Basic Science
Research Paper

OPEN

PAIN REPORTS®

Acute hyperalgesia and delayed dry eye after corneal abrasion injury

Deborah M. Hegarty[a], Sam M. Hermes[a], Michael M. Morgan[b], Sue A. Aicher[a,*]

Abstract

Introduction: Corneal nerves mediate pain from the ocular surface, lacrimation, and blinking, all of which protect corneal surface homeostasis and help preserve vision. Because pain, lacrimation and blinking are rarely assessed at the same time, it is not known whether these responses and their underlying mechanisms have similar temporal dynamics after acute corneal injury.

Methods: We examined changes in corneal nerve density, evoked and spontaneous pain, and ocular homeostasis in Sprague-Dawley male rats after a superficial epithelial injury with heptanol. We also measured changes in calcitonin gene-related peptide (CGRP), which has been implicated in both pain and epithelial repair.

Results: Hyperalgesia was seen 24 hours after abrasion injury, while basal tear production was normal. One week after abrasion injury, pain responses had returned to baseline levels and dry eye symptoms emerged. There was no correlation between epithelial nerve density and pain responses. Expression of both ATF3 (a nerve injury marker) and CGRP increased in trigeminal ganglia 24 hours after injury when hyperalgesia was seen, and returned to normal one week later when pain behavior was normal. These molecular changes were absent in the contralateral ganglion, despite reductions in corneal epithelial nerve density in the uninjured eye. By contrast, CGRP was upregulated in peripheral corneal endings 1 week after injury, when dry eye symptoms emerged.

Conclusion: Our results demonstrate dynamic trafficking of CGRP within trigeminal sensory nerves following corneal injury, with elevations in the ganglion correlated with pain behaviors and elevations in peripheral endings correlated with dry eye symptoms.

Keywords: Pain, Trigeminal, Tears, Blink, CGRP, Immunohistochemistry, Beta-tubulin

1. Introduction

The cornea contains the highest density of sensory nerves of any tissue in the body.[3,41,44] Corneal sensory nerves are predominantly nociceptive and even low-threshold stimulation evokes a sensation of pain.[3] Corneal nerves are also vital for homeostatic reflexes, such as blinking and lacrimation, and epithelial health.[6] Loss of innervation causes a loss of sensation[49] and the development of spontaneous corneal abrasions.[59] Damage to corneal nerves can be caused by diabetes,[30] pharmacotherapeutics such as paclitaxel,[17] vision correction surgery,[38] or accidental injury[21] that causes damage to the ocular surface. Studies examining injury-induced changes in corneal nerves find conflicting and paradoxical results when trying to correlate altered nerve structure with ocular pain. In some cases, ocular sensation is unchanged despite obvious corneal nerve damage, and in other cases, persistent pain may occur.[3,9] The presence of ocular pain may be caused by neuropathic changes in corneal nerves. The present study tested this hypothesis by inducing a unilateral corneal abrasion injury in rats to look at the relationship between changes in corneal nerve density and morphology, neurochemistry, and ocular sensation. We examined temporal changes in ocular homeostatic responses, including blinking and tear production. We also examined molecular changes in corneal sensory cell bodies and peripheral endings, using activating transcription factor 3 (ATF3) as a marker of nerve injury[7] and calcitonin gene-related peptide (CGRP) as a molecule associated with changes in nociceptive processing[8] and epithelial health.[55]

We found distinct temporal dynamics for changes in nociception vs homeostatic ocular responses. Pain increased acutely after superficial corneal epithelial injury, but pain responses returned to normal levels long before nerve density returned to normal; by contrast, tear production was normal acutely after injury, but was impaired 1 week after corneal damage when reinnervation of the corneal epithelium is progressing and pain responses are normal. Together these findings suggest divergent mechanisms underlying ocular pain and homeostatic functions, although sensory nerves and some molecular transducers such as TRPM8 are necessary for both cold pain[4] and homeostatic

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

[a] Department of Physiology and Pharmacology, Oregon Health & Science University, Portland, OR, USA, [b] Department of Psychology, Washington State University, Vancouver, WA, USA

**Corresponding author. Address: Department of Physiology and Pharmacology, Oregon Health & Science University, Mail code: L334, 3181 SW Sam Jackson Park Rd, Portland, OR 97239-3098. Tel.: 503-418-2550; fax: 503-494-4352. E-mail address: aichers@ohsu.edu (S.A. Aicher).*

PR9 3 (2018) e664

http://dx.doi.org/10.1097/PR9.0000000000000664

responses.[50] The trigeminal sensory neurons that innervate the corneal surface are molecularly complex and may differentially traffic materials to central and peripheral sites. These neurons are also integrated into distinct reflex pathways that mediate pain responses, blinking, and lacrimation. The present studies indicate that trafficking of the neuropeptide CGRP to different regions of the sensory nerves innervating the cornea is correlated with distinct functional changes in nociceptive and homeostatic responses.

2. Methods

Methods were approved by Institutional Animal Care and Use Committee (IACUC) at OHSU. Experiments were performed on male Sprague-Dawley rats (250–350 g). Rats used for wheel running studies were bred at Washington State University and were housed individually. Rats studied at OHSU (Charles River Laboratories, Wilmington, MA) were housed in pairs on a 12/12 light/dark cycle and had access to food and water at all times. A total of 52 rats were used for all the experiments; specific group sizes are indicated in the Results section for each study. In the course of measuring behavioral responses, corneal nerve density, and homeostatic responses, some rats at OHSU were assessed for more than one metric when possible.

2.1. Heptanol abrasions and tracer applications

Rats were anesthetized with isoflurane for unilateral corneal abrasion. Heptanol (10 μL) was pipetted into a small metal ring (6-gauge [4.4 mm internal diameter] stainless steel tubing, custom, HTX-06R; Small Parts Inc, Miramar, FL) secured to the cornea with petroleum jelly and left for 90 seconds.[20,25] After 90 seconds, the heptanol was wicked away with cotton swabs, the damaged epithelium was removed, and the corneal surface was rinsed with saline. Rats were returned to their home cage and monitored until they were awake from anesthesia. Previous studies demonstrate that the epithelium is closed within 24 hours after heptanol injury.[53]

In a subset of animals, the tract tracer FluoroGold (FG; Fluorochrome, Denver, CO) was applied to the central cornea in the 6-gauge metal ring immediately after the heptanol abrasion to allow for labeling of nerves that were severed during the initial abrasion. One week after abrasion, 1% Cholera Toxin B (CTb; List Laboratories, Campbell, CA) was applied to the cornea using a similar method, but with a smaller metal ring (7-gauge [3.8 mm internal diameter], HTX-07R; Small Parts Inc). For both tract tracer applications, the dye was left on the cornea for 30 minutes.[23] Animals were perfused 1 week after CTb application as described below, and trigeminal ganglia (TG) were examined for the presence of single (CTb)- or dual (CTb and FG)-labeled neurons (see section 2.5).

2.2. Evoked and spontaneous pain measures: menthol-evoked eye wipes and spontaneous wheel running

To assess evoked pain responses, menthol (10 μL of 50 mM menthol in 10% ethanol/10% Tween-80 in saline) was applied to the corneal surface in awake, lightly restrained rats.[1,12,40] Two observers counted the number of ipsilateral eye wipes for 3 minutes after menthol stimulation.[24] Rats were habituated to handling the testing apparatus and testing room for 40 minutes per day for 2 days before nociceptive testing. Each rat received a single corneal menthol application to avoid sensitization; thus, separate groups were used for 24-hour and 1-week assessments. Individual rats were tested either on the abraded or contralateral eye, but not both.

Home cage wheel running was used to assess spontaneous pain as described previously.[32,33] Rats were given access to a running wheel in the home cage for 23 hours a day for 7 days before corneal abrasion. The number of wheel revolutions measured on the seventh day was used as the baseline value. Rats were randomly assigned to control or abrasion groups. Only rats with a baseline wheel running value of at least 400 revolutions on the baseline day were tested. Testing began approximately 1 hour after induction of a unilateral heptanol abrasion of the cornea as described above. Control rats were briefly anesthetized but not abraded. Rats were returned to the home cage, and wheel running was assessed 23 hours a day for 1 week. No one entered the test room except during the 1 hour when running wheel data were not assessed. Food, water, and the health of the rat were monitored during this hour.

2.3. Spontaneous blinks and winks

The number of blinks (simultaneous eye closures of both eyes) and winks (unilateral eye closures) was counted before (baseline) and 1 week after corneal abrasion. Rats were placed in a Plexiglas chamber and allowed to acclimate for 15 minutes. Blinks and winks were counted by an observer for 5 minutes. Rats were habituated to handling the testing apparatus and testing room for 60 minutes per day for 2 days before eye closure assessment.

Table 1

List of primary and secondary antibodies used for each immunohistochemical experiment.

Tissue experiment	Primary antibodies, concentration, source, catalog number	Secondary antibodies, concentration, source, catalog number
Corneas	Mouse anti-β-tubulin, 1:500, BioLegend (San Diego, CA), Cat no. 801202 Rabbit anti-CGRP, 1:8000, ImmunoStar (Hudson, WI), Cat no. 24112	Donkey anti-Mouse Cy3, 1:800, Jackson ImmunoResearch (West Grove, PA), Cat no. 715-165-151 Donkey anti-Rabbit Alexa Fluor 647, 1:800, Jackson ImmunoResearch, Cat no. 711-605-152
Trigeminal ganglia dual retrograde experiment	Goat anti-CTb, 1:25000, List Laboratories (Campbell, CA), Cat no. 703 Rabbit anti-FluoroGold, 1:20000, Fluorochrome, LLC (Denver, CO), Cat no. *Antibody to Fluoro-Gold*	Donkey anti-Goat Alexa Fluor 488, 1:800, Life Technologies (Carlsbad, CA), Cat no. A11055 Donkey anti-Rabbit Alexa Fluor 647, 1:800, Jackson ImmunoResearch, Cat no. 711-605-152
Trigeminal ganglia assessment of injury and nociceptive markers	Rabbit anti-ATF3 (C-19), 1:1000, Santa Cruz Biotechnology (Dallas, TX), Cat no. sc-188 Sheep anti-CGRP, 1:3000, Abcam (Cambridge, MA), Cat no. ab22560	Donkey anti-Rabbit Alexa Fluor 488, 1:800, Life Technologies, Cat no. A21206 Donkey anti-Sheep Alexa Fluor 647, 1:800, Life Technologies, Cat no. A21448

CGRP, calcitonin gene-related peptide; CTb, Cholera Toxin B.

Table 2

Corneal epithelium volumes.

Injury group	Epithelium volume ($\times 10^6$ μm^3)	N
Uninjured control	6.3 ± 0.8	6
24 h abraded	n/a	4
24 h contralateral	8.0 ± 0.9	3
1 wk abraded	5.7 ± 0.5	4
1 wk contralateral	7.1 ± 1.1	4

Data were expressed as mean ± SEM. All volumes have an exponent of $\times 10^6$.

2.4. Phenol thread test

Tear production was assessed using the phenol thread test (Zone-Quick, Oasis Medical, Glendora, CA) as described previously.[1] This test is equivalent to the Schirmer test.[57] A timed wicking (15 seconds/eye) was used to measure tear production in rats briefly anesthetized with isoflurane (5% in oxygen). At the end of 15 seconds, the length of thread that turned red was measured to the nearest millimeter (mm). Each animal was tested before abrasion (baseline) and at a time point after abrasion (24 hours; 1 week). The phenol thread test was conducted at least 24 hours before nociceptive testing because we have observed that even brief anesthesia interferes with nociceptive testing on the same day.

2.5. Immunohistochemistry

Rats were overdosed with sodium pentobarbital (150 mg/kg) and perfused transcardially through the ascending aorta with 10 mL of heparinized saline (1000 units/mL) followed by 600 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4.[24,27] The eyes were enucleated immediately after perfusion and placed in PB. Using a 5 mm corneal trephine (Ambler Surgical, Exton, PA), the central corneas were removed and placed in fresh PB at 4°C until immunoprocessing. The TG were dissected, placed in fixative for 30 minutes, and then stored in 30% sucrose in PB for at least 24 hours. Trigeminal ganglia were then cryosectioned at 20 μm on a Leica CM1950 cryostat (Leica Microsystems Inc, Buffalo Grove, IL) and mounted directly onto room temperature Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were dried and stored at −20°C until immunoprocessing.

Whole-mount corneas were processed free-floating, whereas TG were processed on slides as previously described.[1,23,24] Tissue was incubated in 1% sodium borohydride solution for 30 minutes before primary antibody incubation. Corneas were incubated for 3 nights, and TG sections were incubated for 2 nights at 4°C in primary antibody cocktails in 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 0.1% bovine serum albumin (Sigma) in 0.1 M Tris-buffered saline pH 7.6 (**Table 1**). Corneas and TG sections were rinsed and incubated in fluorescent secondary antibody cocktails made in 0.1% bovine serum albumin in Tris-buffered saline (**Table 1**) light-protected for 2 hours at room temperature. Corneas were rinsed, blotted dry and placed into individual wells of an 18-well μ-slide (ibidi USA, Inc, Fitchburg, WI), and coverslipped with CFM-1 (Electron Microscopy Sciences, Hatfield, PA), which maintains the natural curvature of the cornea for imaging. Trigeminal ganglia sections were additionally stained with NeuroTrace (NT) Fluorescent Nissl stain 530/615 (1:200, ThermoFisher Scientific, Waltham, MA) for 20 minutes before being rinsed, dried, and coverslipped with Prolong Gold Antifade reagent (Life Technologies, Carlsbad, CA).

2.6. Imaging and image analysis

2.6.1. Corneal nerve density and calcitonin gene-related peptide content

Antibodies to β-tubulin and CGRP (**Table 1**) were used to assess corneal nerve density and CGRP content as previously described.[24] Images of β-tubulin- and CGRP-labeled corneal nerves were taken within the central cornea and included the whorl-like vortex as an anchor. Images were captured on a Zeiss LSM510 or LSM780 confocal microscope with a 20× Plan-Apochromat objective (Carl Zeiss MicroImaging, Thornwood, NY) using the single-pass, multitrack format at a 2048 × 2048 pixel resolution. Optical sectioning produced Z-stacks of 1 μm optical slices that were bounded by the extent of fluorescent β-tubulin and CGRP immunolabeling throughout the thickness of the corneal epithelium.

Volumetric assessments of corneal nerve density and CGRP content were performed using Imaris 8.0 software (Bitplane USA, Concord, MA) on an offline workstation in the Advanced Light Microscopy Core at OHSU by a treatment-blind observer as previously described in detail.[24] Only corneas that did not receive menthol stimulation were assessed. Briefly, we defined the corneal epithelium by the β-tubulin- and CGRP-labeled subbasal and intraepithelial corneal nerves from the anterior surface of the cornea to the epithelial–stromal border. The corneal epithelium was isolated as a region of interest (ROI) using the Surfaces Segmentation tool and its volume was calculated by the software (μm^3). The Mask Channel function was then used to isolate β-tubulin and CGRP labeling within the corneal epithelium ROI for further analysis. β-tubulin labeling within the ROI was determined using the Thresholding function and Surfaces Segmentation tool in Imaris, and a volume (μm^3) was calculated. To account for subtle differences in the corneal epithelium volumes (**Table 2**), the volume of β-tubulin labeling was calculated as a percentage of the corneal epithelium volume for each cornea and expressed as the mean % β-tubulin or the percent of the epithelium that contains β-tubulin–labeled nerves. To measure the CGRP content specifically within the corneal nerves, we established the β-tubulin volume as the ROI and isolated the CGRP labeling using the Mask Channel function and then, CGRP volume (μm^3) was calculated. The volume of CGRP within the volume of the β-tubulin labeling was calculated as a percentage and expressed as the % β-tubulin containing CGRP.

2.6.2. Trigeminal ganglia

In the dual retrograde experiment, TG sections were assessed for the number of CTb-labeled corneal neurons and whether they contained FG tracer. Cryostat sections (13–15 sections per animal, 120 μm apart) throughout the TG were evaluated using an Olympus BX51 epifluorescence microscope equipped with a DP71 camera and associated software (Olympus America, Center Valley, PA). All TG sections with CTb labeling were imaged for CTb, NT, and FG labeling. Each marker was imaged separately using a 10× UPlanFL N objective and the appropriate filter (FITC, TRITC, and Cy5) for the fluorophore or neuronal stain. Trigeminal ganglion neurons (TGNs) were analyzed using the Cell Counter Plug-In in ImageJ (NIH) similar to our previous study.[23] In order for TGNs to be included in each analysis, the nucleus had to be visible in the NT channel. In the dual retrograde experiment, every CTb-labeled TGN was marked with a number using the Cell Counter. The CTb numbers were then transferred to the FG image from the same trigeminal ganglion section, and each numbered neuron was evaluated for FG expression. The total

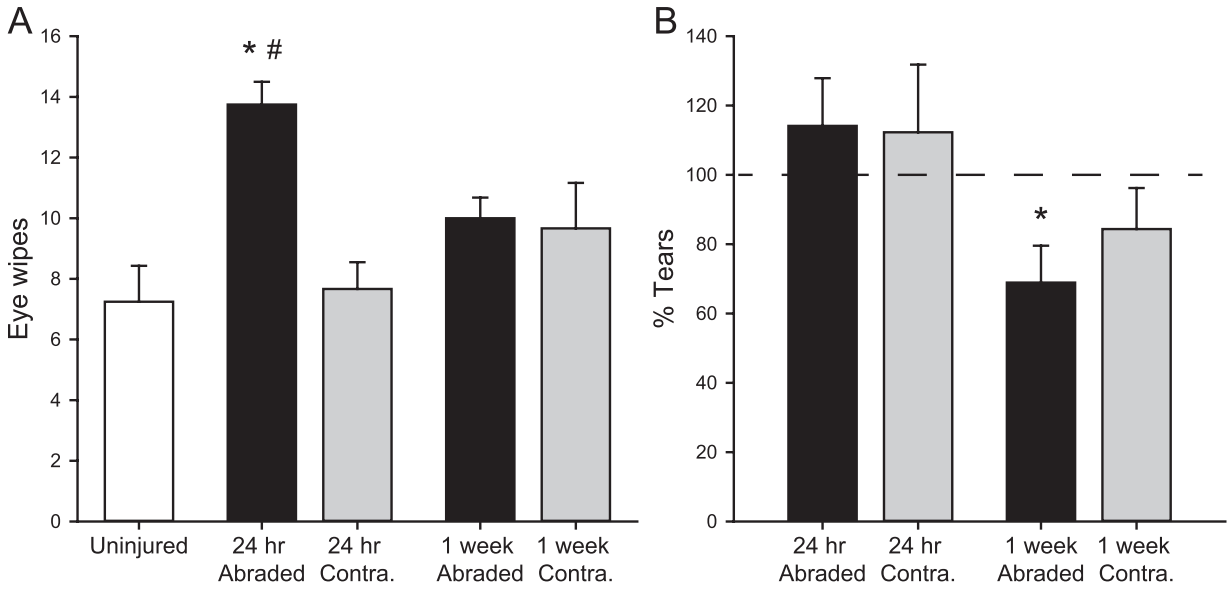

Figure 1. Corneal abrasion produces hyperalgesia at 24 hours, and dry eye at 1 week after abrasion. (A) Abraded rats show more eye wipes to ocular application of noxious menthol 24 hours after injury (black bar, n = 4) compared with uninjured rats (white bar, n = 4) or abraded rats tested at 24 hours on the contralateral side (24 hours Contra., gray bar, n = 3). At 1 week after abrasion, the eye wipe responses to menthol are normal on both abraded (black bar, n = 6) and contralateral eyes (gray bar, n = 6). *$P < 0.05$ vs uninjured; #$P < 0.05$ vs contralateral side. (B) Abrasion injury has a delayed effect on tear production. Phenol thread measurements taken at 24 hours or 1 week after injury are expressed as a percent of preinjury baseline measurements (% tears; baseline: dotted line at 100%). Tear production was not affected 24 hours after abrasion injury on either the injured (24 hours abraded, black bar, n = 8) or unabraded (24 hours Contra., gray bar, n = 8) side. Tear production was significantly reduced 1 week after abrasion injury on the injured side (1 week abraded, black bar, n = 4) as compared to preinjury, but not the unabraded (1 week Contra., gray bar, n = 4) side. *$P < 0.05$ as compared to raw preinjury phenol thread measurements.

number of CTb-labeled TGNs that contained FG was calculated as a percent for each animal and expressed as % CTb-labeled neurons containing FG.

In the assessment of activating transcription factor 3 (ATF3), a nerve injury marker,[7] and CGRP immunohistochemistry, one trigeminal ganglion section from each uninjured control and each abraded animal (24 hours or 1 week later), was evaluated. To avoid counting corneal neurons activated by noxious corneal menthol stimulation,[7] only trigeminal ganglion sections from unstimulated sides were used. Cryostat sections (9–14 sections per animal, 120 μm apart) throughout each trigeminal ganglion were first evaluated with the Olympus epifluorescence microscope. The trigeminal ganglion section that contained peak ATF3 labeling within the ophthalmic region per ganglion was also imaged for NT and CGRP using the 10× UPlanFL N objective and the appropriate filter (FITC, TRITC, and Cy5). Analysis was

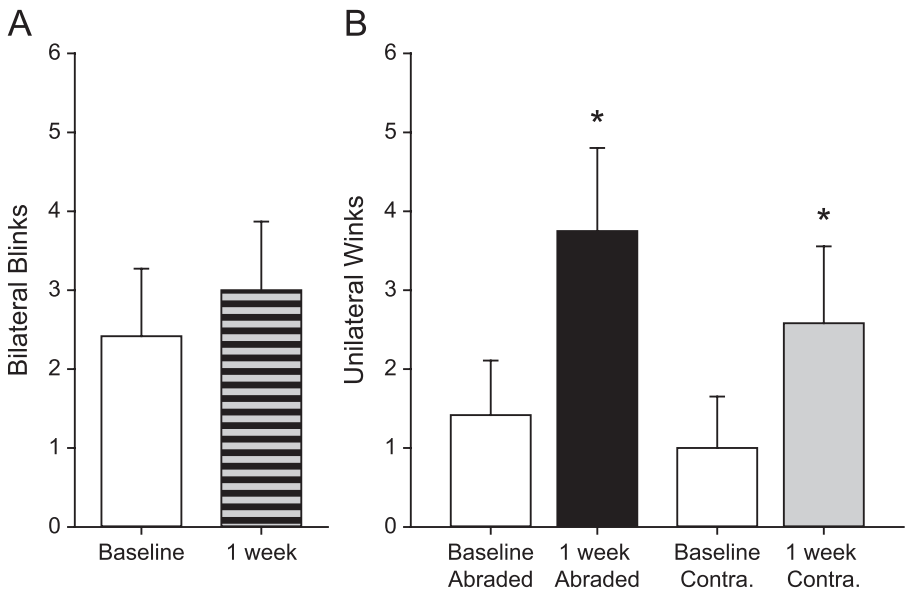

Figure 2. Eye closures were increased after abrasion injury. It was not possible to accurately assess blinks and winks at 24 hours after abrasion because of photophobia that produced prolonged eye closures. (A) Spontaneous bilateral blinks (closure of both eyes) were not different 1 week after abrasion injury (black/gray patterned bar, n = 12) compared with preinjury baseline (white bar, n = 12). (B) Unilateral winks increased 1 week after abrasion in both the abraded (black bar, n = 12) and contralateral eyes (gray bar, n = 12) compared with preinjury baseline (white bars). *$P < 0.05$.

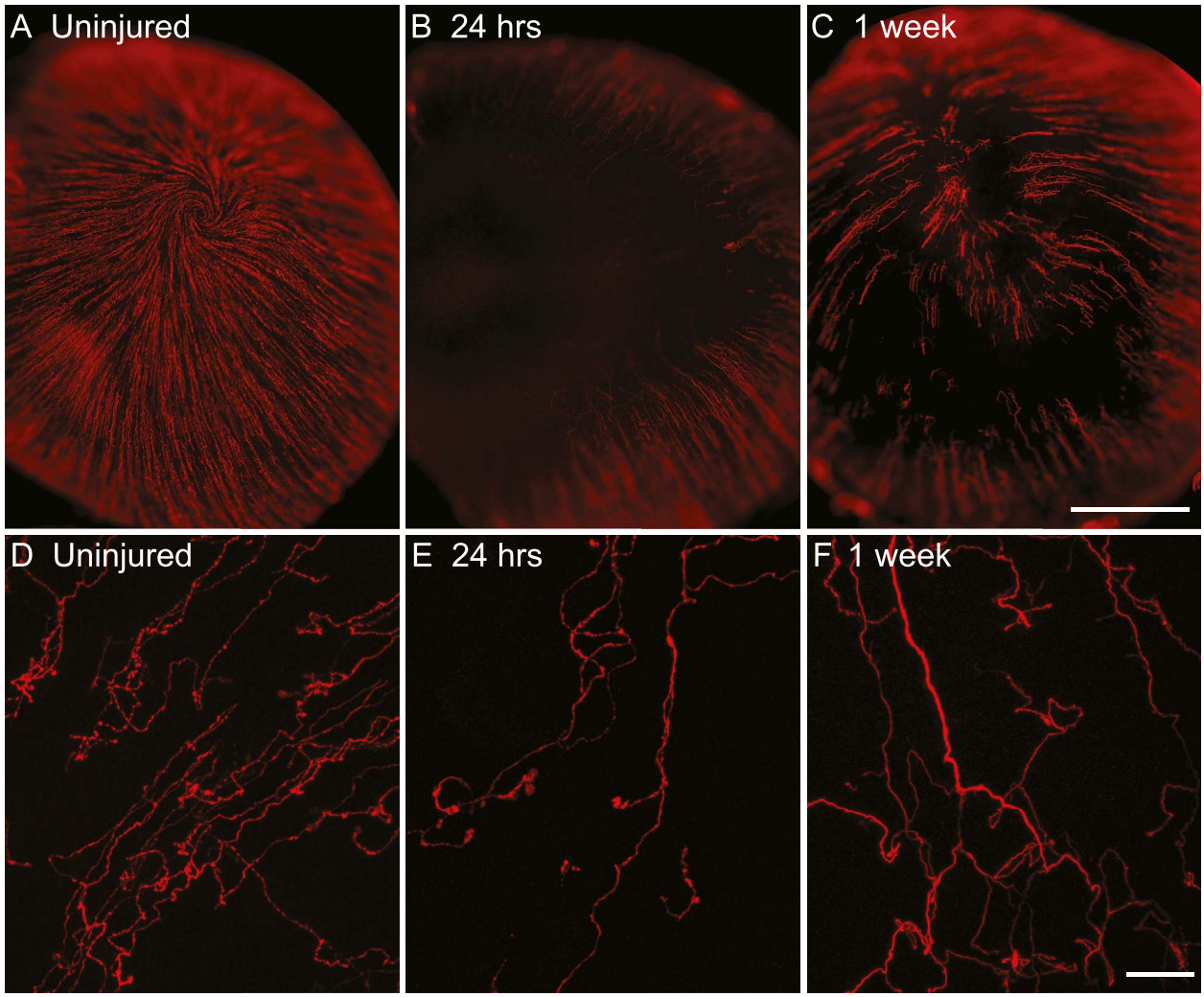

Figure 3. Corneal nerves detected with β-tubulin antibody are damaged after epithelial abrasion. (A–C) Epifluorescent images of whole-mount corneas show the loss of central corneal nerves in the abraded eye 24 hours after abrasion injury (B) compared with an uninjured cornea (A). There is partial recovery of corneal nerve density 1 week after abrasion (C). (D–F) High-magnification images of corneal nerve morphology show changes after abrasion injury. Images are projections of Z-stacks of 0.8 μm optical sections captured with a 63× Plan-Apochromat objective. (D) A high-magnification confocal image of β-tubulin–labeled corneal nerves from an area close to the central whorl of an uninjured cornea shows thin nerves beaded with varicosities. (E) At 24 hours after abrasion, the nerves lining the edge of the abraded area have enlarged nerve endings. (F) One week after injury, the abraded central cornea contains corneal nerves that are disorganized and smoother in appearance. Scale bars = 1 mm (A–C); 20 μm (D–F).

performed by an observer blind to the experimental conditions. Using the ImageJ Cell Counter Plug-In, every NT-stained TGN with a visible nucleus was marked with a number to get the total number of neurons in that section. Trigeminal ganglion neurons that were ATF3-immunoreactive (-ir) or CGRP-ir were evaluated separately using the Cell Counter Plug-In. The number of TGNs that contained ATF3 or CGRP was calculated as a percent for each ganglion and grouped by treatment. Data were expressed as mean % TGNs containing ATF3 or CGRP.

2.7. Statistics

Statistical analyses and graph production were performed using SigmaPlot 12.0 software. Data are expressed as the mean ± SEM unless otherwise noted. A 1-way analysis of variance (ANOVA) test was used when comparing more than 2 experimental groups; post-hoc tests were used during these analyses when applicable. We used a *t* test in those cases in which there were only 2 experimental groups to compare, or a paired *t* test for within-animal comparisons. In all studies, if the data did not meet normality criteria, we used appropriate nonparametric statistical tests. A *P* value of 0.05 was considered significant.

3. Results

3.1. Corneal abrasion causes short-term increase in evoked and spontaneous pain

To assess evoked nociceptive responses, menthol was applied to the left eye, and the number of nocifensive eye wipes was measured.[1] The dose of menthol tested is sufficient to activate TRPM8 channels, as well as other types of nociceptors.[51] Eye wipe responses to ocular application of menthol were increased at 24 hours after abrasion injury (**Fig. 1A**; 1-way ANOVA [$P = 0.002$]) compared with uninjured animals (Holm–Sidak post hoc, $P = 0.004$) and the unabraded contralateral eye at this time point (Holm–Sidak post hoc, $P = 0.006$). There was no change in eye wipe responses to menthol in the unabraded contralateral eye as

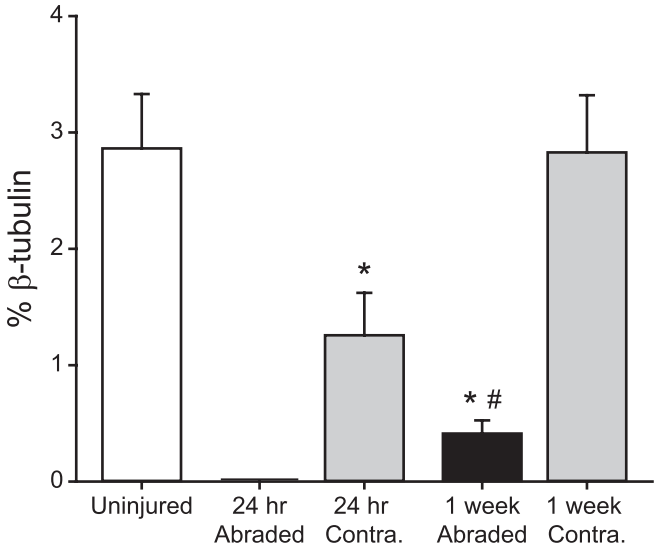

Figure 4. Quantitative changes in corneal epithelial nerve volumes after abrasion injury. Central corneal nerves were absent 24 hours after abrasion in the abraded eye, and there was also a significant nerve loss in the unabraded contralateral eye. Corneal nerve density was reduced in contralateral corneas 24 hours after abrasion (gray bar, n = 3) compared with uninjured control corneas (white bar, n = 6). Corneal nerve density remained reduced 1 week after abrasion (black bar, n = 4) compared with corneas from uninjured rats and unabraded contralateral corneas 1 week after abrasion (1 week Contra., gray bar, n = 4). *$P < 0.05$ vs uninjured corneas; #$P < 0.05$ vs contralateral side.

compared to the uninjured control eye at 24 hours. Responses in both eyes were at baseline levels 1 week after injury (**Fig. 1A**). The presence of hyperalgesia in the abraded eye is consistent with previous studies of increased photophobia using this model of corneal injury.[20]

Separate groups of animals were assessed for spontaneous pain using a wheel-running assay. There was no difference in baseline wheel running rates between abraded and uninjured control animals before corneal injury (abraded baseline: 1418.3 ± 307.7 revolutions; uninjured baseline: 1169.3 ± 205.9 revolutions; the *t* test, $P = 0.306$). A significant decrease in wheel revolutions occurred in the 24 hours after corneal abrasion (abraded 24 hours: 832.8 ± 136.2 revolutions) compared with baseline values (the Wilcoxon signed-rank test, $P = 0.031$), whereas no change in wheel running was evident in uninjured control animals (uninjured 24 hours: 1125.3 ± 394.1 revolutions; the paired *t* test, $P = 0.419$). Wheel revolution values returned to baseline levels in abraded animals by the second day after abrasion and remained at baseline through 1 week after abrasion. These data support the evoked pain data and suggest that spontaneous pain after corneal abrasion peaks 24 hours after injury and resolves by 1 week after corneal injury in abraded animals.

3.2. Corneal abrasion causes delayed reduction in tear production

Corneal abrasions can evoke symptoms of dry eye, including changes in tear production.[2] Tear production in the abraded eye was significantly reduced 1 week after the abrasion (baseline: 13.5 ± 1.5 mm; 1 week abraded: 9.0 ± 1.3 mm; the paired *t* test, $P = 0.0489$), but not 24 hours after abrasion (baseline: 12.8 ± 1.4; 24 hours abraded: 13.9 ± 1.5; the paired *t* test, $P = 0.182$). The tear measurements in the contralateral eye were not different at 24 hours (baseline: 9.5 ± 1.6; 24 hours contralateral: 9.5 ± 1.4; the paired *t* test, $P = 0.5$) or at 1 week (baseline: 8.5 ± 1.0; 1 week contralateral: 7.0 ± 1.1; the paired *t* test, $P = 0.148$), suggesting some independence of the reflex circuits for the 2 eyes. The percent of baseline tear production was calculated for each animal, averaged for each treatment group, and expressed as % tears (**Fig. 1B**). The temporal expression of the changes in tear production is apparently unrelated to nociceptive responses (**Fig. 1A**).

3.3. Corneal abrasion increases unilateral eye closures (winks) but not blinks

We assessed changes in behavioral responses related to ocular surface homeostasis because molecular mechanisms involved in cold nociception[48] have also been implicated in both spontaneous blinking and tear production.[47,50] We assessed bilateral (blinks; **Fig. 2A**) and unilateral (winks; **Fig. 2B**) eye closures in subsets of our corneal injury rats and uninjured control rats. We were not able to accurately quantify blinking and winking behavior at 24 hours after injury because these rats exhibit profound photophobia including extended eye closures.[20] However, rats showed increased ipsilateral winks 1 week after abrasion compared with baseline (**Fig. 2B**; the paired *t* test, $P = 0.0009$). Abraded animals also demonstrated increased winks in the contralateral unabraded eye (**Fig. 2B**; the paired *t* test, $P = 0.01$), although bilateral blink frequency did not change significantly (**Fig. 2A**; the paired *t* test, $P = 0.324$). These results show that the overall rate of eye closure is increased, but the responses are not bilaterally coordinated. This change in eye closure behavior occurs at a time when nocifensive responses have returned to normal. Interestingly, the increased wink behavior was detected in both eyes, whereas tear production was only significantly reduced in the abraded eye.

3.4. Central corneal nerves are absent 24 hours after abrasion and partially restored by 1 week

The extent of nerve injury was assessed at times when nociceptive responses were measured in our abraded rats (**Fig. 3A–C**). Using quantitative assessment of β-tubulin immunohistochemistry, we found that heptanol abrasion removed epithelial nerves from the central cornea at 24 hours after injury (**Fig. 3B**). Some corneal innervation returned at 1 week after injury (**Fig. 3C**), but nerve density was reduced and did not have the classic central whorl seen in intact corneas (**Fig. 3A**). These findings are intriguing and demonstrate that hyperalgesia can be evoked by ocular application of menthol, even in the absence of epithelial nerve endings in the central cornea. Also, nociceptive responses return to normal at a time when innervation is not fully restored.

Careful examination of nerve endings at high magnification (**Fig. 3D–F**) shows enlarged endings resembling neuromas at the border of the corneal abrasion 24 hours after injury (**Fig. 3E**). Some of these endings contained CGRP immunoreactivity (data not shown), supporting the idea that these are nociceptors.[25] These endings should be accessible to topical menthol applied to the eye of an awake rat and appear to be sufficient to support hyperalgesia (**Fig. 1**). Innervation to the abraded central cornea did not return to control levels 1 week after injury (**Fig. 3C, F**), and appeared to lack the organization and morphology of uninjured corneal nerves (**Fig. 3D**). Despite the nerve pathology, nociceptive responses returned to baseline levels by 1 week after injury.[54]

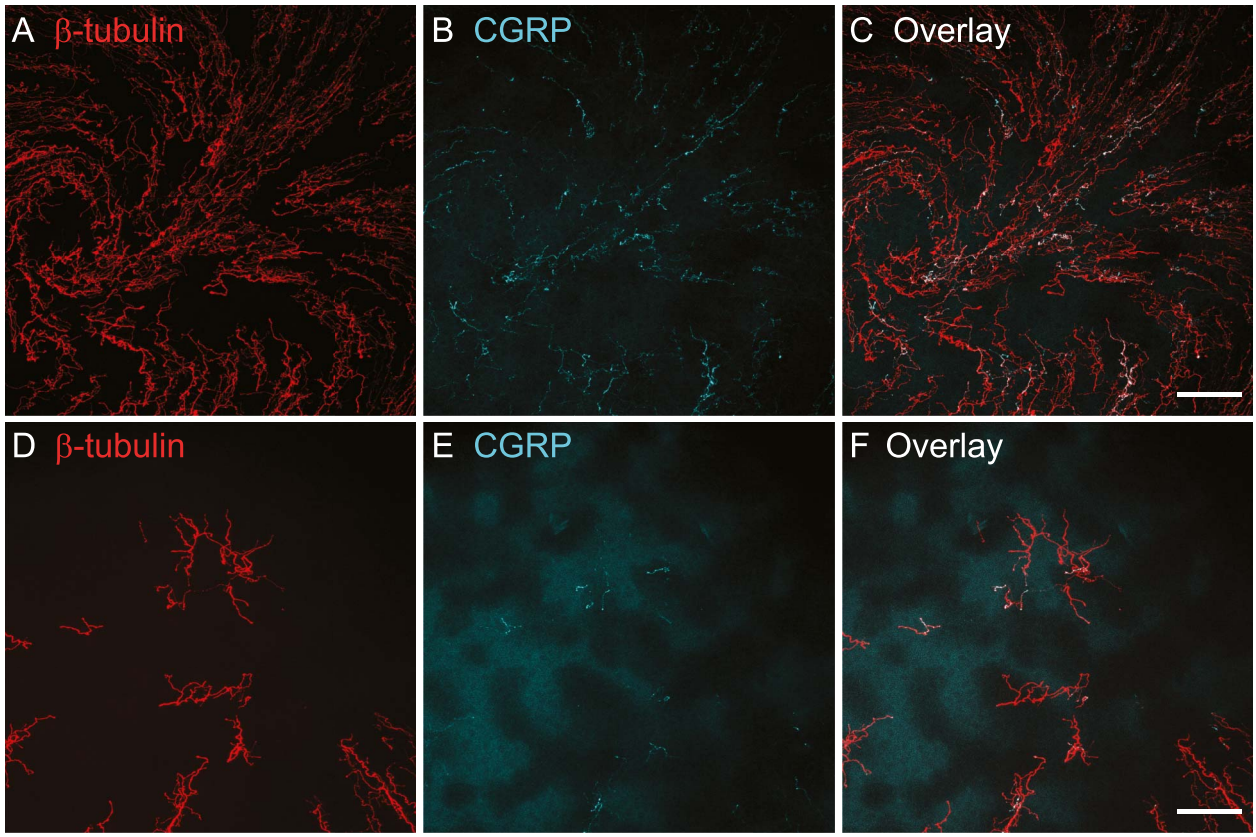

Figure 5. Confocal images of central corneas for uninjured control cornea (A–C) compared with 1 week after abrasion (D–F). β-tubulin (red) and CGRP (blue) signals are shown separately and in overlay (C and F). Both nerve density and CGRP expression remain low at 1 week after abrasion. Scale bar = 100 μm. CGRP, calcitonin gene-related peptide.

3.5. Unilateral abrasion injury alters corneal nerve density in both corneas

We quantitatively assessed nerve density in the corneal epithelium at different times after injury (**Fig. 4**). There was a complete absence of corneal nerves in the central portion of the cornea 24 hours after abrasion (**Figs. 3B and 4**). For the abraded eye, nerve density 1 week after abrasion was reduced by 86% compared with uninjured corneas (**Figs. 3 and 4**; 1-way ANOVA ($P = 0.004$), Holm–Sidak post hoc, $P = 0.006$). More surprising was a 56% reduction in corneal nerve volume in the unabraded contralateral eye 24 hours after injury compared with the uninjured group (**Fig. 4**; the *t* test, $P = 0.0312$). The innervation in the contralateral eye returned to baseline levels 1 week after abrasion (**Fig. 4**; Holm–Sidak post hoc, $P = 0.956$). There were no significant differences in corneal epithelium volume among the treatment groups (**Table 2**; 1-way ANOVA, $P = 0.358$), showing that changes in epithelium volume are not underlying nerve density changes. These findings are noteworthy because there were no detectable changes in nociceptive response in the contralateral eye (**Fig. 1**), despite significant changes in nerve density.

3.6. Calcitonin gene-related peptide content is altered after corneal nerve injury

Calcitonin gene-related peptide is the most abundant peptide in corneal central processes terminating in the trigeminal dorsal horn,[25] as well as in trigeminal ganglion cell bodies.[36,45] We examined the epithelial nerves to determine the CGRP content of the intraepithelial endings of corneal nerves and how the content of this molecule changes after corneal abrasion injury. Calcitonin gene-related peptide is present in a subset of corneal nerve endings (**Fig. 5**) and this can be quantified as a percentage of the nerves by examining CGRP as a portion of β-tubulin volume within the epithelium. Calcitonin gene-related peptide could not be calculated in the abraded corneas 24 hours after injury because intraepithelial nerves were absent from the central cornea, but 1 week after abrasion, the CGRP content of the remaining nerves was increased approximately 4-fold compared with the uninjured control group (**Fig. 6**; Kruskal–Wallis 1-way ANOVA on ranks, $P = 0.003$; the Dunn method post-hoc test, $P < 0.05$). Interestingly, CGRP content of nerves in the contralateral eye was stable at both 24 hours and 1 week time points, despite significant fluctuations in nerve volume in the uninjured eye (**Fig. 4**). This suggests that the neuropeptide content is well regulated during changes in innervation density in the absence of injury to the cornea, in contrast to the injured cornea where CGRP content was elevated even when nerve density was quite low (**Figs. 5 and 6**). It is also noteworthy that CGRP immunoreactivity was present in abraded corneas 1 week after injury in a diffuse pattern (**Fig. 5E**) that was not associated with nerve endings, and therefore not included in our analysis, but could be interpreted as being present in epithelial tissue. We speculate that this could be due to increased peptide release from the corneal nerves. Our findings support the hypothesis that there are phenotypic changes in corneal nerves that occur specifically after peripheral nerve axotomy.

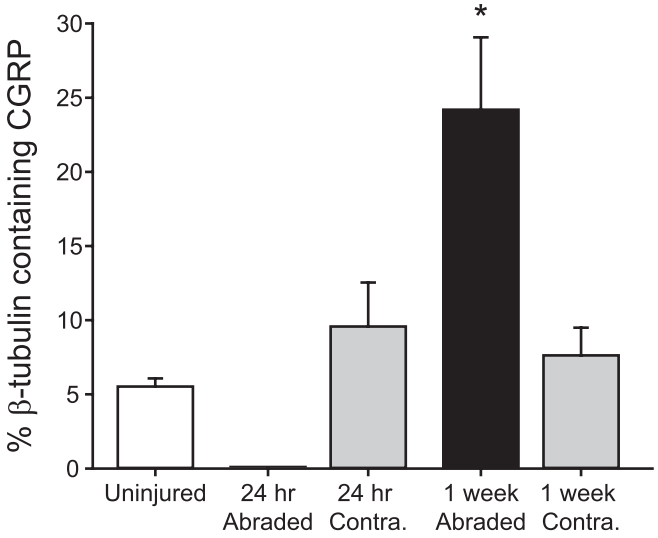

Figure 6. Calcitonin gene-related peptide (CGRP) content of β-tubulin nerves was increased 1 week after abrasion in injured corneas, but not in the nerves of the contralateral eye, despite reductions in nerve density. The volume of CGRP immunoreactivity within the volume of β-tubulin-ir corneal nerves was calculated for each cornea and expressed as the percent of β-tubulin containing CGRP. Calcitonin gene-related peptide content was not measurable at 24 hours in the abraded corneas because intraepithelial nerves were absent in these animals. The percent of β-tubulin volume containing CGRP was increased 1 week after abrasion injury (black bar, n = 4) as compared to uninjured corneas (white bar, n = 6). Calcitonin gene-related peptide levels remained stable within contralateral corneas at 24 hours (gray bar, n = 3) and 1 week (gray bar, n = 4) after abrasion. *$P < 0.05$ compared with the uninjured group.

3.7. Increased expression of ATF3 and calcitonin gene-related peptide in trigeminal ganglia after injury

Our findings show a disconnect between corneal epithelial nerve density, pain responses, and homeostatic responses such as tear production and eye closure. We hypothesize that phenotypic changes in corneal nerve cell bodies are likely induced after frank injury of the peripheral endings in the epithelium. In this case, we would expect to see changes in the expression of molecules in the trigeminal ganglion that happens acutely after injury. We examined the expression of a neuronal injury marker, ATF3,[56] as well as CGRP, the primary neuropeptide in corneal nociceptors[25] within the ophthalmic region of TG (see Methods). Both ATF3 and CGRP are present in subsets of trigeminal ganglion cells after injury (**Fig. 7**). The proportion of trigeminal ganglion cells containing the nerve injury marker ATF3 was increased 24 hours after injury in abraded rats, but only on the injured side (**Fig. 7D**) compared with uninjured control animals (1-way ANOVA [$P = 0.007$], Holm–Sidak post hoc, $P = 0.008$) or the contralateral unabraded side (Holm–Sidak post hoc, $P = 0.027$). ATF3 levels returned to control levels 1 week after abrasion (1-way ANOVA, $P = 0.343$). The proportion of TG neurons that contained ATF3 is high because the region of analysis was centered on the ophthalmic section of the ganglion that contains corneal afferents, but is not 100% because we did not use a tract tracer to confine our analysis to only corneal-projecting neurons.

The nociceptive marker CGRP showed the same pattern of change in the trigeminal ganglion, with increased levels 24 hours after injury on the side ipsilateral to the abrasion (**Fig. 7E**; 1-way ANOVA, $P = 0.046$). Interestingly, the CGRP content of trigeminal ganglion cells returned to baseline levels 1 week after injury (1-way ANOVA, $P = 0.322$) when CGRP content in the peripheral endings was increased (**Fig. 6**). Thus, the elevated CGRP content in the trigeminal ganglion is better correlated with increased pain responses than the CGRP content in the peripheral endings within the cornea, at the 1 week time point when both portions of the trigeminal nerve were assessed. Despite changes in peripheral nerve density in the contralateral eye 24 hours after injury (**Fig. 4**), there was no change in expression of either ATF3 or CGRP in the contralateral trigeminal ganglion at either time point (**Fig. 7D and E**). This difference suggests that the changes in peripheral nerve density seen in the contralateral eye are not of sufficient degree or nature to evoke molecular changes in the trigeminal ganglion, the cell bodies of the peripheral endings in the cornea.

3.8. Corneal nerve restoration is produced by original nerve population

Studies of cutaneous nerves suggest that regions of denervated epithelium are reinnervated by new nerve endings taking over the receptive field of the skin that has lost innervation.[14] We tested whether the nerves appearing in the central cornea at 1 week after abrasion injury were from new nerves taking over the corneal receptive field or from the same neurons sending in new branches. This hypothesis was tested by measuring sequential retrograde labeling from the central cornea at the time of abrasion injury (FG) and again 1 week later (CTb; **Fig. 8A**). A smaller application area for the second retrograde dye placement was used to limit labeling to nerve endings within the most central portion of the cornea after injury. Although this approach underestimates the number of cells innervating the central cornea, it ensures that we are only detecting cells with branches in the central cornea. The vast majority (96.2% ± 1.6%, n = 247 neurons from 4 rats) of trigeminal ganglion cells labeled with CTb 1 week after abrasion (**Fig. 8B**, green) also contained the FG tracer that was applied at the time of injury (**Fig. 8B**, magenta). These results indicate that the innervation in the central cornea 1 week after abrasion injury represents branches from the nerves that originally occupied this region of the epithelium, not from new nerves taking over that territory.

4. Discussion

Our findings show dynamic changes in pain, corneal nerve density, molecular phenotype, and ocular homeostasis after a superficial abrasion of the cornea. Hyperalgesia was seen acutely and was correlated with increased expression of ATF3 and CGRP in the trigeminal ganglion; whereas dry eye symptoms, including increased eye closures and reduced tear production, occurred later when acute pain had resolved. Although TRPM8 channels are involved in mediating nocifensive responses to menthol,[48] as well as ocular homeostatic responses such as blinking,[50] we find that changes in nociception and blinking did not always co-occur in our abrasion injury animals. Our results suggest that either TRPM8 is modulated in different ways over time after injury in our animals, or that it is not directly involved in mediating the changes we see after abrasion injury.

Chemical abrasion of the corneal surface with heptanol virtually abolished the epithelial innervation but resulted in increased pain responses. This paradoxical finding[54] suggests that abrasion injury causes alterations in either nerve function, the central pain pathways mediating corneal sensation, or both. The presence of neuromas at the edge of the abrasion injury may be a local mechanism for increased sensory nerve activation. We found expression of CGRP in these neuromas and others have found accumulation of sodium channels at these sites, coupled with

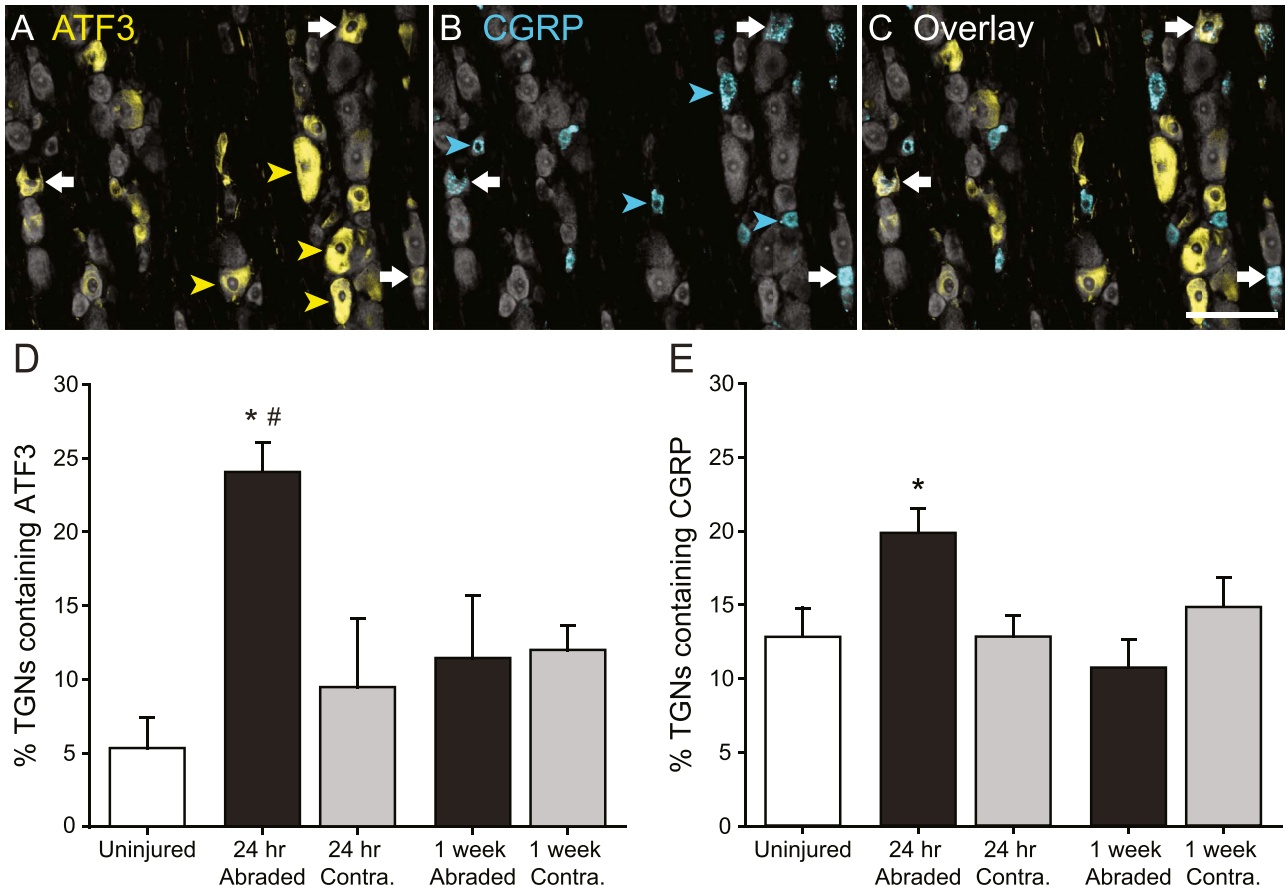

Figure 7. ATF3, a marker of neuronal injury, and the nociceptive marker CGRP are both present in rat trigeminal ganglion neurons (TGNs) and elevated after abrasion injury. (A–C) Subsets of TGNs (gray) contain ATF3 (A, yellow arrowheads) or CGRP (B, blue arrowheads), or in some cases, both markers (C, white arrows). Scale bar = 50 μm (D and E) The percent of TGNs that contain (D) ATF3 or (E) CGRP is increased in ipsilateral TGNs 24 hours after corneal abrasion (black bars, n = 3) compared with uninjured rats (white bars, n = 4). One week after abrasion, the percent of TGNs on the abraded side containing ATF3 (D) or CGRP (E) is similar to controls (black bars, n = 6). There were no significant increases in ATF3 or CGRP in contralateral TGNs at either time point (24 hours, gray bars, n = 3; 1 week, gray bars, n = 6). *$P < 0.05$ vs uninjured; #$P < 0.05$ vs contralateral side. CGRP, calcitonin gene-related peptide.

increased excitability of injured nerves.[3,11,13,42] Blockade of sodium channels with tetrodotoxin prevents the photophobia seen with corneal heptanol injury.[20] Because both the light used to evoke photophobia and our topical menthol application can reach nerve endings at the edge of the corneal injury, our findings are consistent with an acute upregulation of sodium channels in these endings that may mediate hyperalgesia. Changes in corneal nerve morphology, including swellings, have been reported after both nerve injury and application of intense stimuli such as high osmotic concentrations of hypertonic saline,[24,29] suggesting that these enlargements may be a site for aberrant nerve activity. By contrast, responses to stimuli confined to the denervated central cornea are impaired in the hours immediately after heptanol abrasion.[28]

Both CGRP and ATF3 expression in TG cell bodies increased 24 hours after injury and returned to normal levels by 1 week, paralleling the time course of changes in nociceptive responses. The increased expression of CGRP and ATF3 in the trigeminal ganglion may enhance central activation of pain pathways in response to ocular stimulation, leading to hyperalgesia.[37] Surprisingly, the CGRP content of the trigeminal ganglion was normal 1 week after injury, whereas CGRP expression was elevated within the peripheral corneal nerve population that is reinnervating the central cornea. A study from another laboratory reported no changes in CGRP or substance P in trigeminal ganglion cells after corneal injury with sodium hydroxide (NaOH),[16] but responses were assessed 3 days after injury. Our findings suggest that changes in molecular expression within the TG are rapid and dynamic after injury, and the content of cell bodies may be distinct from the content of peripheral or central endings of the trigeminal nerves. A previous study from our laboratory found expression of the transient receptor potential vanilloid 1 (TRPV1) channel in 30% of corneal-projecting cells in the TG but only 2% of their central terminals,[23] consistent with the idea of differential trafficking of molecules within corneal sensory neurons. Nerve injury may further provoke differential changes in peptide trafficking.

Increased CGRP expression in corneal epithelial nerves was seen in the context of severely reduced corneal nerve density, suggesting that there is more CGRP per nerve ending in the ocular surface. Calcitonin gene-related peptide is known to be released on noxious corneal stimulation,[3] and we speculate that there may be increased CGRP release in the epithelium, despite reduced nerve density. Reduced number of nerves and increased release of CGRP may impair corneal epithelial function and eventually destabilize the corneal surface.[34] One long-term consequence of this impaired corneal epithelial function may be reduced tear production,[43] and the reduced tear production may stimulate reflex blinking,[26] and thus cause the increase in eye closure responses at 1 week after injury. These results suggest

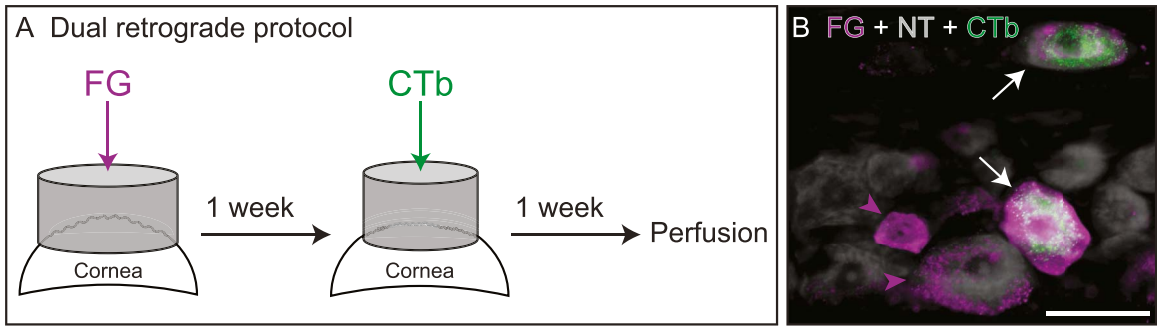

Figure 8. Identification of trigeminal ganglia innervating the central cornea. (A) Heptanol was applied within a small 6-gauge metal ring to abrade an area of the central cornea followed by application of the FG tracer. After 1 week, a shorter heptanol application time within a smaller diameter 7-gauge metal ring was used to lightly abrade the cornea and apply CTb to the central cornea. One week later, trigeminal ganglia were collected for analysis. (B) A representative epifluorescent micrograph demonstrates trigeminal ganglion neurons stained with NeuroTrace (NT, gray) that contains only FG (magenta arrowheads), FG and CTb (white arrows), or no tracer. Scale bar = 50 μm. CTb, Cholera Toxin B; FG, FluoroGold.

that increased CGRP in peripheral nerve endings in the cornea may contribute to the dry eye symptoms (reduced tear production and increased eye closures) seen 1 week after injury.

We saw no evidence of altered nociceptive responses in the contralateral eye, despite a 56% reduction in corneal nerve density.[35] The reduced nerve density could be due to superficial damage to the epithelium during extended intense eye closure during photophobia, but it is of note that there was still no change in nociception. Previous studies report unilateral disease or injury in the cornea causing bilateral effects on corneal nerve density, with no changes in corneal sensation. For example, patients with unilateral microbial keratitis had significant decreases in total number of corneal nerves, total nerve length, number of branches and branch nerve length in the uninfected, contralateral cornea[10] as assessed by in vivo confocal microscopy of subbasal nerves.[58] Despite the decreased nerve density, there was no significant change in corneal sensation. In a mouse model, after unilateral trigeminal axotomy, subbasal nerves from the center of the contralateral cornea were reduced 1 day after axotomy, which caused a loss of sensation on the injured side; on the contralateral side, there was a loss of nerves, but no significant decrease in corneal sensation.[61] The mechanisms underlying these contralateral changes are unclear, especially given that there are other examples of unilateral corneal injury and disease states that affect both contralateral nerve density and sensation.[22] Possible mechanisms of bilateral changes induced by unilateral corneal injury include bilateral increases in dendritic cells that mediate immune responses and alterations in tear cytokines in the context of unilateral bacterial keratitis.[60] Others have suggested that contralateral hyperalgesia, or mirror pain, after nerve injury may be related to changes in the peripheral nervous system, central pathways, and/or the activation of immunocompetent cells such as cytokines, macrophages, microglia, and astrocytes.[15,52] The presence of contralateral sensory deficits have also been reported in various genetic knockout animals, included vasoactive intestinal peptide and tumor necrosis factor alpha receptor knockouts.[18,39] Together, these studies suggest that the causes of contralateral effects of nerve injury may stem from multiple mechanisms, and may depend on the type and severity of the injury or disease state.

Spontaneous pain was evident as a depression in home cage wheel running 24 hours after corneal injury. Previous studies have shown that home cage wheel running is a sensitive method to objectively assess the duration and magnitude of inflammatory pain and migraine-like pain.[32,33] Despite the extent of the nerve injury, the pain resolved quite rapidly with wheel running returning to baseline levels quickly after injury; supporting the argument that pain is largely resolved within a few days, despite the lingering loss of corneal epithelial innervation.

Our animals also exhibited extensive blinking and photophobia 24 hours after abrasion. At 1 week after abrasion, bilateral blinks were at baseline levels, whereas unilateral eye closures were increased in both the injured and contralateral eyes. We speculate that the bilateral blinks may represent the spontaneous blink pattern[31] in these animals, whereas the unilateral eye closures, or winks, may represent corneal reflexes from ongoing spontaneous pain after abrasion injury. Previous studies in humans looking at normal blink rates and blink patterns found that even after topical application of an anesthetic to the cornea, spontaneous blinking was reduced but still present.[46] These and other studies suggest that spontaneous blinks are centrally mediated and modulated by external factors,[5,31] rather than being solely dependent on the state of the peripheral corneal environment. A recent human study found a correlation between higher blink rates and lower thresholds to corneal stimulation, meaning that more corneal pain is correlated with higher rates of blinking.[46] Therefore, if the rat is experiencing bouts of spontaneous pain, this may increase unilateral corneal reflexive eye closures on the injured side. It is unclear why the unabraded eye experienced increased unilateral eye closures as well, but causes of contralateral effects of injury or disease are complicated and require further study.

Corneal nerves are critically important in maintaining the health and integrity of the corneal epithelial environment.[6,44] In light of the reduction in corneal nerve density, we would have predicted that abrasion injury would affect tear production at both time points after injury. However, we only found a reduction in tears at the 1-week time point. It is possible that the extensive blinking observed at 24 hours may have produced reflex tears that would have masked a decrease in normal tear production caused by corneal nerve damage. At 1 week, the decrease in tears may indicate subtle, persistent effects of corneal nerve injury. Our study has demonstrated that the cornea is a complex system in which nociceptive and homeostatic mechanisms are integrated to maintain the important protective barrier. A recent study in humans demonstrated that corneal sensation, tear film stability, tear break-up time, and blink rate are significantly correlated.[46] This integration may underlie the temporal dynamics of corneal injury with immediate paradoxical changes in nerve density and evoked corneal sensation giving way to a dry eye disease

phenotype with changes in spontaneous pain, blink rate, and tear production. This study also suggests that corneal nerve density alone is not a good predictor of pain state.[19]

Disclosures

The authors have no conflict of interest to declare.

The work was supported by Grants from NIH: P30 NS061800 and R03 NS095057; the Medical Research Foundation of Oregon; and Oregon Health & Science University.

Acknowledgments

The authors thank Emma Eikerman, Aveek Ganguly, Clayton Hudson, and Ram Kandasamy for their technical assistance. Aveek Ganguly was supported by Saturday Academy's Apprenticeships in Science & Engineering program.

Article history:
Received 27 February 2018
Received in revised form 12 April 2018
Accepted 28 April 2018

References

[1] Aicher SA, Hermes SM, Hegarty DM. Denervation of the lacrimal gland leads to corneal hypoalgesia in a novel rat model of aqueous dry eye disease. Invest Ophthalmol Vis Sci 2015;56:6981–9.
[2] Ang RT, Dartt DA, Tsubota K. Dry eye after refractive surgery. Curr Opin Ophthalmol 2001;12:318–22.
[3] Belmonte C, Acosta MC, Gallar J. Neural basis of sensation in intact and injured corneas. Exp Eye Res 2004;78:513–25.
[4] Belmonte C, Brock JA, Viana F. Converting cold into pain. Exp Brain Res 2009;196:13–30.
[5] Belmonte C, Nichols JJ, Cox SM, Brock JA, Begley CG, Bereiter DA, Dartt DA, Galor A, Hamrah P, Ivanusic JJ, Jacobs DS, McNamara NA, Rosenblatt MI, Stapleton F, Wolffsohn JS. TFOS DEWS II pain and sensation report. Ocul Surf 2017;15:404–37.
[6] Beuerman RW, Schimmelpfennig B. Sensory denervation of the rabbit cornea affects epithelial properties. Exp Neurol 1980;69:196–201.
[7] Braz JM, Basbaum AI. Differential ATF3 expression in dorsal root ganglion neurons reveals the profile of primary afferents engaged by diverse noxious chemical stimuli. PAIN 2010;150:290–301.
[8] Carlton SM, Westlund KN, Zhang D, Sorkin LS, Willis WD. Calcitonin gene-related peptide containing primary afferent fibers synapse on primate spinothalamic tract cells. Neurosci Lett 1990;109:76–81.
[9] Chao C, Golebiowski B, Stapleton F. The role of corneal innervation in LASIK-induced neuropathic dry eye. Ocul Surf 2014;12:32–45.
[10] Cruzat A, Schrems WA, Schrems-Hoesl LM, Cavalcanti BM, Baniasadi N, Witkin D, Pavan-Langston D, Dana R, Hamrah P. Contralateral clinically unaffected eyes of patients with unilateral infectious keratitis demonstrate a sympathetic immune response. Invest Ophthalmol Vis Sci 2015;56:6612–20.
[11] Davies SL, Loescher AR, Clayton NM, Bountra C, Robinson PP, Boissonade FM. Changes in sodium channel expression following trigeminal nerve injury. Exp Neurol 2006;202:207–16.
[12] de Leeuw AM, Chan KY. Corneal nerve regeneration. Correlation between morphology and restoration of sensitivity. Invest Ophthalmol Vis Sci 1989;30:1980–90.
[13] Devor M, Keller CH, Deerinck TJ, Levinson SR, Ellisman MH. Na+ channel accumulation on axolemma of afferent endings in nerve end neuromas in Apteronotus. Neurosci Lett 1989;102:149–54.
[14] Devor M, Schonfeld D, Seltzer Z, Wall PD. Two modes of cutaneous reinnervation following peripheral nerve injury. J Comp Neurol 1979;185:211–20.
[15] Dubovy P, Tuckova L, Jancalek R, Svizenska I, Klusakova I. Increased invasion of ED-1 positive macrophages in both ipsi- and contralateral dorsal root ganglia following unilateral nerve injuries. Neurosci Lett 2007;427:88–93.
[16] Felipe CD, Gonzalez GG, Gallar J, Belmonte C. Quantification and immunocytochemical characteristics of trigeminal ganglion neurons projecting to the cornea: effect of corneal wounding. Eur J Pain 1999;3:31–9.
[17] Ferrari G, Nalassamy N, Downs H, Dana R, Oaklander AL. Corneal innervation as a window to peripheral neuropathies. Exp Eye Res 2013;113:148–50.
[18] Gallo A, Leerink M, Michot B, Ahmed E, Forget P, Mouraux A, Hermans E, Deumens R. Bilateral tactile hypersensitivity and neuroimmune responses after spared nerve injury in mice lacking vasoactive intestinal peptide. Exp Neurol 2017;293:62–73.
[19] Galor A, Levitt RC, Felix ER, Martin ER, Sarantopoulos CD. Neuropathic ocular pain: an important yet underevaluated feature of dry eye. Eye (Lond) 2015;29:301–12.
[20] Green PG, Alvarez P, Levine JD. Topical tetrodotoxin attenuates photophobia induced by corneal injury in the rat. J Pain 2015;16:881–6.
[21] Grixti A, Sadri M, Watts MT. Corneal protection during general anesthesia for nonocular surgery. Ocul Surf 2013;11:109–18.
[22] Hamrah P, Cruzat A, Dastjerdi MH, Pruss H, Zheng L, Shahatit BM, Bayhan HA, Dana R, Pavan-Langston D. Unilateral herpes zoster ophthalmicus results in bilateral corneal nerve alteration: an in vivo confocal microscopy study. Ophthalmology 2013;120:40–7.
[23] Hegarty DM, Hermes SM, Largent-Milnes TM, Aicher SA. Capsaicin-responsive corneal afferents do not contain TRPV1 at their central terminals in trigeminal nucleus caudalis in rats. J Chem Neuroanat 2014;61–62:1–12.
[24] Hegarty DM, Hermes SM, Yang K, Aicher SA. Select noxious stimuli induce changes on corneal nerve morphology. J Comp Neurol 2017;525:2019–31.
[25] Hegarty DM, Tonsfeldt K, Hermes SM, Helfand H, Aicher SA. Differential localization of vesicular glutamate transporters and peptides in corneal afferents to trigeminal nucleus caudalis. J Comp Neurol 2010;518:3557–69.
[26] Henriquez VM, Evinger C. The three-neuron corneal reflex circuit and modulation of second-order corneal responsive neurons. Exp Brain Res 2007;179:691–702.
[27] Hermes SM, Andresen MC, Aicher SA. Localization of TRPV1 and P2X3 in unmyelinated and myelinated vagal afferents in the rat. J Chem Neuroanat 2016;72:1–7.
[28] Hirata H, Mizerska K, Dallacasagrande V, Guaiquil VH, Rosenblatt MI. Acute corneal epithelial debridement unmasks the corneal stromal nerve responses to ocular stimulation in rats: implications for abnormal sensations of the eye. J Neurophysiol 2017;117:1935–47.
[29] Hirata H, Mizerska K, Marfurt CF, Rosenblatt MI. Hyperosmolar tears induce functional and structural alterations of corneal nerves: electrophysiological and anatomical evidence toward neurotoxicity. Invest Ophthalmol Vis Sci 2015;56:8125–40.
[30] Kallinikos P, Berhanu M, O'Donnell C, Boulton AJ, Efron N, Malik RA. Corneal nerve tortuosity in diabetic patients with neuropathy. Invest Ophthalmol Vis Sci 2004;45:418–22.
[31] Kaminer J, Powers AS, Horn KG, Hui C, Evinger C. Characterizing the spontaneous blink generator: an animal model. J Neurosci 2011;31:11256–67.
[32] Kandasamy R, Calsbeek JJ, Morgan MM. Home cage wheel running is an objective and clinically relevant method to assess inflammatory pain in male and female rats. J Neurosci Methods 2016;263:115–22.
[33] Kandasamy R, Lee AT, Morgan MM. Depression of home cage wheel running: A reliable and clinically relevant method to assess migraine pain in rats. J Headache Pain 2017;18:5.
[34] Ko JA, Mizuno Y, Ohki C, Chikama T, Sonoda KH, Kiuchi Y. Neuropeptides released from trigeminal neurons promote the stratification of human corneal epithelial cells. Invest Ophthalmol Vis Sci 2014;55:125–33.
[35] Koltzenburg M, Wall PD, McMahon SB. Does the right side know what the left is doing? Trends Neurosci 1999;22:122–7.
[36] LaVail JH, Johnson WE, Spencer LC. Immunohistochemical identification of trigeminal ganglion neurons that innervate the mouse cornea: relevance to intercellular spread of herpes simplex virus. J Comp Neurol 1993;327:133–40.
[37] Levine JD, Fields HL, Basbaum AI. Peptides and the primary afferent nociceptor. J Neurosci 1993;13:2273–86.
[38] Levitt AE, Galor A, Weiss JS, Felix ER, Martin ER, Patin DJ, Sarantopoulos KD, Levitt RC. Chronic dry eye symptoms after LASIK: parallels and lessons to be learned from other persistent post-operative pain disorders. Mol Pain 2015;11:21.
[39] Ma F, Zhang L, Oz HS, Mashni M, Westlund KN. Dysregulated TNFalpha promotes cytokine proteome profile increases and bilateral orofacial hypersensitivity. Neuroscience 2015;300:493–507.
[40] Madrid R, Donovan-Rodriguez T, Meseguer V, Acosta MC, Belmonte C, Viana F. Contribution of TRPM8 channels to cold transduction in primary sensory neurons and peripheral nerve terminals. J Neurosci 2006;26:12512–25.

[41] Marfurt CF, Cox J, Deek S, Dvorscak L. Anatomy of the human corneal innervation. Exp Eye Res 2010;90:478–92.
[42] Matzner O, Devor M. Hyperexcitability at sites of nerve injury depends on voltage-sensitive Na+ channels. J Neurophysiol 1994;72:349–59.
[43] 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.
[44] Muller LJ, Marfurt CF, Kruse F, Tervo TM. Corneal nerves: structure, contents and function. Exp Eye Res 2003;76:521–42.
[45] Nakamura A, Hayakawa T, Kuwahara S, Maeda S, Tanaka K, Seki M, Mimura O. Morphological and immunohistochemical characterization of the trigeminal ganglion neurons innervating the cornea and upper eyelid of the rat. J Chem Neuroanat 2007;34:95–101.
[46] Nosch DS, Pult H, Albon J, Purslow C, Murphy PJ. Relationship between corneal sensation, blinking, and tear film quality. Optom Vis Sci 2016;93: 471–81.
[47] Parra A, Madrid R, Echevarria D, del Olmo S, Morenilla-Palao C, Acosta MC, Gallar J, Dhaka A, Viana F, Belmonte C. Ocular surface wetness is regulated by TRPM8-dependent cold thermoreceptors of the cornea. Nat Med 2010;16:1396–9.
[48] Peier AM, Moqrich A, Hergarden AC, Reeve AJ, Andersson DA, Story GM, Earley TJ, Dragoni I, McIntyre P, Bevan S, Patapoutian A. A TRP channel that senses cold stimuli and menthol. Cell 2002;108:705–15.
[49] Perini I, Tavakoli M, Marshall A, Minde J, Morrison I. Rare human nerve growth factor-beta mutation reveals relationship between C-afferent density and acute pain evaluation. J Neurophysiol 2016;116:425–30.
[50] Quallo T, Vastani N, Horridge E, Gentry C, Parra A, Moss S, Viana F, Belmonte C, Andersson DA, Bevan S. TRPM8 is a neuronal osmosensor that regulates eye blinking in mice. Nat Commun 2015;6:7150.
[51] Robbins A, Kurose M, Winterson BJ, Meng ID. Menthol activation of corneal cool cells induces TRPM8- mediated lacrimation but not nociceptive responses in rodents. Invest Ophthalmol Vis Sci 2012;53: 7034–42.
[52] Ruohonen S, Jagodi M, Khademi M, Taskinen HS, Ojala P, Olsson T, Roytta M. Contralateral non- operated nerve to transected rat sciatic nerve shows increased expression of IL-1beta, TGF-beta1, TNF-alpha, and IL-10. J Neuroimmunol 2002;132:11–17.
[53] Sandvig KU, Haaskjold E, Bjerknes R. Regeneration of the rat corneal epithelium after injury. A cell kinetic study comparing the reaction after abrasions made mechanically and with n-heptanol. Acta Ophthalmol (Copenh) 1991;69:717–22.
[54] Simone DA, Nolano M, Johnson T, Wendelschafer-Crabb G, Kennedy WR. Intradermal injection of capsaicin in humans produces degeneration and subsequent reinnervation of epidermal nerve fibers: correlation with sensory function. J Neurosci 1998;18:8947–59.
[55] Tran MT, Ritchie MH, Lausch RN, Oakes JE. Calcitonin gene-related peptide induces IL-8 synthesis in human corneal epithelial cells. J Immunol 2000;164:4307–12.
[56] Tsujino H, Kondo E, Fukuoka T, Dai Y, Tokunaga A, Miki K, Yonenobu K, Ochi T, Noguchi K. Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: a novel neuronal marker of nerve injury. Mol Cell Neurosci 2000;15:170–82.
[57] Vashisht S, Singh S. Evaluation of Phenol Red Thread test versus Schirmer test in dry eyes: a comparative study. Int J Appl Basic Med Res 2011;1:40–2.
[58] Wang EF, Misra SL, Patel DV. In vivo confocal microscopy of the human cornea in the assessment of peripheral neuropathy and systemic diseases. Biomed Res Int 2015;2015:951081.
[59] Yagev R, Levy J, Shorer Z, Lifshitz T. Congenital insensitivity to pain with anhidrosis: ocular and systemic manifestations. Am J Ophthalmol 1999; 127:322–6.
[60] Yamaguchi T, Hamrah P, Shimazaki J. Bilateral alterations in corneal nerves, dendritic cells, and tear cytokine levels in ocular surface disease. Cornea 2016;35(suppl 1):S65–S70.
[61] Yamaguchi T, Turhan A, Harris DL, Hu K, Pruss H, von Andrian U, Hamrah P. Bilateral nerve alterations in a unilateral experimental neurotrophic keratopathy model: a lateral conjunctival approach for trigeminal axotomy. PLoS One 2013;8:e70908.