Physiology and Pharmacology

CGRP Released by Corneal Sensory Nerve Maintains Tear Secretion of the Lacrimal Gland

Linyan Ma,[1,2] Lingling Yang,[2] Xiaolei Wang,[2] Leilei Zhao,[1,2] Xiaofei Bai,[2] Xia Qi,[2] Qing Chen,[3] Ya Li,[2] and Qingjun Zhou[2]

[1]Medical College, Qingdao University, Qingdao, China
[2]State Key Laboratory Cultivation Base, Shandong Provincial Key Laboratory of Ophthalmology, Shandong Eye Institute, Shandong First Medical University & Shandong Academy of Medical Sciences, Qingdao, China
[3]Department of Ophthalmology, West China Hospital of Sichuan University, Chengdu, China

Correspondence: Qingjun Zhou, 5 Yan'er dao Road, Qingdao 266071, China; qjzhou2000@hotmail.com.
Ya Li, 5 Yan'er dao Road, Qingdao 266071, China; liyaleah@126.com.

Received: November 6, 2023
Accepted: March 27, 2024
Published: April 18, 2024

Citation: Ma L, Yang L, Wang X, et al. CGRP released by corneal sensory nerve maintains tear secretion of the lacrimal gland. *Invest Ophthalmol Vis Sci.* 2024;65(4):30. https://doi.org/10.1167/iovs.65.4.30

Purpose. This study aims to elucidate the calcitonin gene-related peptide (CGRP) mediation and primary mechanism of corneal sensory nerves on tear production of the lacrimal gland.

Methods. Mouse corneal denervation models were constructed through surgical axotomy, pharmacologic treatment with capsaicin or resiniferatoxin, and *Trpv1-Cre/DTR* mice with diphtheria toxin injection. The capsaicin-treated mice received subconjunctival injection of CGRP or substance P, while the normal C57BL/6J mice were administered with CGRP receptor antagonist BIBN-4096. Furthermore, double immunostaining of c-FOS$^+$ and choline acetyltransferase was used to evaluate the activation of the superior salivatory nucleus (SSN). Mouse lacrimal glands were collected for transcriptomic sequencing and subsequent RNA and protein expression analysis.

Results. The corneal denervated mice exhibited a significant reduction in corneal sensitivity and tear secretion. In capsaicin-treated mice, tear secretion decreased to 2.5 ± 0.5 mm compared to 6.3 ± 0.9 mm in control mice ($P < 0.0001$). However, exogenous administration of CGRP in capsaicin-treated mice increased tear secretion from 2.6 ± 0.5 mm to 4.5 ± 0.5 mm ($P = 0.0009$), while BIBN-4096 treatment reduced tear secretion to 3.4 ± 0.5 mm when compared to 7.3 ± 0.7 mm in control mice ($P = 0.0022$). Furthermore, c-FOS$^+$ cell number in the SSN increased by twofold ($P = 0.0168$) after CGRP administration compared with capsaicin-treated mice. In addition, the expressions of CCNA2, Ki67, PCNA, and CDK1 in acinar cells of the lacrimal gland were impaired by corneal denervation and alleviated by CGRP administration.

Conclusions. CGRP released by corneal sensory nerves mediates tear secretion of the lacrimal gland, providing a new strategy for improving tear secretion in patients with neurotrophic keratitis.

Keywords: cornea, TRPV1, lacrimal gland, tear secretion, CGRP

Cornea possesses dense sensory nerve fibers originating from the trigeminal ganglion.[1] The abundant innervations are involved in the regulations of corneal sensations, tear secretion, blink reflex, and wound healing.[2–5] As the major tear components, the fluid secreted from lacrimal gland is vital for the aqueous layer of tear film to moisturize and maintain a healthy ocular surface.[6] Tear secretion is tightly controlled by the afferent sensory nerves from the cornea and conjunctiva, as well as the efferent autonomic nerves innervating the lacrimal gland and meibomian glands.[7–9] When the cornea receives external stimulation, sensory afferent nerves transmit impulses to the trigeminal ganglia and integrate in the brain, where the central ending synapses with the superior salivatory nucleus (SSN).[10,11] The SSN projects the efferent parasympathetic signals toward the lacrimal gland to trigger rapid secretion of lacrimal fluid, including water, electrolytes, and proteins.[12–14]

With the tight control of the neural response, the dysfunction of the afferent signal evokes the reduction of tear production and dry eye diseases.[14] The afferent nerve dysfunctions, such as trigeminal ganglia damage, herpes simplex keratitis, and diabetes mellitus, will cause partial and even complete corneal denervation.[15–18] In cornea, approximately 70% of nociceptors are polymodal nociceptors, which respond to chemical, mechanical, and thermal stimuli.[19] Moreover, the activation of corneal polymodal nociceptors evokes an augmented lacrimal secretion, compared with corneal mechanonociceptors and cold receptors.[20] Among corneal polymodal nociceptors, the transient receptor potential vanilloid 1 (TRPV1) plays a crucial role in sensory transmission and reflex tearing.[21,22] Resiniferatoxin (RTX) and capsaicin are potent agonists to stimulate TRPV1$^+$ nerves.[23,24] By using capsaicin and TRPV1 knockout mice, previous research demonstrated that the corneal TRPV1 sensory nerve played a predominant role in the

maturation of neonatal tearing.[22] However, the mechanism of corneal TRPV1 nerves on the regulation of tear secretion remains unclear.

Corneal sensory nerves mediate wound healing, inflammatory responses, and neovascularization by releasing calcitonin gene-related peptide (CGRP) and substance P (SP).[1,25,26] CGRP has been found to accelerate wound healing by promoting the growth of corneal epithelial cells and restore corneal sensitivity.[27–29] CGRP is similarly involved in the conduction of pain transmission and blood vessel dilation.[30,31] Moreover, SP promotes diabetic corneal wound healing by enhancing the proliferation and stemness of limbal stem cells of the mouse neurotrophic keratopathy model.[32] However, as the major neuropeptides secreted by sensory nerves, the role of CGRP and SP in the regulation of tear secretion remains undefined.

Corneal TRPV1 sensory nerve is not only crucial for the regulation of corneal homeostasis and wound healing but also important for corneal sensation and maturation of lacrimal function for tear secretion.[20,22,33] Combined with the major neuropeptides of CGRP and SP, we hypothesize that the CGRP or SP released by the corneal sensory nerve may be involved in the regulation of tear secretion from the lacrimal gland. Therefore, this study aimed to confirm the hypothesis and investigate this potential mechanism by using various corneal denervation models, including surgical denervation, and topical application of RTX or capsaicin, as well as TPRV1 neuronal ablation mice. Moreover, we explored the important neuropeptide in regulating tear secretion by subconjunctival injection of CGRP or SP in capsaicin-treated mice. To identify the functional changes of the lacrimal gland after corneal denervation, we performed transcriptomic sequencing comparative analysis of the lacrimal gland in capsaicin-treated mice with or without CGRP administration.

Materials and Methods

Mice

Adult male C57BL/6J mice (6 to 8 weeks old) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Trpv1-Cre$^{+/+}$ (strain number: #017769) and ROSA26iDTR$^{+/+}$ mice (strain number: #007900) were acquired from the Jackson Laboratory (Bar Harbo, Maine, USA). To deplete TRPV1 neurons, Trpv1-Cre$^{+/+}$ mice were crossbred with ROSA26iDTR$^{+/+}$ mice, resulting in TRPV1 neuron-depleted mice (*Trpv1-Cre/DTR*). The mice were bred and housed in the animal center of the Eye Institute of Shandong First Medical University. All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Ethics Committee of the Eye Institute of Shandong First Medical University.

Experimental Models

The corneal sensory nerve is derived from the short ciliary nerves of the ciliary ganglion and the long ciliary nerves from the ophthalmic branch of the trigeminal ganglion.[1] To induce the surgical denervation model, C57BL/6J mice were anesthetized with by intraperitoneal injection of 0.6% pentobarbital sodium and topical administration of 2% lidocaine. Then, the ciliary nerves were separated at the posterior sclera close to the optic nerve and squeezing for 45 seconds with forceps.[34] The surgical denervation model exhibited an immediate loss of the basal nerve in axotomized eyes and provided a model for investigating the relationship between the sensory nerve and tear secretion. Corneal sensitivity and tear secretion volume were assessed using a Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, Chartres Cedex, France) and phenol red thread (#30059010; Jingming, Tianjin, China).

High concentrations of capsaicin or RTX cause an excessive influx of calcium ions, which lead to the ablation of TRPV1-expressing afferent terminals.[35,36] To induce the TRPV1^{+} sensory denervated models, capsaicin (3 µg/µL; Selleckchem, Houston, TX, USA, dissolved in saline with 0.20% DMSO, 0.13% PEG300, and 1.68% Tween 80) or RTX (0.5 µg/µL; Sigma, St. Louis, MO, USA) was topically applied to C57BL/6J mice.[37] Moreover, CGRP (10.5 µM; Phoenix Pharmaceuticals, Mountain View, CA, USA) or SP (15 µM; Med Chem Express, Princeton, NJ, USA) dissolved in PBS was subconjunctivally injected 24 hours before and immediately prior to capsaicin or RTX treatment to regulate the tear secretion.

Trpv1-Cre/DTR mice express the diphtheria-toxin receptor (DTR) under the control of the TRPV1 regulatory sequence. To induce TRPV1 neuronal ablation models, diphtheria toxin (DT, 8 µg/kg; Sigma) or PBS was intraperitoneally injected to *Trpv1-Cre/DTR* mice for 5 consecutive days, followed by 2 days off, over a 3-week period.[38]

In the RAMP1 antagonistic model, C57BL/6J mice were subconjunctivally injected with BIBN-4096 (2 µg/µL; Med Chem Express) for 3 continuous days. Mice received saline as a vehicle control.

Immunofluorescence Staining

For whole-mount immunostaining, the cornea was dissected and fixed in 4% polyformaldehyde (Biosharp Life Sciences, Guangzhou, China) for 1 hour on ice. The cornea was then permeabilized and blocked in PBS containing 0.3% Triton X-100 (Solarbio, Beijing, China) and 5% bovine serum albumin (BSA; Solarbio) overnight at 4°C. The next day, the cornea was incubated with APC-conjugated anti–βIII-tubulin antibody (BioLegend, San Diego, CA) for 12 hours at 4°C. Finally, the tissues were examined using the Zeiss LSM880 inverted microscope (Carl Zeiss, Jena, Germany).

Mice that received subconjunctival injections of PBS or CGRP 24 hours prior were treated with capsaicin, while the control mice that had subconjunctival injections of PBS 24 hours prior were treated with the vehicle of capsaicin. The mice were then deeply anesthetized with 0.6% pentobarbital sodium 1 hour later and perfused transcardially with PBS (20 mL), followed by fixation with 4% polyformaldehyde (10 mL). The brain tissue was collected and postfixed in 4% polyformaldehyde overnight at 4°C. Afterward, it was dehydrated in 20% sucrose for 2 days at 4°C and embedded in optimal cutting temperature (OCT) compound (Sakura, Torrance, CA, USA). For immunostaining, brain sections (25 µm) were premeabilized with 0.1% Triton X-100 for 15 minutes, followed by blocking with 1% BSA and 10% donkey serum (Solarbio) for 1 hour at room temperature. Then, the sections were incubated overnight at 4°C with rabbit α-c-FOS antibody (ab222699; Abcam, Cambridge, MA, USA) and mouse α-choline acetyltransferase (ChAT) antibody (AMAB91130; Sigma).

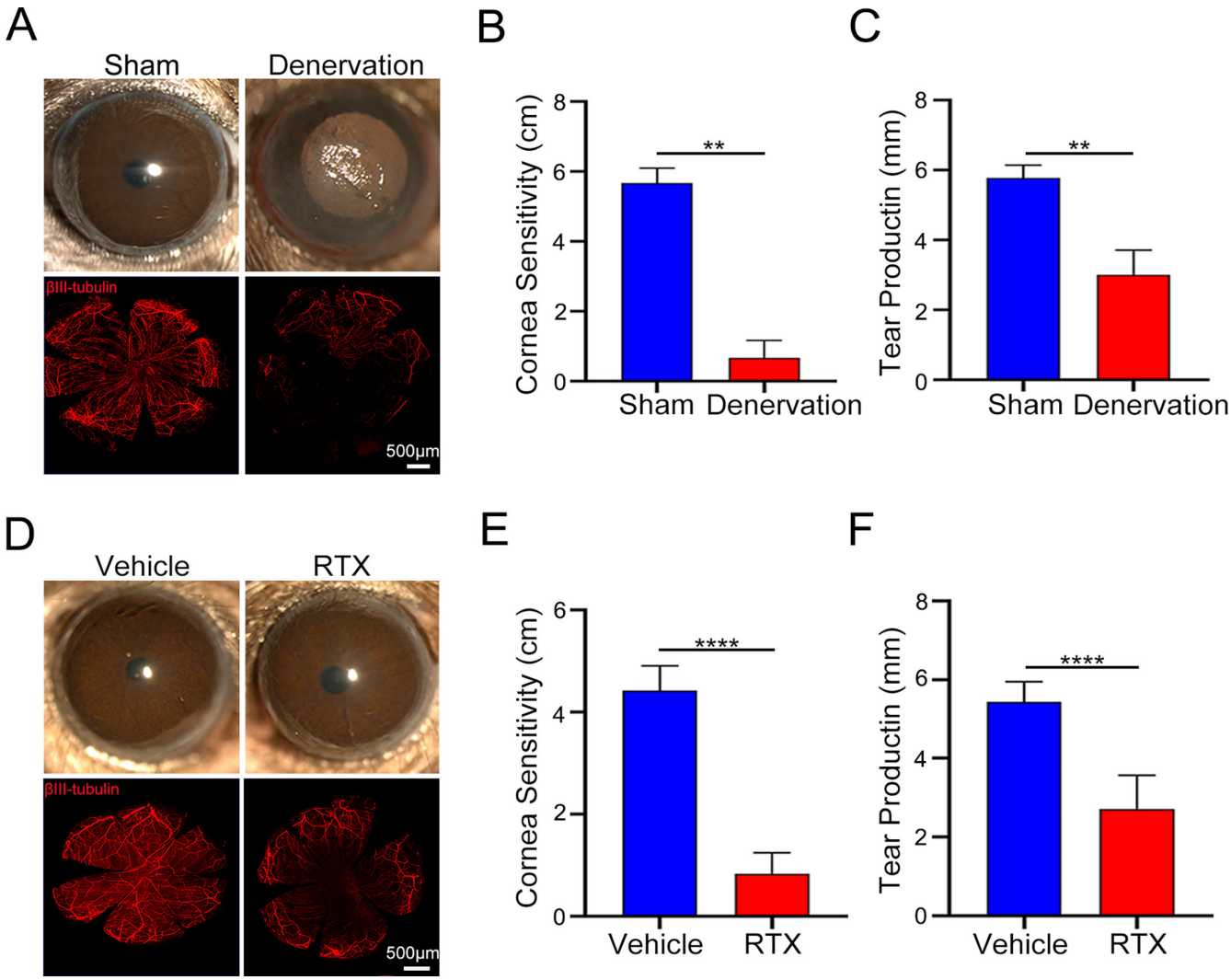

Figure 1. Tear secretion of mice with surgical and pharmacological denervation. (**A–C**) The ocular surface, corneal nerve density, and corneal sensitivity, as well as tear secretion of surgical denervated mice ($n = 6$ per group). (**D–F**) The ocular surface, corneal nerve density, and corneal sensitivity, as well as tear secretion, of RTX-treated mice ($n = 6$ per group). Data are shown as mean ± SD (**$P < 0.01$, *** *$P < 0.0001$).

The extraorbital lacrimal gland was collected and fixed in 4% polyformaldehyde for 1 hour on ice, followed by overnight dehydration in 10% sucrose at 4°C. Subsequently, the lacrimal gland was embedded in OCT compound. The frozen sections (7 μm) were fixed in 4% polyformaldehyde for 15 minutes, premeabilized with 0.3% Triton X-100 for 15 minutes, and then blocked with 5% BSA for 1 hour at room temperature. The sections were incubated overnight at 4°C with mouse α-Mist1 (SC80984; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit α-CCNA2 (18202-1-AP; Proteintech, Chicago, IL, USA), rabbit α-Ki67 (ab16667; Abcam), rabbit α-PCNA (10205-2-AP; Proteintech), or rabbit α-CDK1 (19532-1-AP; Proteintech). Following this, the sections were incubated with Alexa Fluor 488–labeled donkey anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA, USA) and Alexa Fluor 594–labeled donkey anti-mouse IgG antibody (Invitrogen) for 2 hours at room temperature. After staining with 4′,6-diamidino-2-phenylindole (DAPI, C0065; Solarbio) for 5 minutes, all stainings were observed and captured using the Zeiss LSM880 inverted microscope.

ELISA Analysis

At 24 hours after capsaicin treatment, the cornea was collected and the levels of CGRP (Bertin Pharma/SPIbio, Montigny le Bretonneux, France) and SP (Cayman, Ann Arbor, MI, USA) were analyzed by ELISA kits following the manufacturer's instructions. The total protein concentration was measured using the bicinchoninic acid kit (Beyotime, Shanghai, China).

RNA Extraction and PCR Analysis

Total RNA was extracted using Nucleospin RNA Kits (Transgen, Beijing, China) and cDNA was synthesized with the HiScript III RT SuperMix kit (Vazyme, Nanjing, China). Quantitative PCR was performed with SYBR Green reagents (Roche, Mannheim, Germany) on the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers used are listed in Supplementary Table S1.

Western Blotting

The total protein of 40 µg was separated by 10% SDS-PAGE, transferred onto the methanol-pretreated polyvinylidene difluoride membranes (Millipore, Darmstadt, Germany), and immunoblotted with α-CCNA2 antibody, α-PCNA antibody, or α-β-actin antibody (Proteintech) for 2 hours at room temperature. The membranes were then incubated with the horseradish peroxidase–conjugated secondary antibody (Proteintech) and the signal was visualized by chemiluminescence using the ECL kit (Millipore).

RNA Sequencing and Bioinformatics Analysis

Lacrimal glands were individually collected from control, capsaicin-treated, and capsaicin-treated mice supplemented with CGRP. Subsequently, these samples were preserved in liquid nitrogen. The transcriptome sequencing and subsequent analysis were conducted by OE Biotech Co., Ltd. (Shanghai, China). The clean reads were aligned to the mouse genome using HISAT2.[39] Differential expression analysis was carried out using DESeq2 R package.[40] The significance threshold for identifying significantly differentially expressed genes (DEGs) was set at a log base twofold change of $\geq$1 and adjusted P value <0.05. Utilizing the hypergeometric distribution, enrichment analyses of DEGs were conducted for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways, using clusterProfiler R package (version 3.2.0).[41] Additionally, gene set enrichment analysis (GSEA) was performed employing GSEA software.[42] The volcano plots and heatmaps were developed to draw the DEGs.

Statistical Analysis

The statistical analyses were performed with GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). The Mann–Whitney U test was employed to compare the two groups for nonparametric variables. For multiple comparisons, one-way ANOVA was performed, followed by either the Tukey honestly significant difference test or Dunnett test. Differences between measurement variables were considered significant if the P value was $\leq$0.05.

Results

Corneal Denervation Causes the Reduction of Tear Secretion

To explore the correlation of corneal innervations and tear secretion, we first adopted the surgical denervation model and examined corneal lesions. The results showed that surgical denervation led to the corneal surface roughness and reduced corneal nerve fibers (Fig. 1A), accompanied with the significant reduction of corneal sensitivity (Fig. 1B). Furthermore, tear secretion of denervated mice decreased significantly to 2.7 ± 0.6 mm, compared to 5.4 ± 0.4 mm in the

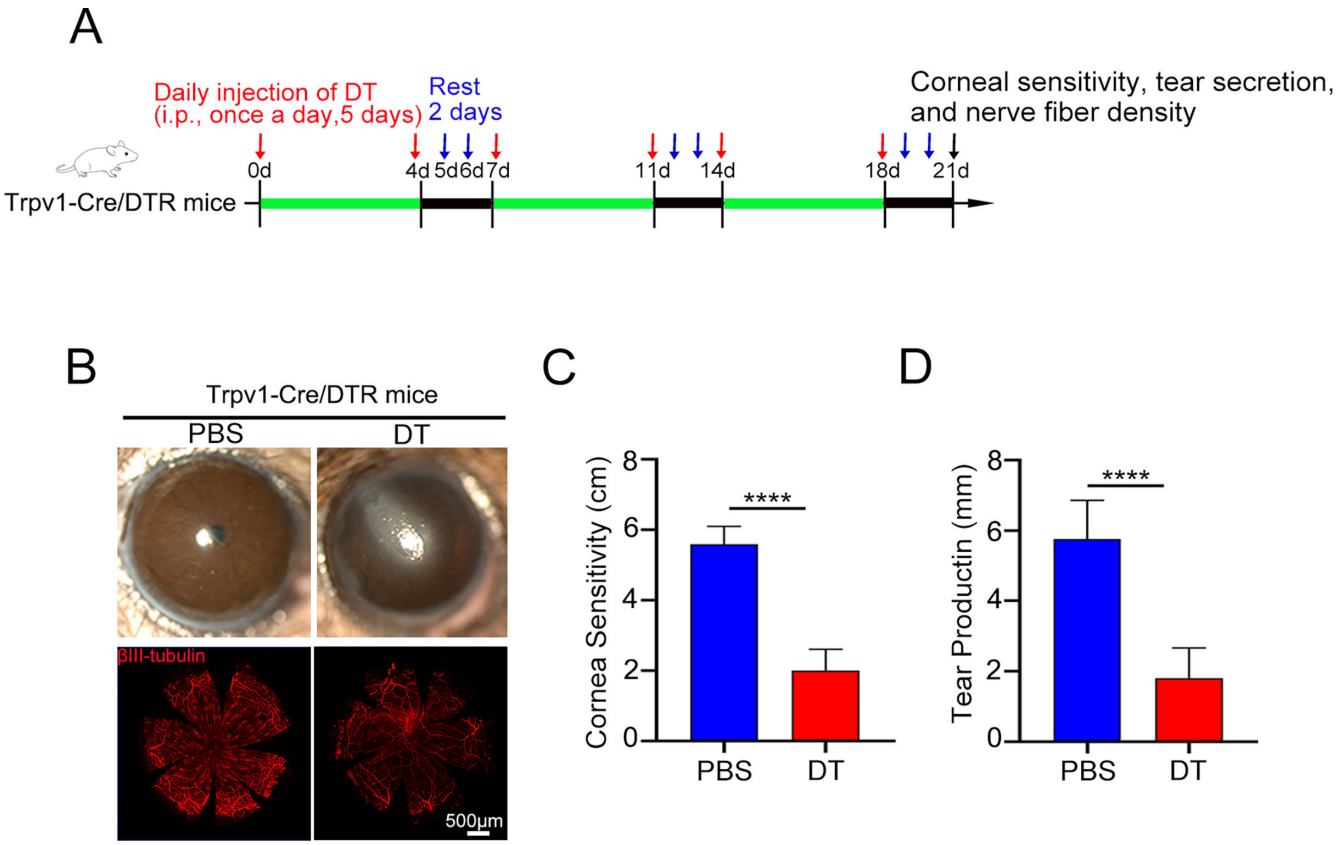

Figure 2. Tear secretion of mice with TRPV1 neuronal ablation. (**A**) Schematic diagram illustrating the intraperitoneal injection of DT into *Trpv1-Cre/DTR* mice. (**B–D**) The ocular surface, corneal nerve density, and corneal sensitivity, as well as tear secretion, of *Trpv1-Cre/DTR* mice injected with PBS or DT (n = 6 per group). Data are shown as mean ± SD (****P < 0.0001).

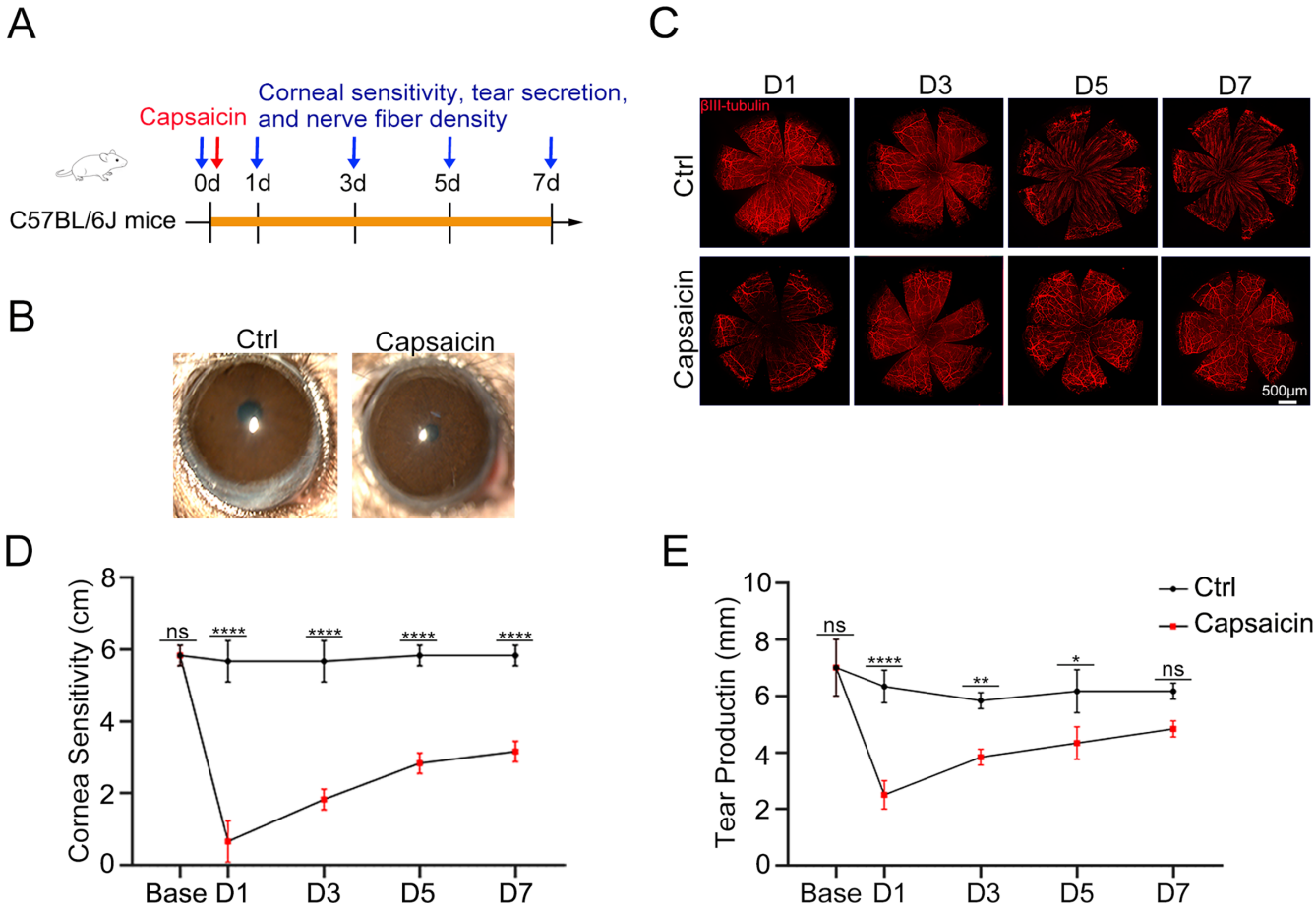

Figure 3. Corneal nerve regeneration and tear secretion in capsaicin-treated mice. (**A**) A schematic diagram illustrating the topically administration of capsaicin in mice. (**B**) The ocular surface of capsaicin-treated mice on day 1. (**C–E**) Corneal nerve density, corneal sensitivity, and tear secretion of mice treated with capsaicin ($n = 3$ per group). Data are shown as mean ± SD (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

control group that underwent sham surgery ($P = 0.0022$) (Fig. 1C).

To specifically ablate TRPV1$^+$ corneal sensory nerves, normal mice were applied with topical RTX treatment according to a previous description.[36] Pharmacologic denervation resulted in significant degeneration of corneal nerves and reduced corneal sensitivity, while maintaining an intact ocular surface (Figs. 1D, 1E). The tear secretion of mice treated with RTX was significantly reduced to 2.7 ± 0.8 mm, compared to 5.4 ± 0.5 mm in the control group ($P < 0.0001$) (Fig. 1F).

TRPV1 Neuronal Ablation Causes the Reduction of Tear Secretion

To understand the correlation of TRPV1 neurons and tear production, we further used the *Trpv1-Cre/DTR* mice with intraperitoneal DT injection to ablate TRPV1 neurons and corneal nerve fibers, with PBS injection as vehicle control (Fig. 2A). The *Trpv1-Cre/DTR* mice with DT injection also exhibited the corneal surface roughness and reduced corneal nerve fibers, as well as the decreased corneal sensitivity (Figs. 2B, 2C). The tear secretion of mice injected with DT was significantly reduced to 1.8 ± 0.5 mm, compared to 5.7 ± 1 mm in the control group injected with PBS ($P < 0.0001$) (Fig. 2D).

Corneal Nerve Regeneration Accompanies Elevated Tear Secretion

To further elucidate the relationship between corneal nerve recovery and tear secretion, we utilized capsaicin to induce temporary regression of corneal TRPV1 nerves (Fig. 3A). Following the treatment of capsaicin, the corneal nerve fiber density and sensitivity were decreased significantly on day 1, but the ocular surface remained intact (Figs. 3B–D). By day 7, the corneal nerve density largely recovered, accompanied by an increase in corneal sensitivity (Figs. 3B–D). Consequently, the tear secretion of capsaicin-treated mice decreased to 2.5 ± 0.5 mm on day 1 compared to 6.3 ± 0.9 mm in control mice ($P < 0.0001$). However, on day 7, the tear secretion of mice treated with capsaicin increased to 4.6 ± 0.2 mm, with no significant difference in tear secretion compared to the control group ($P = 0.765$) (Fig. 3E).

CGRP Mediates Corneal Sensory Nerve–Controlled Tear Secretion

To identify changes of the main neuropeptides secreted by TRPV1 sensory nerves, we analyzed the levels of CGRP and SP after capsaicin treatment (Fig. 4A). The results revealed a reduction in CGRP levels from 762.9 to 267 pg/mg ($P < 0.0001$) after capsaicin treatment, while SP decreased from 305.1 to 216.9 pg/mg ($P = 0.0005$) (Fig. 4B). We then

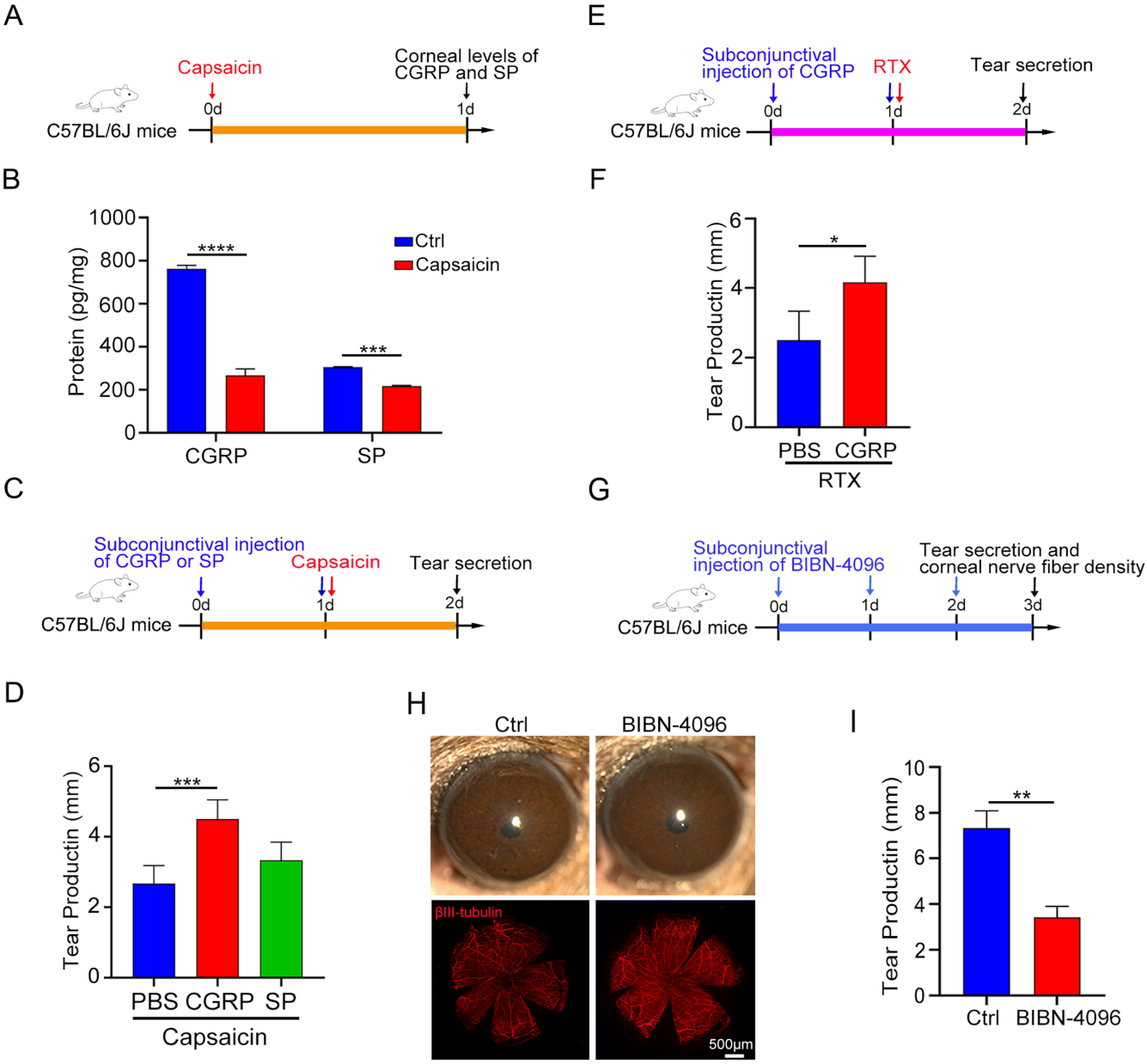

Figure 4. Mediation of CGRP in the regulation of tear secretion. (**A**, **B**) Schematic diagram and corneal levels of CGRP and SP after capsaicin treatment ($n = 3$ per group). (**C**, **D**) Schematic diagram and tear secretion of capsaicin-treated mice with CGRP or SP administration ($n = 6$ per group). (**E**, **F**) Schematic diagram and tear secretion of RTX-treated mice with CGRP administration ($n = 6$ per group). (**G–I**) Schematic diagram, corneal nerve density, and tear secretion of C57BL/6J mice with BIBN-4096 treatment ($n = 6$ per group). Data are shown as mean ± SD (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

assessed the therapeutic effects of CGRP and SP on tear secretion in the capsaicin-treated mice (Fig. 4C). The administration of CGRP in the capsaicin-treated mice led to an increase in tear secretion from 2.6 ± 0.5 to 4.5 ± 0.5 mm ($P = 0.0009$). Conversely, the therapeutic effect of SP appears to be negligible (Fig. 4D). These findings were further validated by utilizing CGRP in RTX-treated mice (Fig. 4E). The administration of CGRP in RTX-denervated mice led to an increase in tear secretion from 2.5 ± 0.8 to 4.2 ± 0.75 mm ($P = 0.0195$) (Fig. 4F). To further define the involvement of CGRP in controlling tear secretion, we adopted the CGRP receptor antagonist BIBN-4096 in the wild-type mice (Fig. 4G). As expected, the tear secretion of BIBN-4096–treated mice decreased to 3.4 ± 0.5 mm compared to 7.3 ± 0.7 mm of control mice ($P = 0.0022$), with a minimal impact on ocular surface and corneal nerves (Figs. 4H–I). Together, these results suggest that CGRP plays an important role in corneal sensory nerve–controlled tear production.

CGRP Activates the Parasympathetic Superior Salivatory Nucleus

To address whether CGRP was involved in the SSN-controlled tear secretion, we analyzed the c-FOS expression in parasympathetic neurons of the SSN. The results revealed

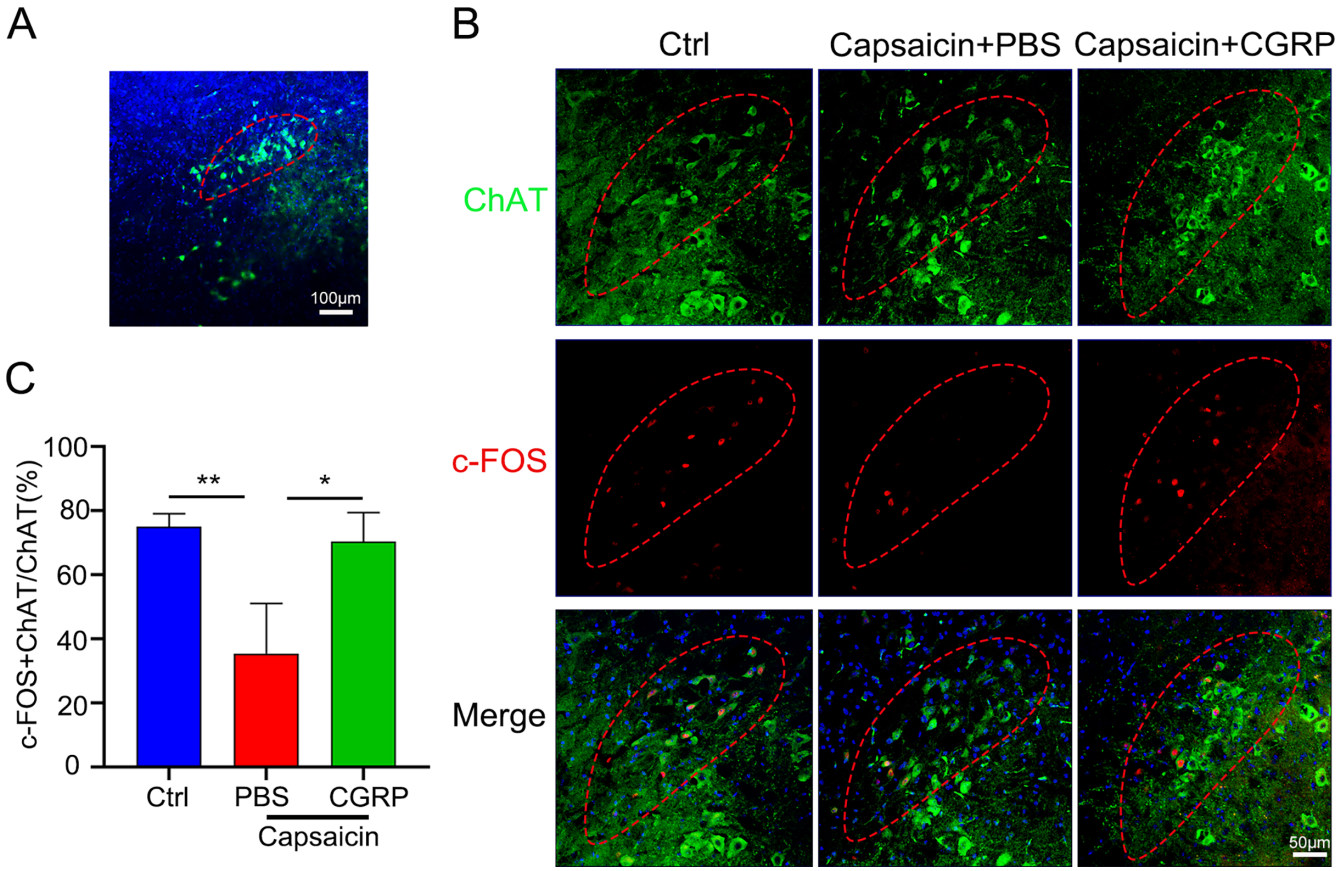

Figure 5. Regulation of CGRP on the activation of the parasympathetic superior salivatory nucleus. (**A**) Representative image of the brain with the immunostaining of ChAT in the SSN. Scale bars: 100 μm. (**B**, **C**) Representative images and quantitative analysis of c-FOS$^+$ neurons in the SSN of capsaicin-treated mice with or without CGRP injection (n = 6 per group). Data are shown as mean ± SD (*P < 0.05, **P < 0.01).

that capsaicin treatment led to a 52% (P = 0.0094) decrease of c-FOS$^+$ cell number in the SSN (Figs. 5A–C). However, c-FOS$^+$ cell number increased by twofold (P = 0.0168) after CGRP administration compared with capsaicin-treated mice (Figs. 5B, 5C). Taken together, these data suggest that CGRP increased tear secretion by activating parasympathetic neurons of the SSN, which projected parasympathetic signals to the lacrimal gland.

CGRP Increases the Proliferation of Lacrimal Acinar Cells

To correlate the tear secretion with the specific changes in the lacrimal gland, we conducted a transcriptomic sequencing comparative analysis of the lacrimal gland. In capsaicin-treated mice, KEGG and GSEA analysis revealed significantly downregulated genes enriched in cell cycle signaling pathway, which exhibited upregulation following CGRP administration (Figs. 6A, 6B). Notably, the displayed distinctive gene expression patterns associated with cell cycle and proliferation were observed by a volcano map analysis and heatmap (Figs. 6C, 6D). Quantitative PCR and Western blot confirmed that proliferation-related genes such as CCNA2 and PCNA were downregulated in capsaicin-treated mice, which exhibited upregulation following CGRP administration (Figs. 6E, 6F).

The lacrimal gland is composed of acinar cells, ductal cells, and myoepithelial cells.[14,43] Our previous study observed abnormal proliferation of acinar cells in scopolamine-induced dry eye mice.[44] To elucidate the specific cell types mediating tear secretion following CGRP administration, we conducted double immunolabeling in lacrimal gland sections with acinar cell markers MIST1 and CCNA2. The results showed that most proliferating cells were acinar cells, and capsaicin treatment impaired the proliferation of acinar cells (Figs. 7A, 7B). However, this effect was alleviated following CGRP administration (Figs. 7A, 7B). Collectively, these results suggest that CGRP increases tear secretion by improving the proliferation of acinar cells.

Discussion

Corneal sensory nerves play a crucial role in rapidly detecting environmental changes and triggering the secretion of electrolytes, water, and proteins from the lacrimal gland.[14] However, various factors such as diabetes, aging, and LASIK surgery can reduce corneal sensitivity, leading to decreased tear secretion.[18,45,46] In this study, we identified the role of the corneal TRPV1 sensory nerve in regulating tear secretion. Subsequently, we discovered that CGRP released by the corneal TRPV1 sensory nerve restored tear secretion by increasing the number of c-FOS$^+$ cells in parasympathetic

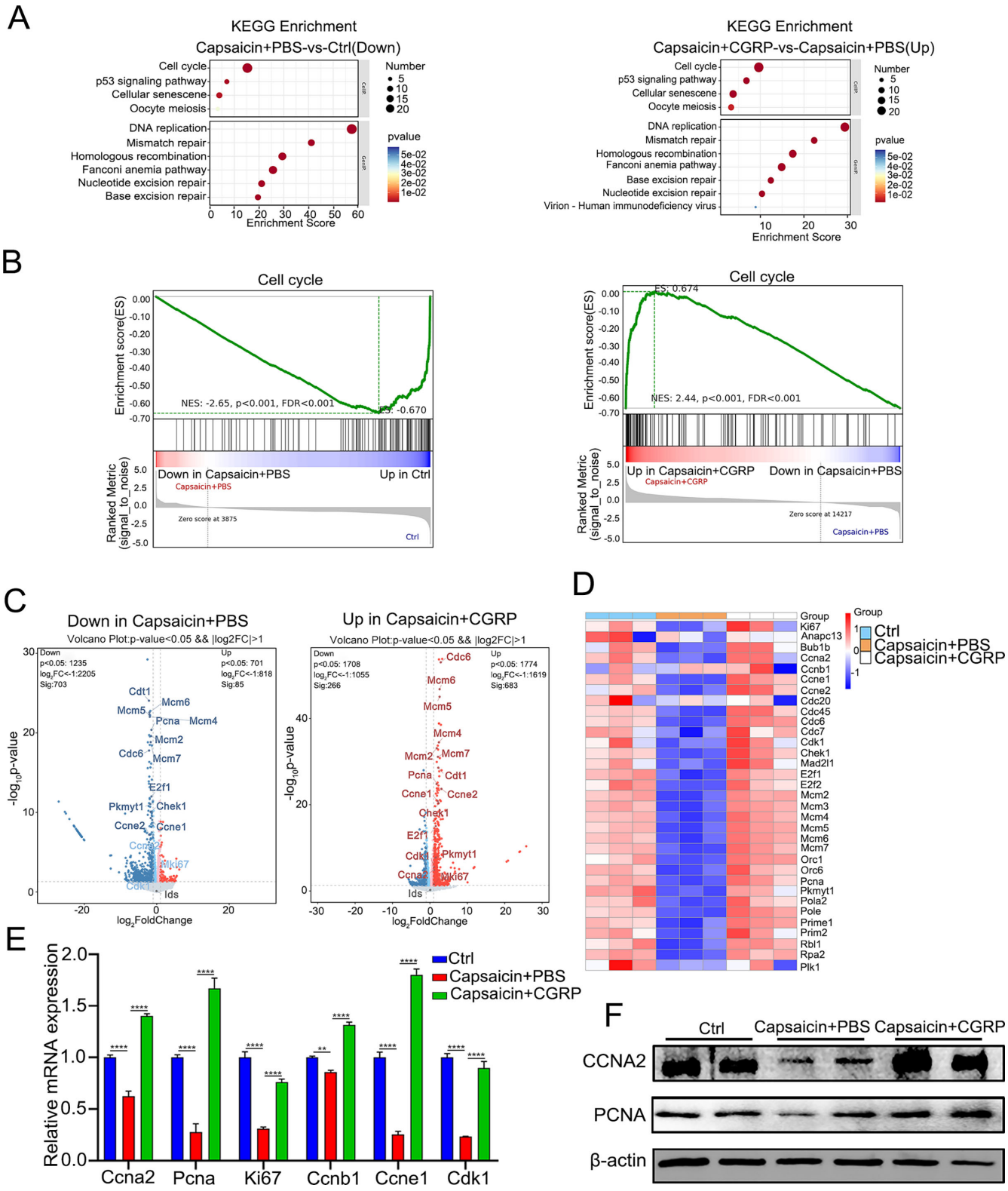

Figure 6. Transcriptomic sequencing analysis of the lacrimal gland following CGRP administration in capsaicin-treated mice. (**A**) KEGG analysis of the downregulated pathways in capsaicin-treated and control mice and upregulated pathways in CGRP-supplemented to capsaicin-treated and capsaicin-treated mice. (**B**) Enrichment profiles of the cell cycle pathway analyzed by GSEA. (**C**) Volcano plot showing the DEGs involving the cell cycle. (**D**) Heatmaps show the expression of genes involved in the cell cycle. The cell cycle–related genes detected by quantitative PCR (**E**) and Western blot (**F**). Data are shown as mean ± SD (**$P < 0.01$, ****$P < 0.0001$).

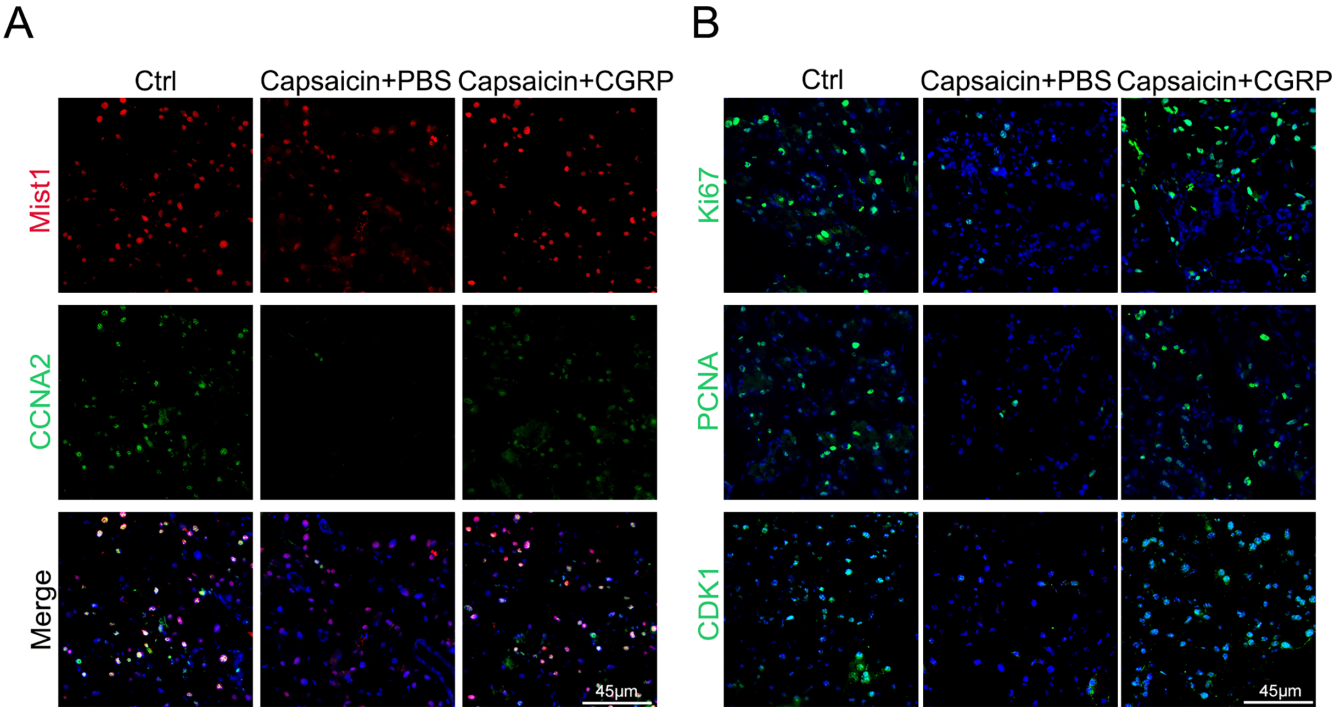

Figure 7. Regulation of CGRP on the proliferation of lacrimal acinar cells. (**A**, **B**) Representative images of immunolabeling in capsaicin-treated mice with or without CGRP administration using anti-MIST1 with anti-CCNA2, anti-Ki67, anti-PCNA, and anti-CDK1 ($n = 6$ per group).

neurons of SSN. Mechanistically, we confirmed that CGRP markedly enhanced the proliferation signaling of lacrimal gland acinar cells. These findings indicated the potential therapeutic effect of CGRP in restoring tear secretion.

Polymodal nociceptors are primarily responsible for the increase in reflex tear secretion following corneal stimulation.[20,33] Moreover, a low dose of capsaicin induces transient reflex tearing through the stimulation of TRPV1 sensory nerves.[22] A recent study has found that the maturation process of lacrimal function also depends on the ocular surface TRPV1 sensory nerve.[22] Through high concentrations of capsaicin and RTX, as well as *Trpv1-Cre/DTR* mice models, we identified a significant decrease in tear secretion after corneal TRPV1 sensory nerve degeneration. More important, we observed an increase in tear production as corneal nerve fibers regenerated following capsaicin treatment. These results suggest the crucial role of corneal TRPV1 nerves in regulating tear secretion.

The corneal sensory afferent nerve transmits impulses through the trigeminal ganglia to the SSN and finally to the lacrimal gland, which in turn regulates tear secretion.[47] Our results demonstrate that corneal TRPV1 nerve denervation leads to a reduction in the number of c-FOS$^+$ cells in the ChAT neurons of the SSN. Importantly, we first identified the function of CGRP in activating the ChAT neurons of the SSN, which further increase tear secretion. Previous studies have shown that the activation of corneal polymodal nociceptor fibers stimulates tear secretion in human models.[20,33] Additionally, the activation of the corneal polymodal nociceptor leads to the release of CGRP,[48,49] indicating that CGRP may also play a role in stimulating tear production. Our study confirmed that CGRP released by the TRPV1 sensory nerve participated in tear secretion of the lacrimal gland. Therefore, modulating the corneal CGRP holds therapeutic potential for disorders associated with impaired tear secretion. Our findings highlight the significant role of CGRP in regulating tear secretion between the corneal afferent sensory nerves and the SSN. Nevertheless, further research is required to elucidate the specific molecular mechanisms and neural pathways involved.

While our study has provided novel insights into the regulation of tear secretion, the limitations of CGRP need to be acknowledged. Neurogenic inflammation acts as the initial line of defense, safeguarding the ocular surface from damage caused by exposure to pathogens, chemical irritants, or mechanical stimulation. Sensory neurons release neuromodulators, such as SP and CGRP, triggering neurogenic inflammation.[50] This process leads to vasodilation and increased permeability, facilitating the extravasation of plasma and immune cells into the tissue.[51,52] More important, the influx of immune cells further releases inflammatory mediators, contributing to aggravated corneal nerve damage and peripheral neuroinflammation.[53,54] The effects of SP on inflammation vary in different corneal diseases. After alkali burn, SP promotes inflammation by facilitating leukocyte recruitment and IL-1β expression through the NK1R pathway.[55] However, in RTX-treated mice, SP exerts an inhibitory effect on inflammatory response.[37] On the other hand, CGRP plays a significant role in anti-inflammatory processes. In RTX-mediated TRPV1 sensory nerve–depleted mice, CGRP decreases inflammation by activating RAMP1 signaling. This activation reduces the accumulation and proinflammatory cytokine expression of $\gamma\delta$ T cells and CCR2$^+$ macrophages in the cornea.[56] Short-term administration of both CGRP and SP has been observed to promote corneal wound healing in RTX-treated mice.[37] Our findings also showed short-term administration of CGRP did not significantly induce inflammation (Supplementary Fig. S1).

However, further study is needed to determine the potential side effects of long-term CGRP administration, such as induction of neurogenic inflammation.

While our study identifies the roles of CGRP released by corneal sensory nerves in regulating tear secretion from the lacrimal gland, several limitations should be acknowledged. First, CGRP stimulates tear secretion under the condition of corneal nerve degeneration. However, further investigation is needed to illustrate whether CGRP regulates the basal tear secretion. Second, while CGRP may provide a basis for stimulating tear secretion in patients with neurotrophic keratitis, it cannot be used to improve tear reduction caused by other factors, such as meibomian gland dysfunction. Third, further research is needed to understand the mechanism of CGRP-dependent afferent nerve stimulation on tear secretion.

In summary, our results reveal that CGRP released by the TRPV1 sensory nerve induces a partial increase in tear secretion by increasing the number of c-FOS$^+$ cells in ChAT neurons of the SSN. These results provide valuable insights into the potential mechanisms underlying CGRP-mediated tear secretion and offer promising avenues for the development of therapeutic interventions for neurotrophic keratitis.

Acknowledgments

The authors thank Shengqian Dou and Jing Feng (Shandong First Medical University & Shandong Academy of Medical Sciences) for the assistance in data analysis on the manuscript.

Supported by the National Natural Science Foundation of China (82070927 and 82000851), the Natural Science Foundation of Shandong Province (ZR2020QH144), and the Key Research and Development Program of Shandong Province (2021ZDSYS14). QZ is supported by the Taishan Scholar Program (tstp20221163) and the Academic Promotion Program and Innovation Project of the Shandong First Medical University (2019RC008).

Disclosure: **L. Ma**, None; **L. Yang**, None; **X. Wang**, None; **L. Zhao**, None; **X. Bai**, None; **X. Qi**, None; **Q. Chen**, None; **Y. Li**, None; **Q. Zhou**, None

References

1. Müller LJ, Marfurt CF, Kruse F, Tervo TM. Corneal nerves: structure, contents and function. *Exp Eye Res*. 2003;76(5):521–542.
2. Heigle TJ, Pflugfelder SC. Aqueous tear production in patients with neurotrophic keratitis. *Cornea*. 1996;15(2):135–138.
3. Al-Aqaba MA, Dhillon VK, Mohammed I, Said DG, Dua HS. Corneal nerves in health and disease. *Prog Retin Eye Res*. 2019;73:100762.
4. Nishida T, Chikama T, Sawa M, et al. Differential contributions of impaired corneal sensitivity and reduced tear secretion to corneal epithelial disorders. *Jpn J Ophthalmol*. 2012;56(1):20–25.
5. Beuerman RW, Schimmelpfennig B. Sensory denervation of the rabbit cornea affects epithelial properties. *Exp Neurol*. 1980;69(1):196–201.
6. Willcox MDP, Argüeso P, Georgiev GA, et al. TFOS DEWS II tear film report. *Ocul Surf*. 2017;15(3):366–403.
7. Stern ME, Beuerman RW, Fox RI, Gao J, Mircheff AK, Pflugfelder SC. The pathology of dry eye: the interaction between the ocular surface and lacrimal glands. *Cornea*. 1998;17(6):584–589.
8. Stern ME, Gao J, Siemasko KF, Beuerman RW, Pflugfelder SC. The role of the lacrimal functional unit in the pathophysiology of dry eye. *Exp Eye Res*. 2004;78(3):409–416.
9. Labetoulle M, Baudouin C, Calonge M, et al. Role of corneal nerves in ocular surface homeostasis and disease. *Acta Ophthalmol*. 2019;97(2):137–145.
10. Belmonte C, Nichols JJ, Cox SM, et al. TFOS DEWS II pain and sensation report. *Ocul Surf*. 2017;15(3):404–437.
11. Meng ID, Kurose M. The role of corneal afferent neurons in regulating tears under normal and dry eye conditions. *Exp Eye Res*. 2013;117:79–87.
12. van der Werf F, Baljet B, Prins M, Otto JA. Innervation of the lacrimal gland in the cynomolgous monkey: a retrograde tracing study. *J Anat*. 1996;188(pt 3):591–601.
13. Tóth IE, Boldogkoi Z, Medveczky I, Palkovits M. Lacrimal preganglionic neurons form a subdivision of the superior salivatory nucleus of rat: transneuronal labelling by pseudorabies virus. *J Auton Nerv Syst*. 1999;77(1):45–54.
14. Dartt DA. Neural regulation of lacrimal gland secretory processes: relevance in dry eye diseases. *Prog Retin Eye Res*. 2009;28(3):155–177.
15. Wong EK, Kinyamu RD, Graff JM, et al. A rat model of radiofrequency ablation of trigeminal innervation via a ventral approach with stereotaxic surgery. *Exp Eye Res*. 2004;79(3):297–303.
16. Rao P, Mckown RL, Laurie GW, Suvas SJEER. Development of lacrimal gland inflammation in the mouse model of herpes stromal keratitis. *Exp Eye Res*. 2019;184:101–106.
17. Misra SL, Patel DV, Mcghee CN, et al. Peripheral neuropathy and tear film dysfunction in type 1 diabetes mellitus. *J Diabetes Res*. 2014;2014:848659.
18. Cousen P, Cackett P, Bennett H, Swa K, Dhillon B. Tear production and corneal sensitivity in diabetes. *J Diabetes Complications*. 2007;21(6):371–373.
19. Belmonte C, Acosta MC, Gallar J. Neural basis of sensation in intact and injured corneas. *Exp Eye Res*. 2004;78(3):513–525.
20. Acosta MC, Peral A, Luna C, Pintor J, Belmonte C, Gallar J. Tear secretion induced by selective stimulation of corneal and conjunctival sensory nerve fibers. *Invest Ophthalmol Vis Sci*. 2004;45(7):2333–2336.
21. Alamri A, Bron R, Brock JA, Ivanusic JJ. Transient receptor potential cation channel subfamily V member 1 expressing corneal sensory neurons can be subdivided into at least three subpopulations. *Front Neuroanat*. 2015;9:71.
22. Jin K, Imada T, Nakamura S, et al. Corneal sensory experience via transient receptor potential vanilloid 1 accelerates the maturation of neonatal tearing. *Am J Pathol*. 2019;189(9):1699–1710.
23. Bates BD, Mitchell K, Keller JM, et al. Prolonged analgesic response of cornea to topical resiniferatoxin, a potent TRPV1 agonist. *Pain*. 2010;149(3):522–528.
24. Caterina MJ, Schumacher MA, Tominaga M, et al. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature*. 1997;389(6653):816–824.
25. Asiedu K, Markoulli M, Bonini S, et al. Tear film and ocular surface neuropeptides: characteristics, synthesis, signaling and implications for ocular surface and systemic diseases. *Exp Eye Res*. 2022;218:108973.
26. Puri S, Kenyon BM, Hamrah P. Immunomodulatory role of neuropeptides in the cornea. *Biomedicines*. 2022;10(8):1985.
27. Zidan AA, Zhu S, Elbasiony E, et al. Topical application of calcitonin gene-related peptide as a regenerative, antifibrotic, and immunomodulatory therapy for corneal injury. *Commun Biol*. 2024;7(1):264.

28. Mikulec AA, Tanelian DL. CGRP increases the rate of corneal re-epithelialization in an in vitro whole mount preparation. *J Ocul Pharmacol Ther*. 1996;12(4):417–423.
29. Cortina MS, He J, Li N, Bazan NG, Bazan HE. Recovery of corneal sensitivity, calcitonin gene-related peptide-positive nerves, and increased wound healing induced by pigment epithelial-derived factor plus docosahexaenoic acid after experimental surgery. *Arch Ophthalmol*. 2012;130(1):76–83.
30. McCoy ES, Taylor-Blake B, Zylka MJ. CGRPα-expressing sensory neurons respond to stimuli that evoke sensations of pain and itch. *PLoS One*. 2012;7(5):e36355.
31. Brain SD, Grant AD. Vascular actions of calcitonin gene-related peptide and adrenomedullin. *Physiol Rev*. 2004;84(3):903–934.
32. Xu P, Lin X, Dong X, Liu Y, Wang Z, Wang S. Trigeminal nerve-derived substance P regulates limbal stem cells by the PI3K-AKT pathway. *iScience*. 2023;26(5):106688.
33. Situ P, Simpson TL. Interaction of corneal nociceptive stimulation and lacrimal secretion. *Invest Ophthalmol Vis Sci*. 2010;51(11):5640–5645.
34. Yamaguchi T, Turhan A, Harris DL, et al. Bilateral nerve alterations in a unilateral experimental neurotrophic keratopathy model: a lateral conjunctival approach for trigeminal axotomy. *PLoS One*. 2013;8(8):e70908.
35. Arora V, Campbell JN, Chung MK. Fight fire with fire: neurobiology of capsaicin-induced analgesia for chronic pain. *Pharmacol Ther*. 2021;220:107743.
36. Brown DC. Resiniferatoxin: the evolution of the "molecular scalpel" for chronic pain relief. *Pharmaceuticals (Basel)*. 2016;9(3):47.
37. Zhang Y, Gao N, Wu L, et al. Role of VIP and sonic hedgehog signaling pathways in mediating epithelial wound healing, sensory nerve regeneration, and their defects in diabetic corneas. *Diabetes*. 2020;69(7):1549–1561.
38. Zhao L, Chen R, Qu J, et al. Establishment of mouse model of neurotrophic keratopathy through TRPV1 neuronal ablation. *Exp Eye Res*. 2024;240:109814.
39. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods*. 2015;12(4):357–360.
40. Ge SX, Son EW, Yao R. iDEP: an integrated web application for differential expression and pathway analysis of RNA-Seq data. *BMC Bioinformatics*. 2018;19(1):534.
41. Kanehisa M, Araki M, Goto S, et al. KEGG for linking genomes to life and the environment. *Nucleic Acids Res*. 2008;36(Database issue):D480–D484.
42. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. 2005; 102(43):15545–15550.
43. Makarenkova HP, Dartt DA. Myoepithelial cells: their origin and function in lacrimal gland morphogenesis, homeostasis, and repair. *Mol Biol Rep*. 2015;1(3):115–123.
44. Chen Q, Qu M, Zhang B, et al. Involvement of aberrant acinar cell proliferation in scopolamine-induced dry eye mice. *Exp Eye Res*. 2023;227:109391.
45. Murphy PJ, Patel S, Kong N, Ryder RE, Marshall J. Noninvasive assessment of corneal sensitivity in young and elderly diabetic and nondiabetic subjects. *Invest Ophthalmol Vis Sci*. 2004;45(6):1737–1742.
46. Konomi K, Chen LL, Tarko RS, et al. Preoperative characteristics and a potential mechanism of chronic dry eye after LASIK. *Invest Ophthalmol Vis Sci*. 2008;49(1):168–174.
47. Dieckmann G, Fregni F, Hamrah P. Neurostimulation in dry eye disease-past, present, and future. *Ocul Surf*. 2019;17(1):20–27.
48. Fehrenbacher JC, Sun XX, Locke EE, Henry MA, Hargreaves KM. Capsaicin-evoked iCGRP release from human dental pulp: A model system for the study of peripheral neuropeptide secretion in normal healthy tissue. *Pain*. 2009;144(3):253–261.
49. Pan XQ, Gonzalez JA, Chang S, et al. Experimental colitis triggers the release of substance P and calcitonin gene-related peptide in the urinary bladder via TRPV1 signaling pathways. *Exp Neurol*. 2010;225(2):262–273.
50. Beuerman RW, Stern ME. Neurogenic inflammation: a first line of defense for the ocular surface. *Ocul Surf*. 2005;3(4, suppl):S203–S206.
51. Choi JE, Di Nardo A. Skin neurogenic inflammation. *Semin Immunopathol*. 2018;40(3):249–259.
52. Chiu IM, Hehn CA, Woolf CJ. Neurogenic inflammation and the peripheral nervous system in host defense and immunopathology. *Nat Neurosci*. 2012;15(8):1063–1067.
53. Ferrari G, Bignami F, Giacomini C, et al. Ocular surface injury induces inflammation in the brain: in vivo and ex vivo evidence of a corneal-trigeminal axis. *Invest Ophthalmol Vis Sci*. 2014;55(10):6289–6300.
54. Lasagni Vitar RM, Rama P, Ferrari G. The two-faced effects of nerves and neuropeptides in corneal diseases. *Prog Retin Eye Res*. 2022;86:100974.
55. Bignami F, Giacomini C, Lorusso A, et al. NK1 receptor antagonists as a new treatment for corneal neovascularization. *Invest Ophthalmol Vis Sci*. 2014;55(10):6783–6794.
56. Liu J, Huang S, Yu R, et al. TRPV1^{+} sensory nerves modulate corneal inflammation after epithelial abrasion via RAMP1 and SSTR5 signaling. *Mucosal Immunol*. 2022;15(5):867–881.