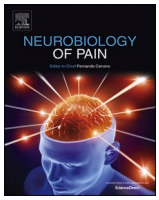
Neurobiology of Pain 16 (2024) 100165

Contents lists available at ScienceDirect

Neurobiology of Pain

journal homepage: www.sciencedirect.com/journal/neurobiology-of-pain



Activating transcription factor 3 (ATF3) and calcitonin gene-related peptide (CGRP) increase in trigeminal ganglion neurons in female rats after photorefractive keratectomy (PRK)-like corneal abrasion

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ARTICLE INFO

Keywords:
CGRP
ATF3
Trigeminal
Pain
Sex
Corneal
Nerve Injury
Immunocytochemistry

ABSTRACT

Photorefractive keratectomy (PRK) is a type of eye surgery that involves removal of the corneal epithelium and its associated nerves, which causes intense acute pain in most people. We used a rat model of corneal epithelium removal (corneal abrasion) to examine underlying cellular and molecular mechanisms. In this study, we used immunohistochemistry of trigeminal ganglion (TG) to assess neuronal content of CGRP and ATF3, as well as orbital tightening (OT) to assess spontaneous pain behaviors. CGRP is an important neuropeptide in pain modulation and ATF3 is often used as a nerve injury marker. We found dynamic changes in CGRP and ATF3 in TG; both increased significantly at 24 h following corneal abrasion and females had a more pronounced increase at 24 h compared to males. Interestingly, there was no sex difference in OT behaviors. Additionally, the number of cells containing either CGRP or ATF3 in each animal correlate significantly with their OT behavior at the assessed timepoint. Since CGRP increased most in females, we tested the effectiveness of Olcegepant, a CGRP antagonist, at reducing OT behaviors following corneal abrasion in female rats. Olcegepant (1 mg/kg) was given prior to and again at 24 h after abrasion but did not change OT behaviors at any time over a 1-week period. Examination of CGRP and ATF3 together in TG showed that they rarely colocalized, indicating that the cells with upregulated CGRP are distinct from those responding to epithelial nerve injury. The studies also show that underlying molecular responses may be sex specific.

1. Introduction

The cornea is a highly specialized ocular tissue that forms the outer anterior surface of the eye, is an essential component of the visual system, and acts as a protective barrier to other vulnerable ocular tissues. The integrity of the cornea is, in part, maintained by a highly concentrated network of nerves that innervate the corneal epithelium and contribute to both sensory and homeostatic mechanisms designed to protect and nourish this important component of the ocular surface (Aicher et al., 2013; Marfurt and Del Toro, 1987; Belmonte et al., 2004; Shaheen et al., 2014; Beuerman and Schimmelpfennig, 1980; Dartt and Willcox, 2013). Damage to the corneal epithelium due to accidental injury, disease, or vision correction surgeries can compromise these functions, resulting in changes sensation and tear production (Hegarty et al., 2018).

The corneal abrasion model used in this study has previously been used to simulate the initial step of the photorefractive keratectomy (PRK) vision correction procedure (Hegarty et al., 2018; Hegarty et al., 2022; Green et al., 2015). In one of our previous studies using male rats, this model demonstrated dynamic behavioral and lacrimation changes following corneal abrasion, as well as increases in the number of trigeminal ganglion (TG) neurons expressing calcitonin gene-related peptide (CGRP) and activating transcription factor 3 (ATF3) at 24 h post-abrasion (Hegarty et al., 2018). In a subsequent study using this model, both male and female rats were used to assess the therapeutic effectiveness of an ophthalmic treatment after corneal abrasion and we found sex differences in spontaneous pain behavior after treatment (Hegarty et al., 2022). This study and other preclinical and clinical

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https://doi.org/10.1016/j.ynpai.2024.100165
Received 2 August 2024; Received in revised form 28 August 2024; Accepted 3 September 2024
Available online 11 September 2024

studies have reported sex differences in different pain states, highlighting the necessity of including both sexes in pain studies (Shansky and Murphy, 2021; Russo and Hay, 2023).

CGRP is an abundant and multifunctional neuropeptide that has been implicated in a wide variety of processes, such as pain, inflammation, and injury recovery (Russo and Hay, 2023; Brain and Cambridge, 1996; Dieterle et al., 2011; Ko et al., 2014). There is also extensive evidence of a sex difference in the involvement and effect of CGRP on pain mechanisms, especially those mediated by the trigeminal nerve, including migraine (Russo, 2015; Ji et al., 2019; Avona et al., 2019; Ahmad and Rosendale, 2022). ATF3 is a marker of nerve injury and stress and has been shown to be upregulated in sensory ganglion cells after peripheral nerve injury (Braz and Basbaum, 2010; Hai et al., 2010; Launay et al., 2016; Vitoux et al., 2020). Assessment of the number and distribution of ATF3+ trigeminal neurons and colocalization with CGRP after corneal abrasion will help determine the TG neuronal response to acute injury of the ocular surface and intraepithelial nerves. Our experimental design also allows us to determine if there is a relationship between the TG neuronal response and pain behaviors after corneal injury in individual animals and between sexes.

2. Materials and methods

2.1. Animals

Sprague-Dawley rats (9–10 weeks old; males: 250–350 g; females: 180–215 g; Charles River Laboratories, Wilmington, MA) were used for these studies. A total of 36 rats (12 male; 24 female) were included; see Results section for group sizes in each experiment. Behavioral data from six rats 1 week post-abrasion (3M (male), 3F (female)) were published as controls in a previous study (Hegarty et al., 2022). Trigeminal ganglion tissue from these animals was used for immunohistochemical analysis and has not been published elsewhere. Rats were housed in pairs and were kept on a 12/12 light/dark cycle with lights on at 0600. All behavioral testing was done between 0900 and 1200. Rats had access to food and water *ad libitum*. All protocols were approved by the OHSU Institutional Animal Use and Care Committee.

2.2. Corneal abrasion

Corneal abrasion was performed as previous described. (Hegarty et al., 2018; Hegarty et al., 2022). Each rat was initially anesthetized in a Plexiglas chamber with 5% isoflurane in oxygen, delivered via an Isotex Tec3 vaporizer (Datex-Ohmeda; Madison, WI). Rats were then transferred to a heating pad (36 °C) and positioned on their side with their left cornea perpendicular to the table surface. Anesthesia was maintained by administration of 2.5–3.5% isoflurane in oxygen delivered via a nose cone. Two drops of 0.5% proparacaine hydrochloride were applied to the left ocular surface to reduce spontaneous eye movements. After drying the corneal surface with a surgical swab, a small 6-gauge metal ring (4.4 mm internal diameter, stainless steel tubing, custom, HTX-06R; Small Parts Inc, Miramar, FL) was used to limit heptanol exposure on peripheral areas of the cornea. Petroleum jelly was applied to the edge of the metal ring that contacts the ocular surface to secure the ring centrally on the cornea over the pupil. The outer epithelial layer was chemically abraded by applying 10 μL of 1-heptanol (99%; Alfa Aesar, Ward Hill, MA) for 90 s before being rinsed profusely with sterile saline. The heptanol-treated area was then debrided by gently rubbing the epithelium with a surgical spear (BVI, Irvine, CA). Following the procedure, rats were removed from anesthesia and monitored on the heating pad as they recovered before being returned to their home cage.

2.3. Spontaneous orbital tightening behavior

Orbital Tightening (OT) behavioral analysis, adapted from the rat grimace scale (Sotocinal et al., 2011) was used to assess spontaneous pain in rats as previously described (Hegarty et al., 2022). Behavioral analyses were conducted on videos of freely moving rats to assess OT of the injured eye at select times. OT was measured prior to corneal abrasion to establish the Baseline OT score and then measured again at 24 h, 72 h, and 1 week following corneal abrasion. The OT data from the 1 week abraded male and female rats (n = 6) were previously published in another study (Hegarty et al., 2022).

Each rat was habituated to handling and the behavior room prior to Baseline OT assessment. Handling and behvaioral assessments were conducted by one researcher throughout all time points. Animals were placed into a clear Plexiglas chamber (8.25″ L x 4.24″ W x 6.0″ H) for 10 min on two separate days. On each testing day, rats were acclimatized to the behavior room for at least 30 min. Individual rats were then placed in the observation chamber for a 5-minute acclimation period, immediately followed by a 5-minute recording session. Behavioral recordings were captured under ambient room lighting (500 – 550 lx; UrceriMT-912 light meter) using two cameras (GoPro Hero 7 and Hero 8, San Mateo, CA; 1440 pixels, 30 frames/sec, wide FOV) in fixed positions on either side of the chamber, as well as mirrors positioned to ensure continuous viewing of the abraded left eye.

For analysis, screen shots were extracted from the 5-minute recordings at approximately 30-second intervals (excluding grooming bouts and spontaneous bilateral blinking), resulting in a total of 10 images per rat at each time point. Images were compiled across all rats and time points, randomized, and assigned a unique identifier. Images were then scored by two independent reviewers who were blinded to treatment and time point. OT behavior was scored on a scale from 0 to 2, where 0 indicates no observable orbital tightening, and 2 represents complete or nearly full orbital tightening. OT scores across the 10 images for each rat were summed, thus total OT score can range from 0 (open at all times) to 20 (eye closed at all times).

2.4. Olcegepant treatment

A subset of female rats (n = 12) was utilized to evaluate the efficacy of the CGRP receptor antagonist Olcegepant in mitigating pain behaviors subsequent to corneal abrasion. Olcegepant was procured from Tocris Bioscience (Bristol, UK, cat no. 4561), dissolved in 5% DMSO and 2% Tween-80, then diluted to a target concentration of 1 mg/mL using 0.9% sterile saline. The solution for the drug and vehicle comprised 93% saline, 5% DMSO, and 2% Tween-80. Both drug and vehicle solutions were subjected to sterile filtration and preserved at −80 °C until use. These solutions were not subjected to multiple freeze–thaw cycles and were utilized within a month of preparation. Rats were administered 1 mg/kg Olcegepant (n = 6) or vehicle (n = 6) via intraperitoneal (IP) injection 30 min prior to corneal abrasion and again 30 min prior to behavioral assessments at 24 h post-abrasion. Corneal abrasion and orbital tightening recordings were conducted following the same protocols as described above.

2.5. Immunohistochemistry

Transcardial perfusions and immunohistochemistry on trigeminal ganglia tissue were performed as previously described (Hegarty et al., 2018; Hegarty et al., 2014). Rats were overdosed with sodium pentobarbital (150 mg/kg Sigma-Aldrich cat # P3761). Once rats no longer had nociceptive withdrawal reflex to foot or tail pinch, the chest cavity was opened, and the rat was perfused transcardially through the ascending aorta with 10 mL heparinized saline (1000 units/mL) followed immediately followed by 600 mL of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB). Trigeminal ganglia (TGs) were dissected from each rat, being careful to preserve the mandibular branch of the trigeminal nerve (V3) to aid in orientation. TGs were then post-fixed in 4% paraformaldehyde in PB for 30 min, rinsed in PB, excess connective tissue was removed, and TGs were then stored in 30% sucrose in PB for at least 24 h at 4 °C to cryoprotect the tissue. Each TG

ipsilateral to abrasion (left) was embedded in Tissue-Tek ® optimal cutting temperature (O.C.T.) compound, frozen and sectioned longitudinally at 20 μm on a Leica CM-1950 cryostat (Leica Microsystems Inc, Buffalo Grove, IL). TG sections were mounted directly onto room temperature Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Sections were allowed to dry and slides were stored at –20 °C until immunoprocessing.

TG sections were processed for dual-labeling immunohistochemistry for Calcitonin Gene-Related Peptide (CGRP) and Activating Transcription Factor 3 (ATF3) as previously described (Hegarty et al., 2018). Sections were thawed at room temperature and were circumscribed on the slide with a hydrophobic barrier (ImmEdge pen, Vector Laboratories, Inc, Newark, CA). TG sections were incubated in 1% sodium borohydride in PB for 30 min, then in 0.5% bovine serum albumin (BSA) in 0.1 M Tris-buffered saline, pH 7.6 (TS) for 30 min and then incubated in a cocktail of primary antibodies in 0.1% BSA, 0.25% Triton-X 100 in TS for 2 nights at 4 °C. The primary antibody cocktail was comprised of Rabbit anti-ATF3 (1:1000; cat no. NBPI-85816, Novus Biologicals, Centennial, CO) and Goat anti-CGRP (1:2000; cat no. ab36001, Abcam, Waltham, MA). Tissue was rinsed and incubated in a fluorescent secondary antibody cocktail consisting of Donkey anti-Rabbit Alexa Fluor (AF) 488 (1:800; cat no. A-21206, ThermoFisher Scientific (TFS), Waltham, MA) and Donkey anti-Goat AF 546 (1:800; cat no. A-11056, TFS) in 0.1% BSA in TS for 2 h at room temperature. Tissue sections were rinsed at least 3 times with PB or TS between each incubation step. Slides were cover slipped with ProLong™ Gold Antifade Mountant (TFS).

2.6. Image analysis

The V1/V2 area of TG sections was imaged at low magnification using a 4x UPlanFLN objective on an Olympus BX51 epifluorescence microscope with an attached DP74 camera and associated CellSens software (Olympus/Evident, Tokyo, Japan). For each TG, 8 – 20 sections that were 60 μm apart were imaged and analyzed. The orientation of the section was the same in each image so that the approximate V1 and V2 regions could be identified. The ATF3 and CGRP markers were imaged separately using the FITC and TRITC filter cubes, respectively. The number of Total, CGRP+ and ATF3+ TG cells in each image were manually counted using the Fiji Cell Counter plugin with Grid overlay (Schindelin et al., 2012). Background labeling in the red (TRITC, 546 nm) channel was used to count total cells (Dieterle et al., 2011). Nuclear ATF3 immunolabeling was confirmed in Fiji by tracing the membrane of the cell and using the histogram tool to determine the intensity of the nuclear labeling. Cells with a difference of 50 or more in intensity between background labeling of the cell body and the labeled nucleus were scored ATF3+. The number of ATF3+ or CGRP+ TG cells counted in all sections for each TG within a sex (Male, Female) and within a treatment group (Control, 24 h post-abrasion, 72 h post-abrasion, 1 week post-abrasion) were summed and divided by the total number of cells counted in those TGs and were expressed as a proportion for statistical analysis or as %ATF3 or %CGRP for graphing.

A subset of images was used to measure the cross-sectional areas of CGRP+ TG cells. One section per 24 h abraded TG that contained the highest number of ATF3+ cells was chosen. Using Fiji, measurements were calibrated to the scale bar, the circumference of each CGRP+ cell was traced using the Freehand Tracing tool and the area of the cell was calculated. Cells were sorted into small (<400 μm), medium (400–800 μm) and large (>800 μm) cells as in Bae et al (Bae et al., 2015). For ATF3+ cell distribution assessment, a 4x7 grid was placed over each TG image and the number of ATF3+ cells in each box was recorded. The central line of the grid was used to distinguish between the V1 and V2 areas, similar to Dieterle et al (Dieterle et al., 2011). Grid boxes were a 370 μm x 370 μm square.

2.7. Statistics

Statistical analyses and graph production were performed using Sigmaplot 14.5 software. Z-tests were used to compare the proportions of ATF3+, CGRP+ or double-labeled TG cells among sexes, treatment groups and time points in which higher z values indicate larger differences, and P values < 0.05 indicate statistical significance (Hegarty et al., 2010). Kruskal-Wallis one-way ANOVA on ranks was used to assess differences in OT scores in abrasion rats across post-abrasion time points. Mann-Whitney Rank Sum tests were used to assess differences in control animal baseline and endpoint OT scores, and differences in the measured areas of CGRP+ cells in TGs. Linear regressions were used to assess the relationship between OT scores and %ATF3 or %CGRP. In the Vehicle-/Olcegepant-treated cohort of rats, a two-way ANOVA was used to analyze OT behavior.

3. Results

3.1. Male and female rats demonstrate spontaneous pain behaviors after corneal abrasion

Corneal abrasion caused acute increases in OT scores in both male and female rats that peaked at 24 h and returned to near-baseline values over a 1-week period (Fig. 1). OT data from the 1-week post-abrasion males and females were published in our previous study (Hegarty et al., 2022), but are included here for additional neurochemical analyses of TG. There were no significant differences between male and female OT scores at any time point, therefore, OT scores were combined for statistical analysis. In abraded rats, there was a main effect of time (Kruskal-Wallis one way ANOVA on Ranks, $p < 0.001$), with OT scores significantly increased at 24 h post-abrasion (16.7 ± 0.7 (mean ± SEM), $n = 18$) as compared to baseline (1.4 ± 0.5, $n = 18$) scores (Dunn's post hoc multiple comparisons, $p < 0.001$) and remaining significantly increased at 72 h (10.4 ± 2.1; $n = 12$, $p = 0.008$), although there was a range of scores at this time point. At 1 week (10.0 ± 5.6, $n = 6$), OT scores were not significantly different than baseline values ($p = 0.26$), although there was also a range of scores at this time point. There was no significant difference between baseline and endpoint OT scores of control, non-abraded rats (baseline: 0 ± 0; endpoint: 1.7 ± 1.1; $n = 6$; Mann-Whitney Rank Sum test, $p = 0.18$). Also, baseline OT scores between controls (0 ± 0, $n = 6$) and pre-abrasion animals ($n = 18$) were not significantly different (Mann-Whitney Rank Sum test, $p = 0.08$). Taken together, these data show that corneal abrasion induces acute pain behaviors in most rats and sustained OT behavior in some rats.

3.2. Trigeminal ganglia neurons in V1 and V2 regions contain ATF3 and CGRP

Immunocytochemical labeling for both ATF3 (Fig. 2**A, 2C**) and CGRP (Fig. 2**B, 2C**) was present in V1/V2 TG cells. In total, 202,876 cells from 24 TGs (1 TG/animal, with an average of 8453 ± 566 cells per animal) were counted, representing male and female control TGs and TGs from three time points after abrasion. Cells were assessed for ATF3 and/or CGRP immunoreactivity.

3.3. ATF3 in TG neurons increases following corneal abrasion

The number of ATF3+ cells in V1/V2 TG (Fig. 2**A**) was assessed from both sexes in control TGs and in TGs from time points after abrasion (Fig. 3). The number of ATF3+ cells from all TGs in a treatment group (control, abraded), at a particular time point (baseline, post-abrasion time points), within each sex (male, female) was summed and expressed as a proportion of the total TG cells counted within that group. Proportions were then compared statistically using z-tests. There were no significant differences in the proportion of ATF3+ neurons between in male and female control TGs (z-test, $z = 0.14$, $p = 0.890$). After

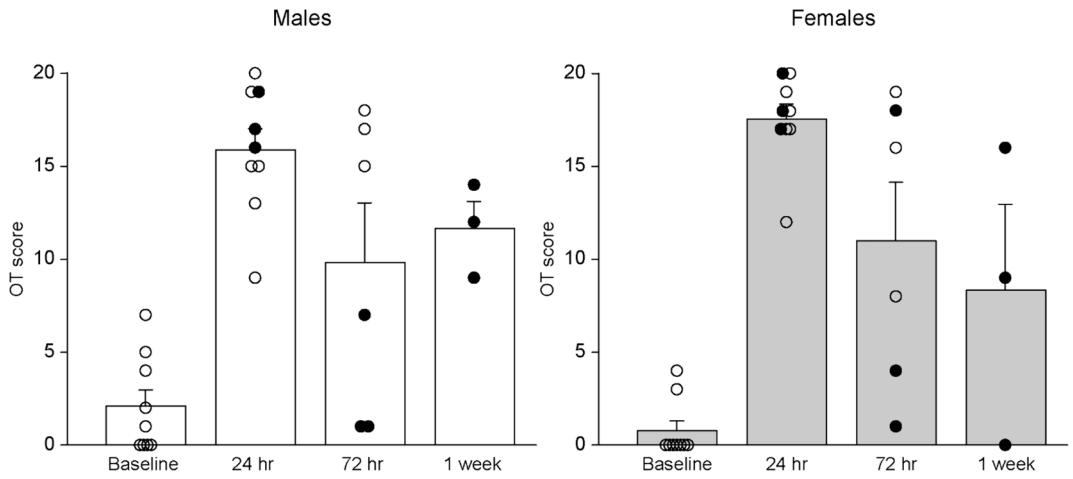

Fig. 1. Spontaneous pain behaviors peak at 24 h after corneal abrasion in male and female rats. There were no sex differences in orbital tightening (OT) at any time point. After abrasion, there was an increase in OT scores at 24 and 72 h as compared to baseline scores in males and females. Filled circles represent rats that were sacrificed at that time point for TG cell analysis; open circles represent OT scores for rats sacrificed at a later time point. Detailed statistical analyses be found in section 3.1. Due to the absence of sex differences, male and female scores were pooled for statistical analysis. N=24 rats; Controls: 3M, 3F; Abrasion: 3M, 3F rats per post-abrasion time point.

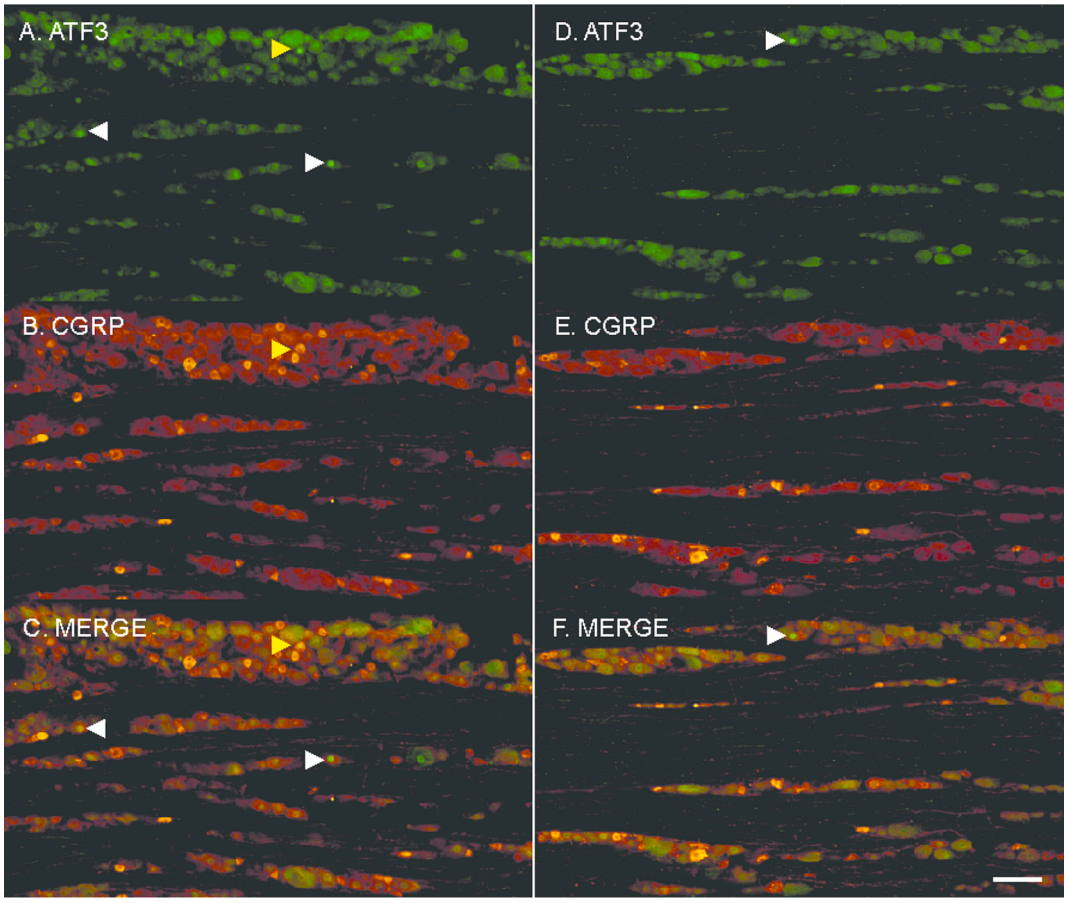

Fig. 2. ATF3 and CGRP labeling in TG cells of a female rat at 24 h after abrasion (A-C) and in a female control rat (D-F). Representative images demonstrate ATF3+ neurons (A, D, green), CGRP+ neurons (B, E, red) and the merged images (C, F). White arrowheads indicate some ATF3+ nuclei (A, D). One neuron that was both ATF3+ and CGRP+ (yellow arrowhead) is indicated. No colocalized neurons are present in the control merged image. Scale bar = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

abrasion, male and female rats both experienced an increase in the number of ATF3+ cells at 24 h (z-test vs control, males: $z = 9.70$, $p < 0.001$, power = 1.00; females: $z = 21.506$, $p < 0.001$, power = 1.00) and 72 h (z-test vs control, males: $z = 9.147$, $p < 0.001$, power = 1.00; females: $z = 11.231$, $p < 0.001$, power = 1.00) after abrasion compared to controls. At 1 week, however, the proportion of ATF3+ TG neurons was still significantly higher in females compared to controls (z-test, $z = 3.267$, $p = 0.001$, power = 0.89), while the proportion of ATF3+ TG

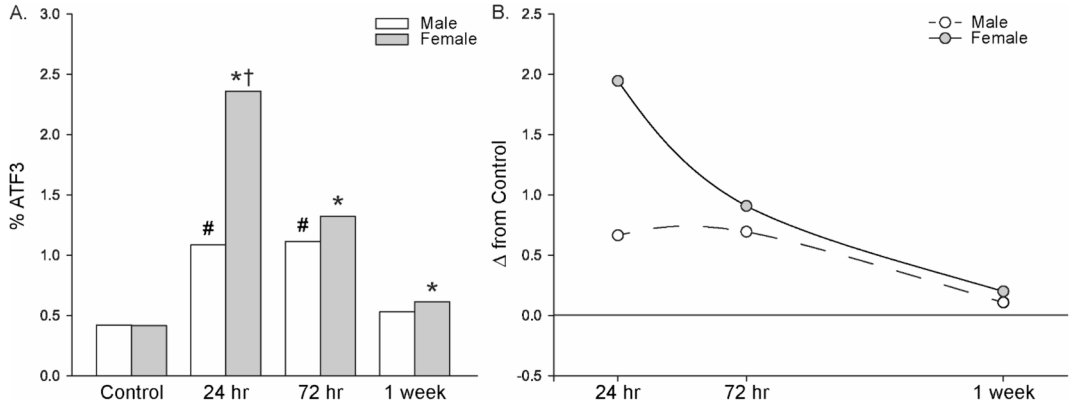

Fig. 3. ATF3 is acutely increased after corneal abrasion. (A) Each bar represents the proportion of ATF3+ TG neurons (%ATF3) for each group at each time point. ATF3 was significantly increased in TGs from abraded males and females compared to control rats at 24 and 72 h, with a significant sex difference at 24 h. At 1 week, %ATF3 was still elevated in females, but not males. * P<0.001 compared to female control, # P<0.001 compared to male control, † P<0.001 compared to males within the same time point. N=24, Controls: 3M, 3F, Abrasion: 3M, 3F per time point (24 h, 72 h, 1 week). (B) %ATF3 represented as % change from control (solid horizontal line).

neurons in males was back to control levels. Females also had a significantly higher proportion of ATF3+ TG neurons at 24 h post-abrasion than males (z-test, $z = 11.44$, $p < 0.001$, power = 1.00), but there were no other sex differences (z-tests, 72 h: $z = 1.69$, $p = 0.092$, power = 0.39; 1 week: $z = 1.21$, $p = 0.225$, power = 0.229). These data suggest that corneal abrasion injures corneal nerves in all rats, but that there are sex differences in the magnitude of that injury response.

When assessing the number of ATF3+ neurons, the relative location of each ATF3+ neuron in TG was recorded via a grid overlay, collapsed over depth (Fig. 4). ATF3+ neurons were distributed throughout the V1 and V2 regions of the TG, with a higher concentration found in the center of the TG. These data suggest that neurons impacted by corneal abrasion may be more widely distributed in TG beyond the V1 distribution reported in studies of neurons retrogradely labeled from the corneal surface (Launay et al., 2015).

3.4. CGRP+ cell number in TG changes dynamically after corneal abrasion

The number of CGRP+ cells in V1/V2 regions of TG (Fig. 2**B**) was assessed as a proportion of total TG cells in control TGs and in TGs from time points after abrasion in both sexes (Fig. 5**A**). In control TGs, there was a sex difference in the proportion of CGRP+ TG cells, with females having a significantly higher proportion than males (z-test, $z = 6.897$, $p < 0.001$, power = 1.00). After abrasion, the proportion of CGRP+ TG cells was significantly greater than the proportion in control TGs at every time point, but the pattern and magnitude of this change differed by sex. In females, the proportion of CGRP+ TG cells significantly increased at 24 h (z-test vs control, $z = 22.69$, $p < 0.001$, power = 1.00) and then fell at 72 h post abrasion (z-test vs control, $z = 5.52$, $p < 0.001$, power = 1.00), remaining lower through 1 week (z-test vs control, $z = 8.83$, $p < 0.001$, power = 1.00) (Fig. 5**B**) as compared to control females. In males, there was an acute increase at 24 h (z-test vs control; $z = 7.65$, $p = 0.001$, power = 1.00), and CGRP+ cell number remained elevated through 1 week (z-test v control, 72 h: $z = 8.21$, $p < 0.001$, power = 1.00; 1 week: $z = 11.87$, $p < 0.001$, power = 1.00). At 24 h post-abrasion, females had a significantly higher proportion of CGRP+ TG cells than males (z-test, $z = 19.93$, $p < 0.001$, power = 1.00). At 72 h and 1 week post-abrasion, the CGRP+ proportion in females was significantly less than males (72 h: z-test, $z = 6.95$, $p < 0.001$, power = 1.00; 1 week: $z = 13.09$, $p < 0.001$, power = 1.00). These data suggest that dynamic changes in CGRP within TG after abrasion are distinct for males and females.

To determine whether the changes in the number of CGRP+ TG neurons immediately after abrasion were limited to a subset of TG neurons, we measured the cross-sectional area of CGRP+ neurons in the section with the highest number of ATF3+ cells in control (n = 492 CGRP+ TG neurons from 3M, 3F control animals) and 24 h post-abrasion (n = 693 CGRP+ neurons from 3M, 3F abrasion animals) TGs (Fig. 6). Overall, the average cross-sectional area of CGRP+ TG neurons decreased in 24 h abraded TGs as compared to controls (Fig. 6**A**; Mann-Whitney Rank Sum test, $p < 0.001$), indicating an increase in the number of small CGRP+ neurons after abrasion. The frequency of CGRP+ cell sizes was determined for both the control and 24 h abraded TGs (Fig. 6**B**). There was a higher frequency of small neurons (<400 μm^2) that were CGRP+ in the abraded animals than the controls. For medium (500 μm^2 – 800 μm^2) and large (>800 μm^2) cell sizes, there were more CGRP+ TG cells in the controls than abraded TGs. These data suggest that dynamic sex-specific changes in TG cells that contain CGRP occur after abrasion, and that more small TG neurons contain CGRP after

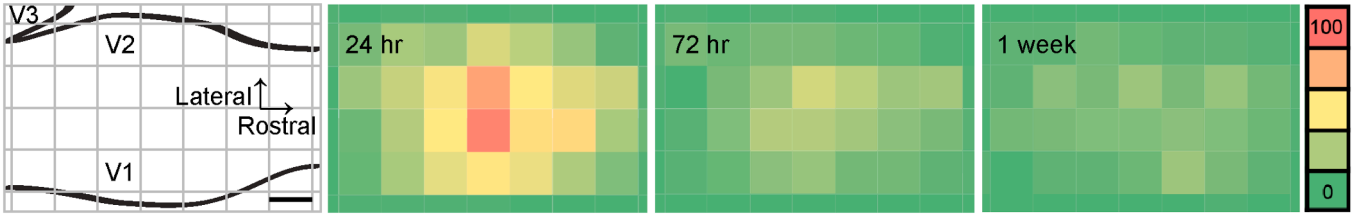

Fig. 4. Heat maps of ATF3+ cell locations in the TG after abrasion. Results from male and female rats were pooled for this analysis (n = 3 per sex per time point, total n = 18). (Left panel) Diagram represents an approximate map with grid lines used for analysis of the V1/V2 area of the TG, rostral from the mandibular branch (V3). (Right panels) Heat map shows the number of ATF3+ neurons per grid square at 24 h, 72 h, and 1 week times post-abrasion. The number of ATF3+ neurons was counted at intervals through the depth of each TG. Color scale ranges from green (0 cells) to red (100 cells) per grid square. Scale bar = 370 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

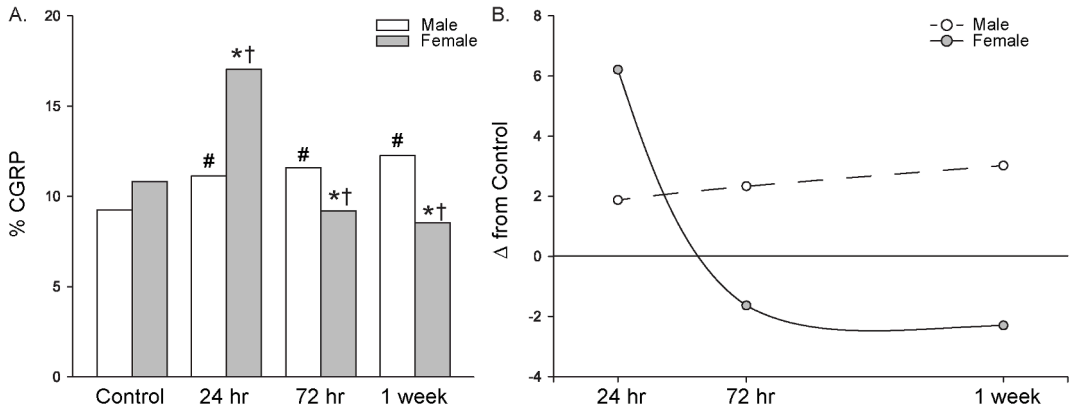

Fig. 5. Male and female rats show dynamic sex-specific changes in CGRP cell number in TG after corneal abrasion. (A) The proportion of CGRP+ cells (%CGRP) in females was increased at 24 h after abrasion, and then decreased at 72 h and 1 week. In males, %CGRP demonstrated a less robust but steady increase in the post-abrasion time. * P<0.001 compared to female control, # P<0.001 compared to male control, † P<0.001 compared to males within the same time point. N=24, Controls: 3M, 3F, Abrasion: 3M, 3F per time point (24 h, 72 h, 1 week). (B) %CGRP represented as % change from control (solid horizontal line).

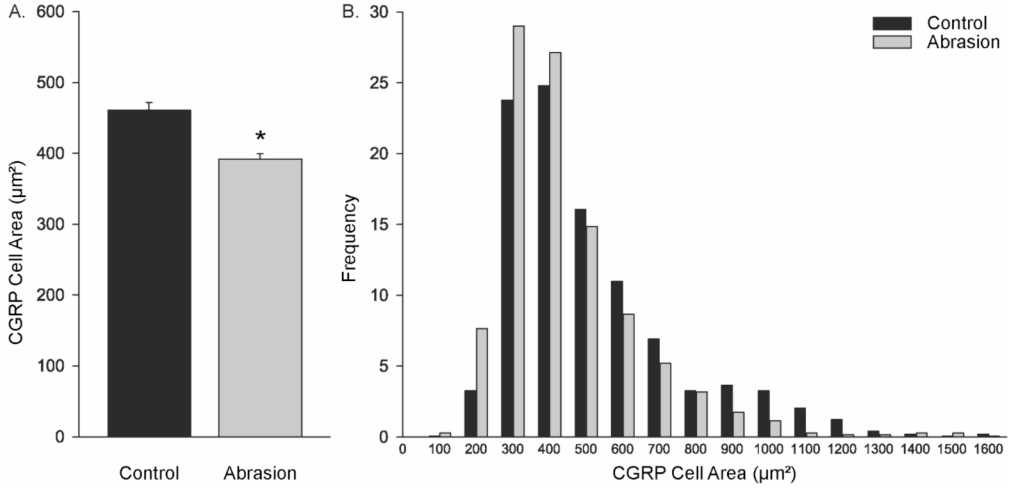

Fig. 6. Corneal abrasion acutely increased the number of small diameter TG neurons that were CGRP+. (A) The average cross-sectional area of CGRP+TG neurons decreased in 24 h abraded TGs as compared to controls. (B) The frequency of small CGRP+ TG cells was increased in 24 h abraded TGs as compared to controls, whereas the frequency of medium and large CGRP+ cells in abraded TGs was reduced. Results from controls males and females were pooled for this analysis. Controls: 3M, 3F, Abrasion: 3M, 3F. * P<0.001 compared to control.

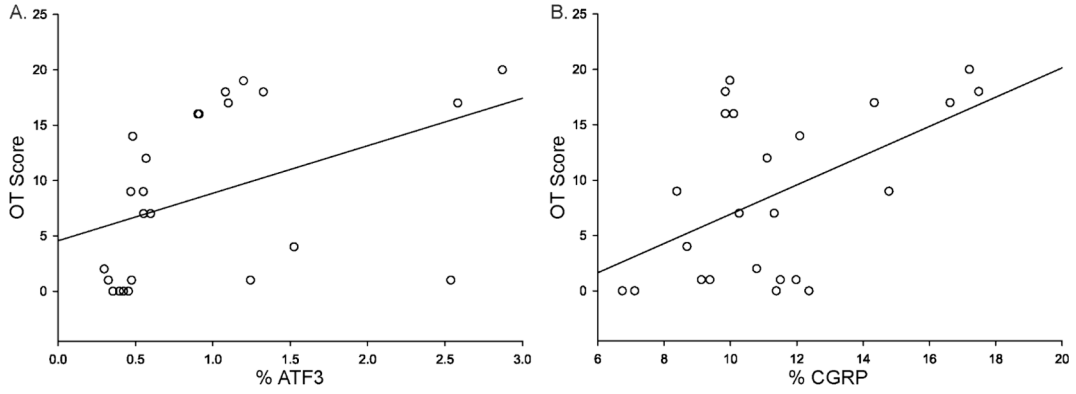

Fig. 7. Both %ATF3 and %CGRP correlate with OT score. Only OT score at time of sacrifice was used to appropriately represent the pain state associated with the histological data. Results from male and female rats were pooled for this analysis. N=24, Controls: 3M, 3F, Abrasion: 3M, 3F per time point (24 h, 72 h, 1 week). Linear regression between (A) %ATF3 or (B) %CGRP and OT scores demonstrate a positive correlation, with OT scores increasing as the %ATF3 or %CGRP increases.

injury.

3.5. Changes in ATF3+ and CGRP+ TG cells correlate with OT scores

Given the dynamic nature of both behavioral and neurochemical measurements after corneal abrasion, we tested whether there was a statistical relationship between OT score and ATF3+ or CGRP+ cell number in TG by looking for correlations across all animals at their individual experimental end point (Fig. 7, n = 24). Both %ATF3 and %CGRP were positively correlated with OT score. The %CGRP was more strongly correlated with OT score (R = 0.514, R^2 = 0.264, Adj R^2 = 0.231, p = 0.010) than the %ATF3 (R= 0.436, R^2 = 0.190, Adj R^2 = 0.154, p = 0.033). This is notable because while OT does significantly increase following abrasion, it varies widely between individuals, particularly at 72 h and 1 week after abrasion (Fig. 1). These data indicate that these molecules may be induced by corneal abrasion and have a relationship with spontaneous pain behaviors.

3.6. ATF3 and CGRP rarely colocalized in TG

The proportion of ATF3+ cells that also contained CGRP within TG was assessed (Fig. 8). There were no significant differences between male and female control rats in the very low percentages of ATF3+ TG cells that also contain CGRP (z-test, z = 1.385, p = 0.166). After abrasion, only the TGs from female rats 24 h post-abrasion demonstrated an increase in the proportion of ATF3+ cells containing CGRP as compared to female control TGs (z-test, z = 2.11, p = 0.035, power = 0.58). There was also a sex difference when compared to male TGs at the same time point (z-test, z = 3.13, p = 0.002, power = 0.93). There were no changes in the proportion of ATF3+ TG cells containing CGRP at any time point in male rats (z-test vs control, 24 h: z = 1.285, p = 0.199, power = 0.21; 72 h: z = 1.387, p = 0.166, power = 0.27; 1 week: z = 1.477, p = 0.140, power = 0.32) (Fig. 8).

3.7. Systemic CGRP receptor antagonist does not block spontaneous pain after corneal abrasion in female rats

Since CGRP was elevated in TG after abrasion, particularly in female rats, we tested whether administration of a CGRP antagonist could block spontaneous pain behaviors. Olcegepant, a CGRP receptor antagonist, had no effect on OT behavior in females that received a corneal abrasion (Fig. 9). There was an overall effect of time (two-way ANOVA, p < 0.001, power = 0.99), but no effect of drug treatment (p = 0.49, power = 0.05) and no interaction of time and treatment (p = 0.886, power = 0.05). OT scores were significantly increased as compared to within-group baseline scores in both vehicle- and Olcegepant- treated animals

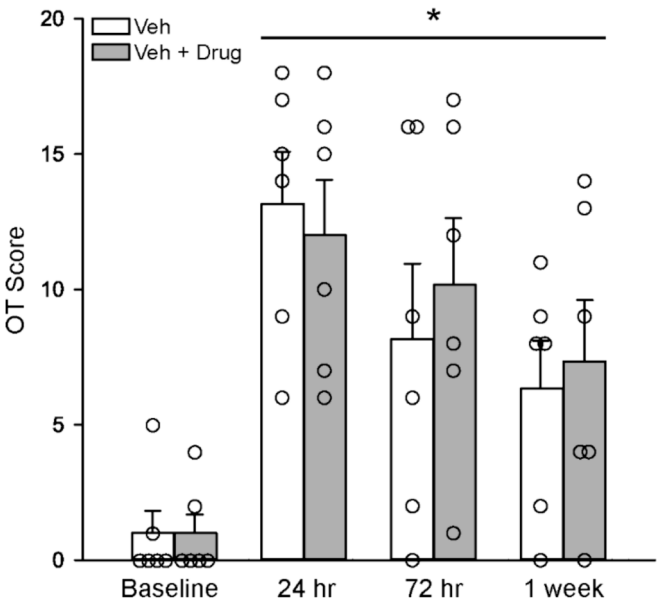

Fig. 9. Olcegepant had no effect on OT behavior following corneal abrasion in female rats. OT scores of rats given vehicle alone (Veh) were compared to rats given vehicle containing Olcegepant (1 mg/kg IP; Veh + Drug) both prior to abrasion and OT testing at 24 h. OT scores at all post-abrasion time points were significantly increased from baseline, but Olcegepant-treated (Veh + Drug) and vehicle-treated (Veh) animals had similar OT scores at each time point. N=12 female rats, 6 Olcegepant, 6 Vehicle. * P< 0.05.

at all post-abrasion time points (Holm-Sidak post hoc comparisons, p ≤ 0.001 at 24 h and 72 h; p = 0.032 at 1 week), but there were no significant differences between Olcegepant- and vehicle-treated rats at any time point (Fig. 9).

4. Discussion

We found that a unilateral corneal abrasion increased orbital tightening (OT) in both male and female rats, consistent with the interpretation that OT is a viable metric of spontaneous pain (Hegarty et al., 2022; Harris et al., 2017; Rea et al., 2022; Rea et al., 2018; Reijgwart et al., 2017). Acute, spontaneous pain and photophobia are also seen in most patients who receive a similar procedure during PRK for refractive correction (Colin and Paquette, 2006; Gaeckle, 2021; Palochak et al., 2020; Ripa et al., 2020; Shetty et al., 2019; Zarei-Ghanavati et al., 2019). The time course for spontaneous pain that we observed in this study was similar to our previous studies, and also consistent with our findings that

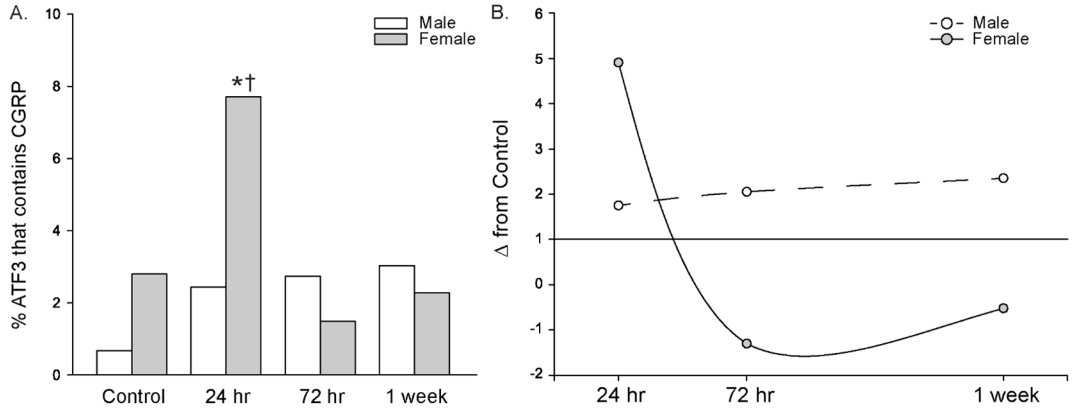

Fig. 8. Colocalization of ATF3 and CGRP is rare in both controls and abraded TGs. Within the small proportion of ATF3+ cells that contain CGRP, there was a sex-specific significant increase in the percentage of ATF3+ cells that contain CGRP in 24 h post-abrasion females. * P<0.05 compared to female control, † P<0.05 compared to males within the same time point. N=24, Controls: 3M, 3F, Abrasion: 3M, 3F per time point (24 h, 72 h, 1 week).

both suppression of spontaneous wheel running and evoked pain to noxious ocular stimuli also peak at 24 h after corneal abrasion (Hegarty et al., 2018; Hegarty et al., 2022). Overall, these findings support the use of corneal abrasion as a translation model of acute ocular pain after corneal injury.

We used this model to study the relationship between spontaneous pain behaviors and molecular changes in trigeminal ganglion neurons which provide innervation to the corneal surface. We examined CGRP since it is the most abundant neuropeptide in corneal afferents terminating in the trigeminal dorsal horn (Hegarty et al., 2010) as well as in the trigeminal ganglion cell bodies (LaVail et al., 1993; Nakamura et al., 2007). CGRP has also been implicated in trigeminal pain disorders including migraine (Russo and Hay, 2023; Lundy and Linden, 2004; Kamm et al., 2021; Diel et al., 2021). Migraine is a disorder of the trigeminovascular system and is more prevalent in women than men. Therefore, many studies relating to sex differences in the TG, CGRP, and pain are conducted in the context of migraine. For instance, Paige et al. (Paige et al., 2022) demonstrated that Olcegepant, a CGRP receptor antagonist, effectively reduced IL-6 induced hypersensitivity exclusively in female mice. Ji et al. (Ji et al., 2019) reported higher levels of receptor component protein (RCP, a CGRP receptor component), in female rats. A sex-specific relationship between peripheral CGRP and pain was demonstrated in a recent study in which dural injections of CGRP decreased facial withdrawal thresholds and increased facial grimace scores in female rats and mice, but not male rodents (Avona et al., 2019; Mason et al., 2017). The exact nature of the interaction between female sex, CGRP, and pain remains unclear, compounded by the underutilization of female animals in research (Shansky and Murphy, 2021). A review of neuropeptides in TG neuropathic pain found that of 19 studies, 16 used exclusively male animals, one did not list the sex of animals used, and only 2 used female animals (Chuinsiri et al., 2021). We sought to determine if the neurochemical sex differences found in other models are also found in the abrasion model of corneal injury.

In this study, we compared the molecular content in TG neurons of male and female rats following corneal abrasion. We found increased numbers of CGRP+ cells in TG following corneal abrasion, with females showing greater increases at 24 h than males although there were no sex differences in OT pain behaviors. There was a significant correlation between CGRP+ TG content and OT behaviors when analyzed for all individual animals across all time points at which TG content was measured for each animal. We also noted that baseline levels of CGRP+ were greater in females than males, consistent with prior studies.

Despite the finding of increased CGRP+ cells in TG of female rats, particularly after corneal abrasion, administration of the CGRP antagonist Olcegepant did not alter OT behaviors. This finding contrasts with several studies showing that CGRP antagonists can reduce pain responses, particularly in females (Paige et al., 2022). This may be due, in part, to differences in dose, route of administration, and/or different behavioral assessments. However, not all studies find CGRP antagonists to be effective against pain and further studies are needed to understand these dynamic responses.

Our finding that CGRP+ cells were largely distinct from cells that contained ATF3 suggests that CGRP is not a significant neuropeptide in corneal nociceptors, but this is inconsistent with prior studies showing that CGRP is found in corneal nerves (Hegarty et al., 2017), corneal TG cell bodies (Alamri et al., 2015; Murata and Masuko, 2006; Mosconi et al., 2001; Felipe et al., 1999) and in corneal nerve endings projecting to trigeminal dorsal horn (Hegarty et al., 2010). These disparate findings could be due to differential trafficking of neuropeptides to central and peripheral endings, as we have seen for TRPV1 in corneal nerves (Hegarty et al., 2014), and may be distinct for different tissues. It is also possible that corneal injury prompts the depletion of CGRP from injured neurons and thus our low levels of colocalization could be due to depletion. However, we did see increased numbers of CGRP+ cells in TG at 24 h, which would not be expected if the injury caused widespread depletion. Females did show lower numbers of CGRP+ neurons at later timepoints, which could reflect peptide depletion and/or reduced synthesis after injury. It is possible that CGRP is playing a chemotaxis or modulatory role in TG (Russo and Hay, 2023), rather than mediating ocular pain behaviors. These intriguing findings require further exploration. Our results emphasize the need to examine both sexes in all biological studies; even when behavioral differences are not seen there can still be distinct molecular responses.

We used ATF3 as a marker to indicate which TG neurons innervate the cornea, since this has been used consistently to label neurons after peripheral nerve injury (Braz and Basbaum, 2010; Tsujino et al., 2000). We found that the number of ATF3+ neurons in TG correlates with OT behavior, supporting the notion that this molecular response is tracking with pain caused by damage to corneal epithelial nerves. However, we found ATF3 cells in both V1 and V2 regions of TG which is a wider distribution than expected based on tract tracing or viral studies that describe a more circumscribed corneal niche in V1 (Launay et al., 2015; Arvidson, 1977; Lopez de et al., 2000). This could be due to corneal nerve injury and epithelial removal causing activation and stress to a wider distribution of TG neurons through indirect mechanisms in addition to direct damage of the peripheral ending of an individual TG neuron. For example, studies report activation or influx of immune cells in TG after various types of injury and other studies have implicated an increase in communication among sensory ganglion neurons and small satellite glial cells as a mechanism for cellular changes beyond those neurons directly affected by a peripheral injury (Hossain et al., 2017; Takeda et al., 2009; Guerrero-Moreno et al., 2020; Ren and Dubner, 2010; Thalakoti et al., 2007). Future studies will explore if these mechanisms cause activation of neurons in TG that do not project directly to the cornea but may play a role in supporting pain responses.

5. Conclusions

We found that corneal abrasion produces acute pain-like behaviors in both male and female rats, as well as increased expression of ATF3 in trigeminal ganglion neurons consistent with nerve injury. CGRP was elevated in trigeminal ganglion neurons and significantly correlated with pain-like behaviors, however CGRP was more elevated in females than males. Thus, while we saw no sex differences in the behavioral responses, we did find sex differences in underlying molecular changes in trigeminal ganglion. Overall, these findings emphasize the importance of examining sex as a biological variable, even when behavioral responses are not different between the sexes.

Funding sources

This work was supported by U01 EY034680 (SAA, TLM, CWM).

CRediT authorship contribution statement

Clem Gunter: Writing – original draft, Visualization, Investigation, Formal analysis. **Cody L. Jiang:** Writing – original draft, Visualization, Investigation, Formal analysis. **Shae O. Zeimantz:** Writing – review & editing, Investigation, Formal analysis. **Deborah M. Hegarty:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Formal analysis, Conceptualization. **Catherine W. Morgans:** Writing – review & editing, Conceptualization. **Tally M. Largent-Milnes:** Writing – review & editing, Funding acquisition, Conceptualization. **Sue A. Aicher:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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