups. The relative abundance of each genus is indicated by a gradient of color from green (low abundance) to red (high abundance). (D) Crystallins family members were significantly up-regulated in SCF treated retinas. (E) Among top10 DEGs in SCF exposed mice, several neuro-protective factors were up-regulated, including Wnt7 related genes, Foxe3/Pitx3, and gap junction protein coding genes Gja8/Gja3.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [FigureS1.png](#)

- [FigureS2.png](#)
- [FigureS3.png](#)
- [TableS1.xlsx](#)