

The Impact of Aging and Sex on Corneal Nerve Density and Function

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PURPOSE. To quantify the role of aging and sex in corneal nerve morphology and function and to identify cellular and molecular mechanisms involved in corneal nerve aging in male and female mice.

METHODS. This study included young (8-week-old) and old (52-week-old) C57BL/6N male and female mice. Corneal nerve function was measured using eye wiping and Cochet-Bonnet tests. Corneal nerve density was analyzed after β 3-tubulin staining in the sub-basal nerve plexus, the vertical nerve projections, and the superficial nerve terminals. Neuronal and neuroinflammatory markers were quantified in the trigeminal ganglia using immunofluorescence and real-time PCR.

RESULTS. Aging significantly reduces nerve density and the corneal nociceptive response ($P < 0.0001$). Mechanical sensitivity was decreased in male mice ($P < 0.0001$), but not in females. Female mice showed milder nerve fiber degeneration compared to males ($P = 0.0453$). Moreover, we identified an age-dependent decrease of substance P-positive neurons in the ophthalmic branch of the trigeminal ganglion ($P = 0.0003$), which was milder in females ($P = 0.0005$). Gene expression analysis in the trigeminal ganglion revealed a higher expression of neuroprotective and pro-regenerative markers in female mice. Immunofluorescence analysis revealed an age-dependent increase in the uptake of leukocytes in the trigeminal ganglion ($P = 0.0068$). M2 anti-inflammatory macrophage numbers were preserved in aging females versus males ($P = 0.0008$).

CONCLUSIONS. Aging was associated with sensory corneal neuropathy, altered sensory abnormalities, and neuroinflammation. Interestingly, female mice appeared to be protected from nerve degeneration compared to males.

Keywords: cornea, sex, aging, nerves, neuroinflammation

Corneal nerves are instrumental in detecting mechanical, thermal, and chemical stimuli. They also contribute to the maintenance of corneal transparency by providing trophic support, regulating tear secretion, and initiating the blink reflex.¹ In addition to their well-known sensory and trophic activities, corneal nerves regulate the immune response by modulating leukocyte diapedesis and activation following infections and injuries.^{2–4} Complete denervation is clinically epitomized by the development of neurotrophic keratitis. However, milder and/or partial nerve dysfunction can be found in most ocular surface diseases, including highly prevalent disorders such as dry eye disease (DED) and ocular neuropathic pain. Aging is a notable risk factor for many of these corneal disorders,^{5,6} and, in fact, corneal nerve density, morphology, and function are significantly affected by aging. For example, it is known that the sub-basal nerve density decreases together with mechanical, chemical, and thermal sensitivity in older subjects.^{7–11} These morphological and functional changes could be the consequence of altered corneal nerve expression of certain neuropeptides

and neurotransmitters, including reductions in the levels of substance P (SP) and calcitonin gene-related peptide (CGRP).^{9,12} Aging appears to affect not only the density of peripheral corneal fibers but also trigeminal neurons, where corneal nerves are generated, as it has been reported that transient receptor potential melastatin 8 (TRPM8)-positive trigeminal neurons undergo morphological and functional changes in aging animal corneas.¹³ It should be noted, however, that it is not known if and how aging has a different impact on corneal nerve morphology and/or function in males as opposed to females. This has relevant clinical implications because, for example, DED has a higher prevalence in female subjects,¹⁴ who are also more susceptible to nociceptive pain.¹⁵ Finally, limited knowledge is available on the potential cellular and molecular mechanisms underlying sex-based differences in corneal nerves during aging.

For these reasons, we aimed to characterize corneal nerve morphology and function of male and female mice during aging. Finally, we attempted to identify age- and sex-

dependent molecular changes associated with the aging of trigeminal sensory neurons.

MATERIALS AND METHODS

Animals

Eight-week-old ($n = 20$ males, 20 females) and 52-week-old ($n = 20$ males, 20 females) C57BL/6N mice (Charles River Laboratories S.r.l., Lecco, Italy) were included in this study. Old mice were obtained from retired matings. All mice used were free of clinically observable ocular surface disease at baseline. The housing conditions were the following: controlled temperature (22°C), light from 07:00 AM to 7:00 PM, stable humidity (50%–60%), and fresh food and water provided ad libitum. The study was performed considering both eyes. The experiments were performed at the IRCCS San Raffaele Scientific Institute, Milan, Italy. All experimental procedures were approved by the Animal Care and Use Committee, following the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were euthanized by carbon dioxide inhalation and cervical dislocation. After a mouse was killed, the eyeballs and trigeminal ganglia (TG) were collected and dissected for immunofluorescence analyses.

Ocular Nociceptive Response

Corneal nociception of mice was measured as described by Farazifard and colleagues.¹⁶ Animals were individually placed in an empty cage for a minimum of 3 minutes to become acclimatized. Mice then received 10- μ L drops of 5-M NaCl topically in each eye, and the number of eye wipes was measured in the subsequent 30 seconds. All measurements were performed by two independent observers, and the eye-wiping count was averaged after collecting all data.

Corneal Sensitivity Test

Corneal mechanical sensitivity was assessed by using a Cochet-Bonnet esthesiometer (Compagnia Ottica Italiana S.r.l., Milan, Italy) with a 0.12-mm nominal nylon filament diameter.¹⁰ Briefly, each cornea was touched with the nylon filament perpendicularly to the central corneal apex. A blink reflex response was considered a positive signal for the detection of mechanical stimulus. All measurements were performed starting with a nylon filament length of 6 cm and gradually reduced by 0.5-cm steps until reaching the positive blink response. Five repeated trials were performed for each filament length, and a minimum of three positive sensation responses were considered.

Corneal Nerve Morphology Analysis

To measure corneal nerve fiber density, cornea whole-mount samples were freshly excised, washed in PBS, and fixed in acetone at 4°C for 20 minutes. Nonspecific staining was removed with 2% bovine serum albumin (BSA) and 5% normal donkey serum (NDS). Corneas were then incubated with a rabbit anti- β 3-tubulin (1:400; BioLegend, San Diego, CA, USA) primary antibody overnight at 4°C. The secondary antibody used was the Alexa Fluor 488 donkey anti-rabbit secondary antibody (1:1000; Invitrogen, Carlsbad, CA, USA), incubated for 2 hours at room temperature (RT) and mounted with VECTASHIELD mounting medium

(Vector Laboratories, Burlingame, CA, USA). Six peripheral and three central fields (40 $\times$ magnification) in the cornea were acquired using a confocal microscope (TCS SP5; Leica Microsystems, Wetzlar, Germany). For the nerve imaging analysis, confocal z-projections were generated for the sub-basal nerve plexus (SBNP; 5- μ m z-stack), the vertical nerve projections (VNPs; single-frame images from the center of the epithelium), and the superficial nerve terminals (SNTs; 3.5- μ m z-stack from the superficial epithelium). For the SBNP and the SNTs, the percentage of β 3-tubulin⁺ signal per field was determined. For the VNPs, the number of axonal fibers projecting vertically was counted and normalized per square millimeter.¹⁰

Trigeminal Sensory Neuron Quantification

For measuring the number of sensory neurons innervating the ocular surface, TG were fixed with 4% PFA for 20 minutes, and the sections were permeabilized using 0.1% Triton X-100 for 5 minutes. Nonspecific staining was blocked using 2% BSA, 5% NDS, and 10% Goat F(ab) Anti-Mouse IgG H&L (Abcam, Cambridge, UK). Sensory neurons were detected by staining transversal TG slices ($n = 5$ mice/group; six serial slices with a thickness of 8 μ m per sample) with a mouse anti- β 3-tubulin (1:100; BioLegend) and a rat anti-SP antibody (1:200; Novus Biologicals, Centennial, CO, USA), incubated at 4°C overnight. Secondary antibody included Alexa Fluor 546 anti-mouse and Alexa Fluor 488 anti-rat (1:1000; Invitrogen). All pictures were taken with a DeltaVision Ultra microscope (20 $\times$, 5- μ m z-stack; GE Healthcare, Chicago, IL, USA), focusing on the ophthalmic branch of the TG (V1).

Immune Cell Quantification in the Cornea and TG

Cornea. To quantify immune cells infiltrating the cornea, whole-mounted samples were freshly excised, washed in PBS, and fixed in acetone at 4°C for 20 minutes. Nonspecific staining was removed with 2% BSA and 5% NDS. Corneas were then incubated with a goat anti-CD45 primary antibody (1:200; R&D Systems, Minneapolis, MN, USA). The secondary antibody used was Alexa Fluor 546 donkey anti-rabbit secondary antibody (1:1000; Invitrogen). The corneas were incubated for 2 hours at RT and mounted with VECTASHIELD mounting medium. Six peripheral and three central fields (20 $\times$ magnification, 5- μ m z-stack) in the cornea were acquired using the Leica TCS SP5 confocal microscope. Finally, the number of leukocytes was calculated in the cornea and limbus using ImageJ (National Institutes of Health, Bethesda, MD, USA).

To characterize the immune cell population in the cornea, we performed F4/80 staining to quantify macrophages and CD3 staining to assess lymphocytes. Briefly, the eyeballs were harvested and frozen in an optimal cutting temperature compound (Killik O.C.T. Compound; Bio-Optica, Milan, Italy). Mouse corneal cross-sections were obtained after sectioning eyeballs at a 10- μ m thickness, collecting at least six serial sagittal slices for each cornea ($n = 5$ mice/group). To measure macrophage and lymphocyte infiltration, the corneas were fixed in acetone at 4°C for 20 minutes. Corneas were blocked with 2% BSA and 5% NDS. For staining macrophages, corneas were incubated with rat anti-F4/80 (1:400; BioLegend) and goat anti-CD45 (1:400; Invitrogen) primary antibodies for 2 hours at RT. For staining lymphocytes, corneas were incubated with rabbit anti-CD3 (1:100;

Abcam) and goat anti-CD45 (1:400; Invitrogen) primary antibodies for 2 hours at RT. As a secondary antibody, we used anti-goat Alexa Fluor 546 (1:1000; CD45) and anti-rabbit Alexa Fluor 488 (1:1000; F4/80 or CD3) (Invitrogen).

Images were acquired from limbus to limbus, including two pictures of each limbal side, and at least six pictures of the cornea. All pictures were taken with a Leica CTR5500 fluorescence microscope at 20 $\times$ magnification. The cell number was assessed by using ImageJ. For the macrophage and lymphocyte analysis, the number of F4/80⁺ and CD3⁺ cells per field was colocalized with CD45⁺ cells, and the percentage of double-positive cells out of the total number of CD45⁺ cells was calculated.

Trigeminal Ganglion. To quantify immune cell infiltration in the TG, cross-sectioned TG samples were harvested and frozen in an optimal cutting temperature compound (Killik O.C.T. Compound; Bio-Optica). Mouse TG cross-sections were obtained after sectioning at least six serial transversal TG slices at an 8- μ m thickness. For staining macrophages, corneas were fixed with acetone at 4°C for 20 minutes. TG sections were incubated with rat anti-CD11b (1:200; Bio-Rad Laboratories, Hercules, CA, USA) together with rabbit anti-iNOS (1:200; Invitrogen) or rabbit anti-CD206 (1:500; Abcam) antibodies. For staining lymphocytes, corneas were incubated with rabbit anti-CD3 (1:100; Abcam) and goat anti-CD45 (1:400; Invitrogen) primary antibodies for 2 hours at RT. As a secondary antibody, we used anti-goat Alexa Fluor 546 (1:1000; CD45, iNOS, or CD206) and anti-rabbit Alexa Fluor 488 (1:1000; CD11b or CD3) (Invitrogen).

Images were acquired focusing on the ophthalmic branch of the TG, collecting at least three pictures per slice. All pictures were taken with the Leica CTR5500 fluorescence microscope at 20 $\times$ magnification. The cell number was assessed by using ImageJ.

Real-Time Polymerase Chain Reaction Analysis

After mouse sacrifice, four to six TG of different animals for each group were harvested. Total RNA extraction, DNase treatment, retrotranscription, and real-time polymerase chain reaction (RT-PCR) were performed as previously described.¹⁷ TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) for *Tacr1* (Mm01166996_m1), *Tacr1* (Mm00436892_m1), *Calca* (Mm00801463_g1), *Ramp1* (Mm00489796_m1), *Sox11* (Mm01281943_s1), *Gap43* (Mm00500404_m1), *Gdnf* (Mm00599849_m1), *Atf3* (Mm00476033_m1), *Trpv1* (Mm01246300_m1), *Trpm8* (Mm01299593_m1), *Piezo2* (Mm01265861_m1), *Trpa1* (Mm01227437_m1), *Ngf* (Mm00443039_m1), *Bdnf* (Mm04230607_s1), and glyceraldehyde 3-phosphate dehydrogenase (*Mm99999915_g1*) as the housekeeping gene were performed. Results are presented as a relative expression ($\Delta\Delta$ Ct method).

Statistical Analysis

The statistical significance of the differences between young (8 weeks old) and old (52 weeks old) mice and between male and female mice for clinical endpoint, immunohistochemical, and RT-PCR analyses was calculated by using unpaired *t*-tests. Cell population studies were analyzed with one-way ANOVA and Tukey's post hoc test. All of the analyses were performed considering both eyes, and the data collected were included as independent data for each group. $P < 0.05$

was considered statistically significant. Prism 10.4.0 (GraphPad Software, Boston, MA, USA) was used to perform the statistical analyses. Data are expressed as mean $\pm$ standard error of the mean (SEM).

RESULTS

Aging Generates Sex-Dependent Changes in Nerve Morphology and Function in the Mouse Cornea

To assess the effect of aging on corneal nerve function, we measured ocular pain and mechanical sensitivity through the eye-wiping test and with the Cochet–Bonnet esthesiometer, respectively. These tests were performed on male and female mice at 8 and 52 weeks of age ($n = 20$ mice/group). Our findings show that corneal pain was significantly reduced by aging in both sexes (males, from 21.6 ± 0.79 to 10.5 ± 0.59 , -50.4% , $P < 0.0001$; females, from 23.9 ± 0.78 to 10.65 ± 1.14 , -55.4% , $P < 0.0001$). Moreover, young female mice had higher eye-wiping counts than males (females, 23.9 ± 1.14 ; males, 21.6 ± 0.79 ; $P = 0.0114$) (Fig. 1A). In line with these results, we found reduced corneal mechanical sensitivity during aging in male mice (from 5.69 ± 0.05 cm to 4.49 ± 0.15 cm, -21.1% , $P < 0.0001$). Interestingly, female mice did not show the same outcome, as they showed no effects of aging in Cochet–Bonnet measurements (from 5.69 ± 0.07 cm to 5.79 ± 0.08 cm, $+1.8\%$; $P = 0.2523$) (Fig. 1B).

Next, we quantified β 3-tubulin⁺ nerve fibers ($n = 4$ –8 eyes/group) in whole-mounted corneas, measuring the nerve density in the SBNP, VNPs, and SNTs. When compared with 8-week-old mice, 52-week-old mice showed a notable decrease of β 3-tubulin signal in the SBNP in both males and females (males, from $4.04\% \pm 0.19\%$ to $2.14\% \pm 0.1\%$, -47% , $P < 0.0001$; females, from $4.39\% \pm 0.19\%$ to $2.91\% \pm 0.25\%$, -33.7% , $P = 0.0007$) (Figs. 1C, 1D). Of note, 52-week-old female mice had higher nerve density compared to age-matched males ($P = 0.0453$). Regarding VNP density, we found a notable age-dependent decrease of β 3-tubulin⁺ fibers in male mice (from 378.7 ± 10.7 to 169.87 ± 16.9 fiber projections per mm², -55.2% ; $P < 0.0001$). Interestingly, female mice did not show any significant change in VNP during aging (from 391.4 ± 34.5 to 248.8 ± 50.3 fiber projections per mm², -36.4% ; $P = 0.0572$) (Figs. 1E, 1F). Finally, we quantified the SNT nerve density in young and older mice of both sexes. Our data revealed that aging induced a significant reduction of β 3-tubulin⁺ fibers in the SNTs (males, from $0.28\% \pm 0.03\%$ to $0.16\% \pm 0.02\%$, -44.6% , $P = 0.0063$; females, from $0.31\% \pm 0.01\%$ to $0.23\% \pm 0.01\%$, -24.4% , $P = 0.0042$) (Figs. 1G, 1H). Similar to the previous evidence, older female mice had a higher SNT density compared to older males ($P = 0.0089$).

Age- and Sex-Related Changes in Substance P⁺ Neurons in Mouse Trigeminal Sensory Neurons

We stained mouse TG with β 3-tubulin and SP antibodies to quantify the number of SP⁺ neurons, focusing on the ophthalmic branch (V1) in male and female mice at 8 and 52 weeks. Our data revealed that aging generated a significant loss of β 3-tubulin⁺ sensory neurons only in male mice (males, from 173.3 ± 9.42 to 132.7 ± 12.35 , $P = 0.0414$; females: from 183.0 ± 9.11 to 160.1 ± 16.84 , $P = 0.3066$) (Figs. 2A, 2B). Interestingly, aging induced a significant SP⁺ neuron loss in the V1 area of the TG (males, from 65.22 ± 4.53 to 30.03 ± 3.06 , $P = 0.0003$; females, from 72.48 ± 2.18

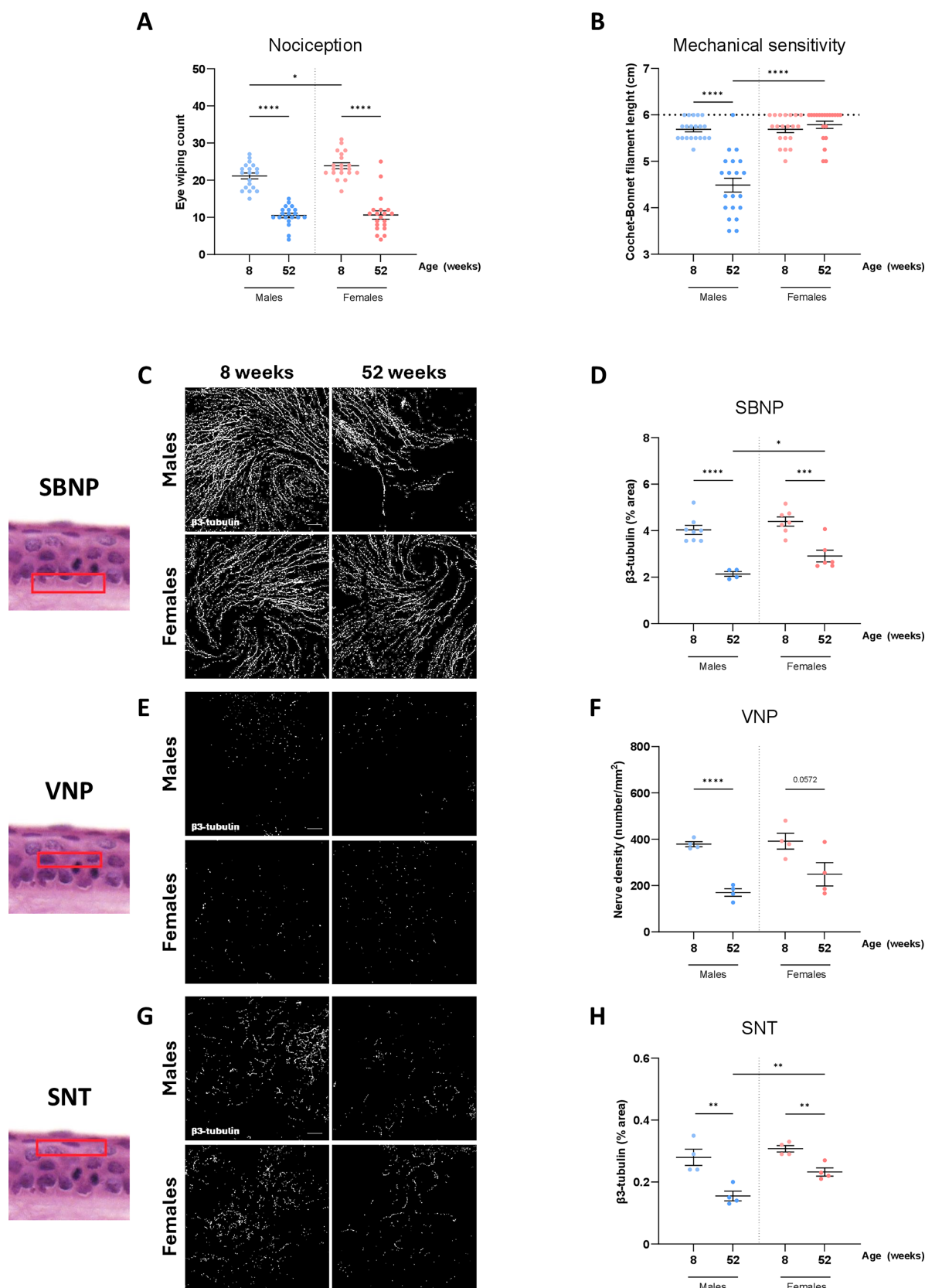


FIGURE 1. Aging affects mouse corneal nerve function and morphology differently in males and females. (A) Quantification of ocular surface pain in male versus female mice ($n = 20$ mice/group). Eye wipes were measured at 8 and 52 weeks. (B) Graph illustrating results of corneal mechanical sensitivity measurements in mice of both sexes during aging ($n = 20$ mice/group). (C, E, G) Mouse whole-mounted corneas were stained for $\beta 3$ -tubulin ($n = 4$ –8 eyes/group), with a focus on the SBNP (C), VNPs (E), and SNTs (G). (D, F, H) Quantification of nerve density in the cornea of male and female mice during aging, focused on the SBNP (D), VNPs (F), and SNTs (H). The graphs show the percentage of $\beta 3$ -tubulin⁺ nerves (D, H) and the number of fibers per mm² (F). Data are expressed as average $\pm$ SEM. Statistical analysis was performed using unpaired Student's t -tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

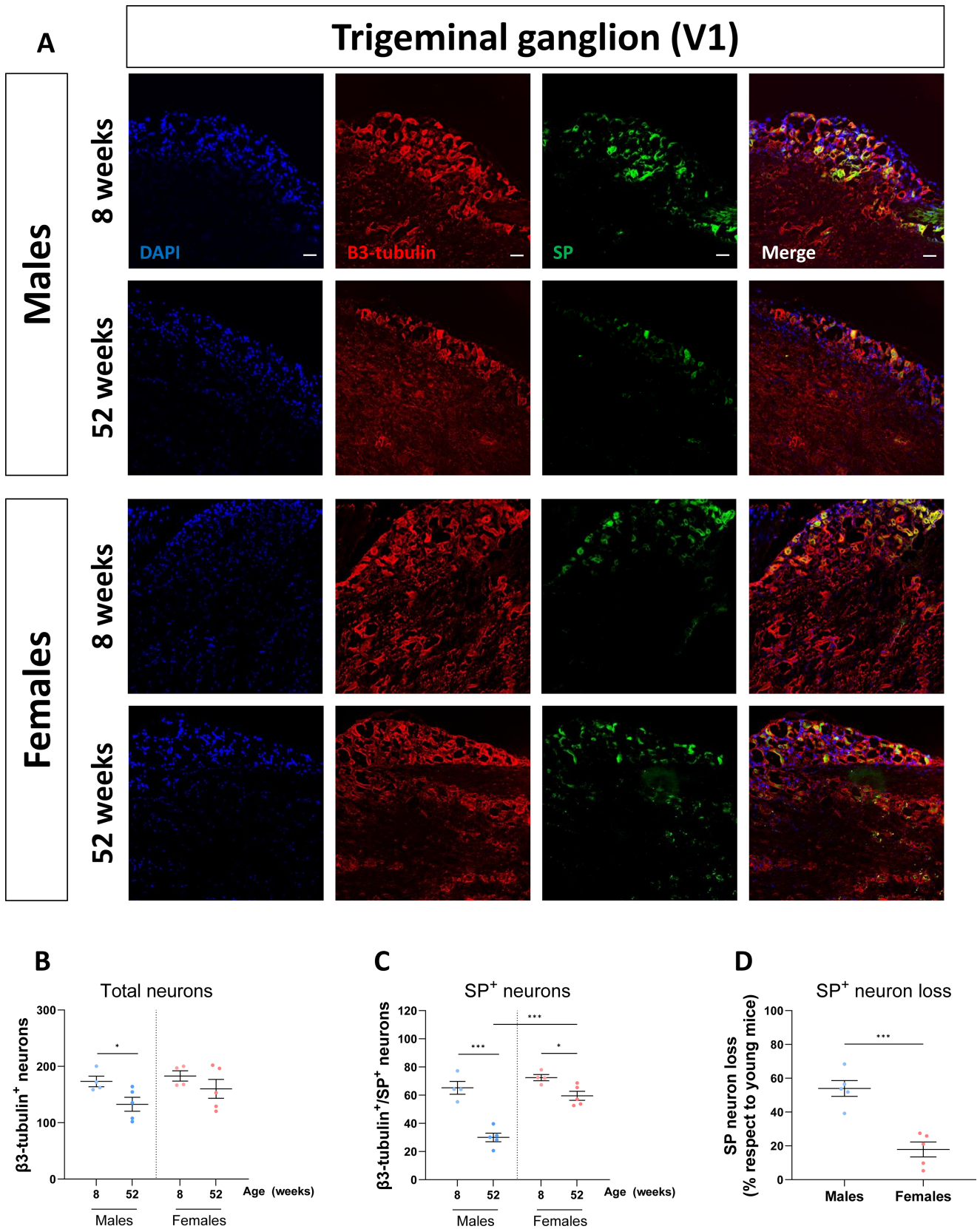


FIGURE 2. Age-induced reduction of sensory SP⁺ neurons in the ophthalmic branch of the TG. (A) Representative pictures showing β 3-tubulin (red) and SP (green) staining in the ophthalmic branch of the TG (V1) at 8 and 52 weeks, focusing on mouse sex ($n = 5$ mice/group). (B, C) Graph illustrating results in the total number of β 3-tubulin⁺ (B) and SP⁺ (C) neurons in the TG (V1). (D) Graph showing the percentage of SP neuron loss in aged male and female mice with respect to young mice. Scale bar: 75 μ m. The graphs represent mean values $\pm$ SEM. Statistical analysis was performed using unpaired Student's *t*-tests. * $P < 0.05$, *** $P < 0.001$.

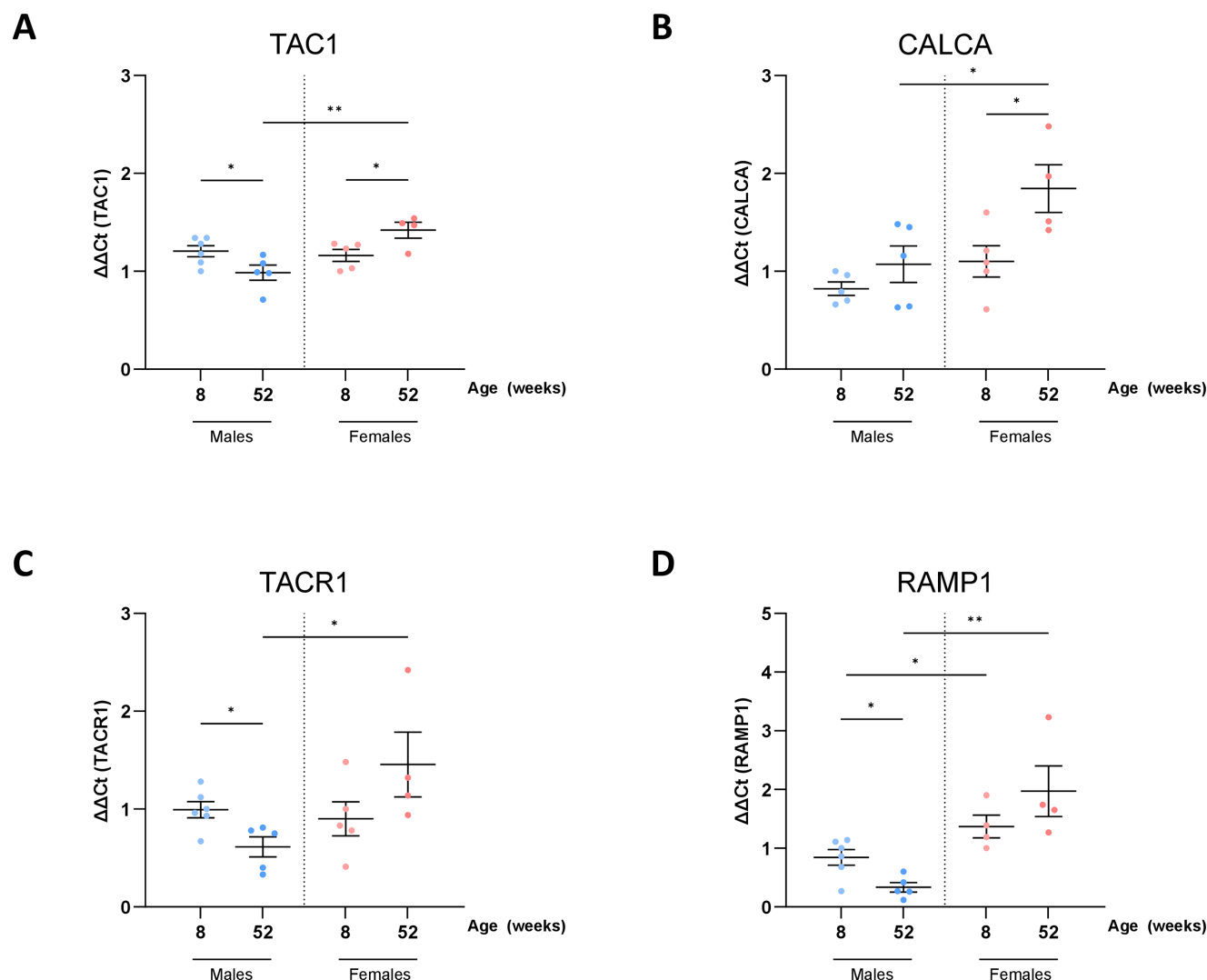


FIGURE 3. Aging impacts the gene expression of trigeminal pain biomarkers in a sex-dependent way. (A–D) Quantification of SP (TAC1) (A), CGRP (CALCA) (B), NK1R (TACR1) (C), and RAMP1 (D) gene expression in the TG of male and female mice 8 and 52 weeks old ($n = 4$ –6 mice/group). The graphs represent mean values $\pm$ SEM. Statistical analysis was performed using unpaired Student's *t*-tests. * $P < 0.05$, ** $P < 0.01$.

to 59.52 ± 3.2 ; $P = 0.016$) (Figs. 2A–C). Of note, female mice showed a milder age-dependent SP neuron loss compared to male mice (males, $53.95\% \pm 4.68\%$; females: $17.88\% \pm 4.42\%$; $P = 0.0005$) (Fig. 2D).

Aging Impacts the Expression of Pain Biomarkers in the TG Differently in Male and Female Mice

We measured the gene expression of sensory neuron biomarkers in the TG of male and female mice at 8 and 52 weeks. Here, we quantified the gene expression of SP (TAC1), NK1R (TACR1), CGRP (CALCA), and RAMP1 using RT-PCR analysis. We found that aging induced sex-dependent changes in the gene expression of TAC1, with reduced expression in males ($P = 0.00448$) and increased expression in females ($P = 0.0355$). Elderly females showed higher TAC1 expression than age-matched males ($P = 0.0063$) (Fig. 3A). Similarly, we found that the gene expression of TACR1 was inhibited in male mice during aging ($P = 0.0175$), whereas it was stable and higher in older females

($P = 0.313$) (Fig. 3C). Regarding CGRP expression, our data revealed that female mice increased the gene expression in the TG during aging ($P = 0.033$). Moreover, 52-week-old females had statistically higher CALCA gene expression when compared to age-matched males ($P = 0.0372$) (Fig. 3B). Finally, we detected reduced RAMP1 expression in male mice during aging ($P = 0.0132$), with no differences in young versus older females (Fig. 3D). Interestingly, females had higher RAMP1 expression at both 8 and 52 weeks than males (8 weeks, $P = 0.049$; 52 weeks, $P = 0.004$).

Neurotrophic and Nerve-Regenerative Markers Are Influenced by Aging and Sex

Subsequently, we assessed the role of aging and sex in modulating the gene expression of relevant neurotrophic and nerve-regenerative markers. Specifically, we observed that older female mice had a higher expression of the pro-regenerative neuronal markers SRY-box transcription factor 11 (SOX11; $P = 0.0177$) and growth-associated protein

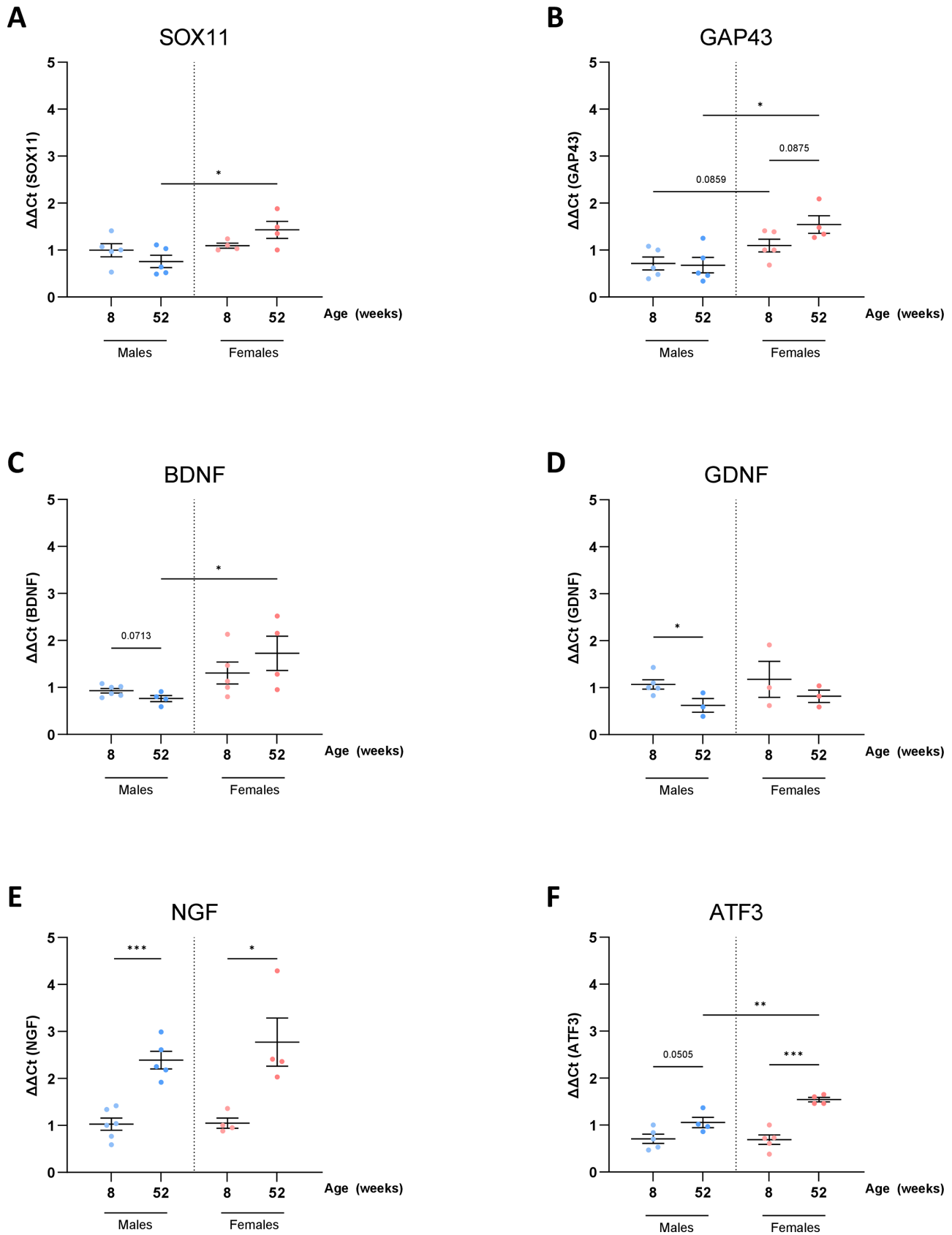


FIGURE 4. The gene expression of neurotrophic and neuroregenerative markers is influenced by age and sex. (A–F) Quantification of SOX11 (A), GAP43 (B), BDNF (C), GDNF (D), NGF (E), and ATF3 (F) in the TG of male and female mice 8 and 52 weeks old ($n = 3–6$ mice/group). The graphs represent mean values $\pm$ SEM. Statistical analysis was performed using unpaired Student's t -tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

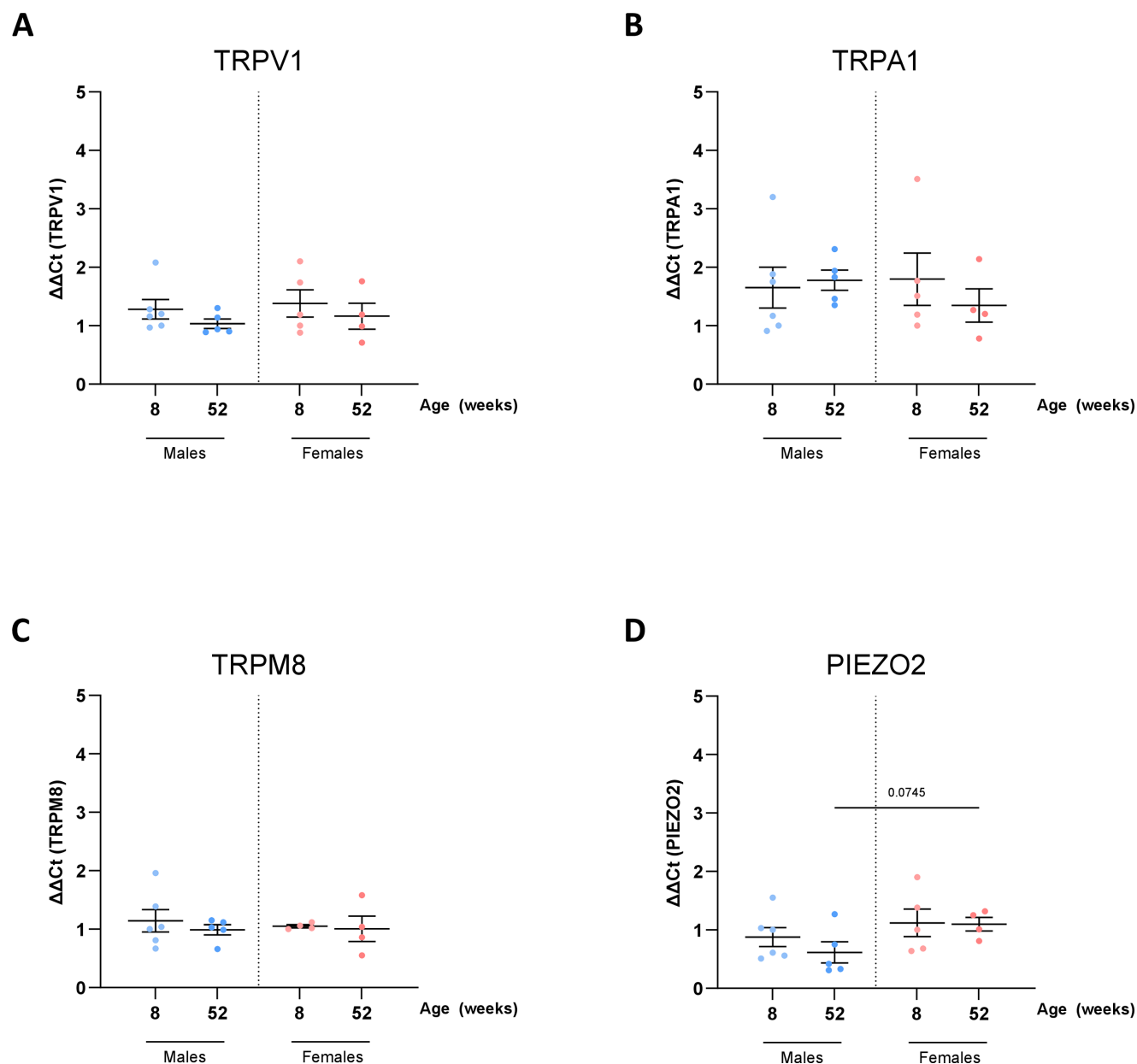


FIGURE 5. Aging does not affect the gene expression of corneal sensory receptors in the TG. (A–D) Quantification of TRPV1 (A), TRPA1 (B), TRPM8 (C), and PIEZO2 (D) gene expression in the TG of male and female mice 8 and 52 weeks old ($n = 4$ –6 mice/group). Graphs represent mean values $\pm$ SEM; Statistical analysis was performed using unpaired Student's *t*-tests.

43 (GAP43; $P = 0.0101$) when compared to older male mice (Figs. 4A, 4B). Of note, female mice slightly upregulated GAP43 gene expression during aging ($P = 0.0875$). Regarding neurotrophic factors involved in neuron function survival, we found a slight, albeit not significant, decrease in brain-derived neurotrophic factor (BDNF) expression in the TG of male mice ($P = 0.0713$) during aging, whereas no changes were measured in young versus older females (Fig. 4C). Of note, 52-week-old female mice showed a higher expression of BDNF compared to age-matched males ($P = 0.0415$). Similarly, glial cell line-derived neurotrophic factor (GDNF) was also reduced in the TG of male mice during aging ($P = 0.0394$), with preserved gene expression in females (Fig. 4D). Moreover, we observed a significant age-dependent increase in the expression of nerve

growth factor (NGF) in both male and female mice at the TG (males, $P = 0.0002$; females, $P = 0.0166$), with no sex-based differences (Fig. 4E). Finally, we determined that aging induced activating transcription factor 3 (ATF3) gene expression in both male ($P = 0.0505$) and female ($P = 0.0002$) mice, with higher levels in older females when compared with age-matched males ($P = 0.0068$) (Fig. 4F).

Aging Does Not Affect Gene Expression of Sensory Receptors in the TG

We measured the expression of well-known receptors involved in the sensation of chemical, mechanical, and

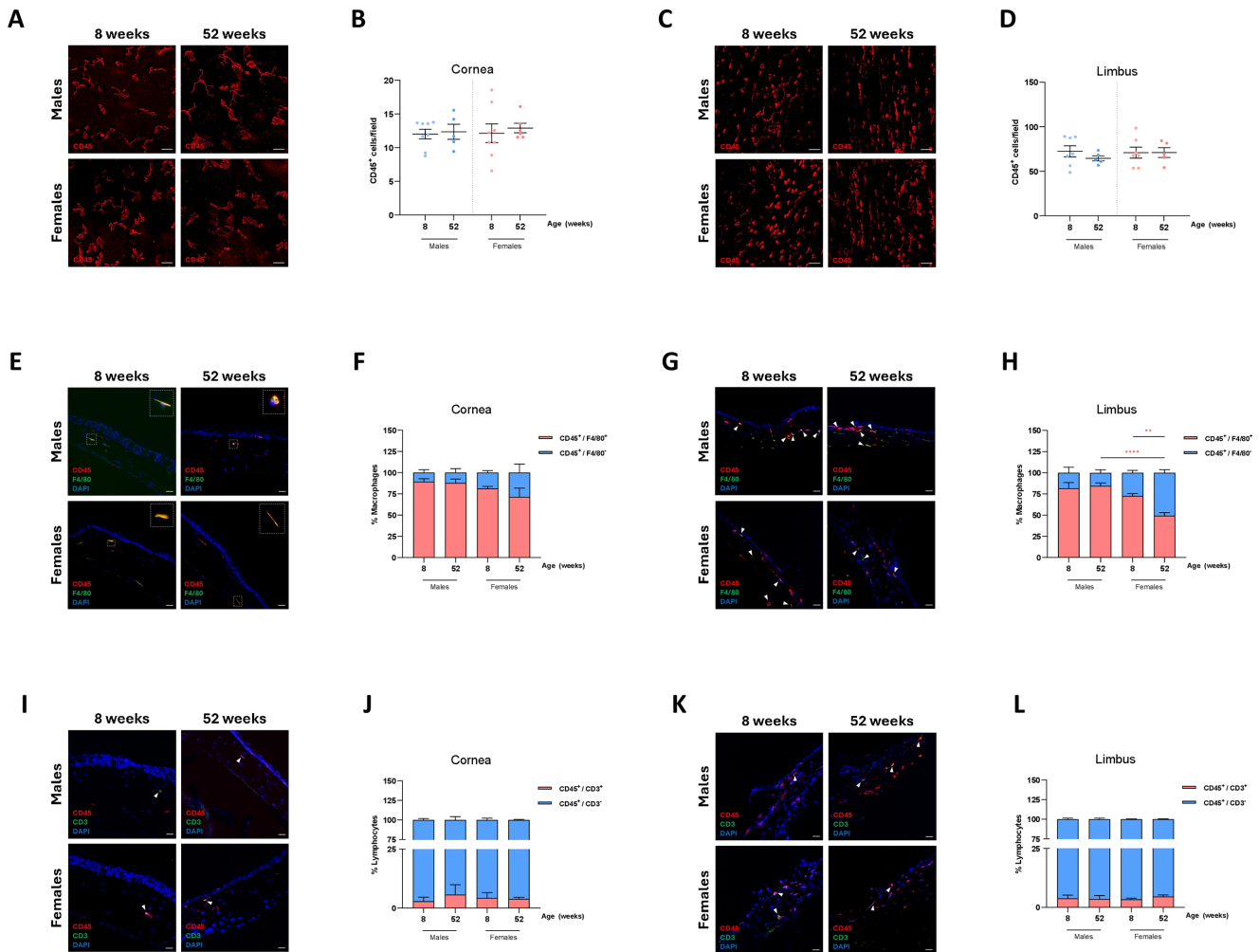


FIGURE 6. Infiltration of immune cells into the cornea of male and female mice. (A, C) Representative pictures showing CD45 staining in whole-mounted corneas of male and female mice 8 and 52 weeks old ($n = 5-8$ eyes/group), with a focus on the cornea (A) and in the limbus (C). (B, D) Graphs quantify the number of CD45⁺ cells/field in the cornea (B) and in the limbus (D) of young (8 weeks old) and older (52 weeks old) mice of both sexes. The graphs represent mean values $\pm$ SEM. Statistical analysis was performed using unpaired Student's *t*-tests. (E, G) Corneal cross-sections stained for CD45 (red) and F4/80 (green) in the cornea (E) and in the limbus (G); arrowheads indicate CD45⁺/F4/80⁺ cells. (F, H) Graphs show the percentage of CD45⁺/F4/80⁺ cells out of the total number of CD45⁺ cells in the cornea (F) and in the limbus (H) ($n = 4$ or 5 eyes/group). (I, K) Corneal cross-sections stained for CD45 (red) and CD3 (green) in the cornea (I) and in the limbus (K); arrowheads indicate CD45⁺/CD3⁺ cells. (J, L) Graphs illustrate the percentage of CD45⁺/CD3⁺ cells out of the total number of CD45⁺ cells in the cornea (J) and in the limbus (L) ($n = 4$ or 5 eyes/group). Scale bar: 50 μ m. The graphs represent mean values $\pm$ SEM. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. ** $P < 0.01$, **** $P < 0.0001$.

thermal stimuli. Our findings revealed no age- or sex-dependent differences in the expression of transient receptor potential vanilloid 1 (TRPV1), transient receptor potential melastatin 8 (TRPM8), transient receptor potential ankyrin 1 (TRPA1), or PIEZO-type mechanosensitive ion channel component 2 (PIEZO2) (Figs. 5C–F). However, we measured slightly higher expression of PIEZO2 in 52-week-old female mice compared with age-matched males ($P = 0.0745$).

Leukocyte Infiltration in the Cornea Is Not Affected by Aging

We performed leukocyte staining of corneas using the CD45 antibody, searching for age- and sex-dependent abnormalities. We quantified the percentages of macrophages (M Φ) and lymphocytes infiltrating the cornea by means of

F4/80 and CD3 staining, respectively. We found no age-dependent changes in the number of leukocytes, in either the cornea (Figs. 6A, 6B) or the limbus (Figs. 6C, 6D). We next focused on estimating the percentage of macrophages in the cornea and in the limbus of male and female mice during aging. Our data revealed no age- or sex-dependent changes in the central cornea (Figs. 6E, 6F). However, we found a significant reduction of macrophage-like cells in the limbus of female mice during aging (from 72.7% to 49.3%; $P = 0.0041$), with no differences in male mice (Figs. 6G, 6H). Old females showed a lower percentage of macrophages when compared to age-matched males (males, 84.54%; females, 49.3%; $P < 0.0001$). Finally, we quantified the percentages of lymphocytes infiltrating the cornea in young versus older mice of both sexes. Our findings revealed that aging did not induce significant age- or sex-dependent changes in the percentage of lymphocytes

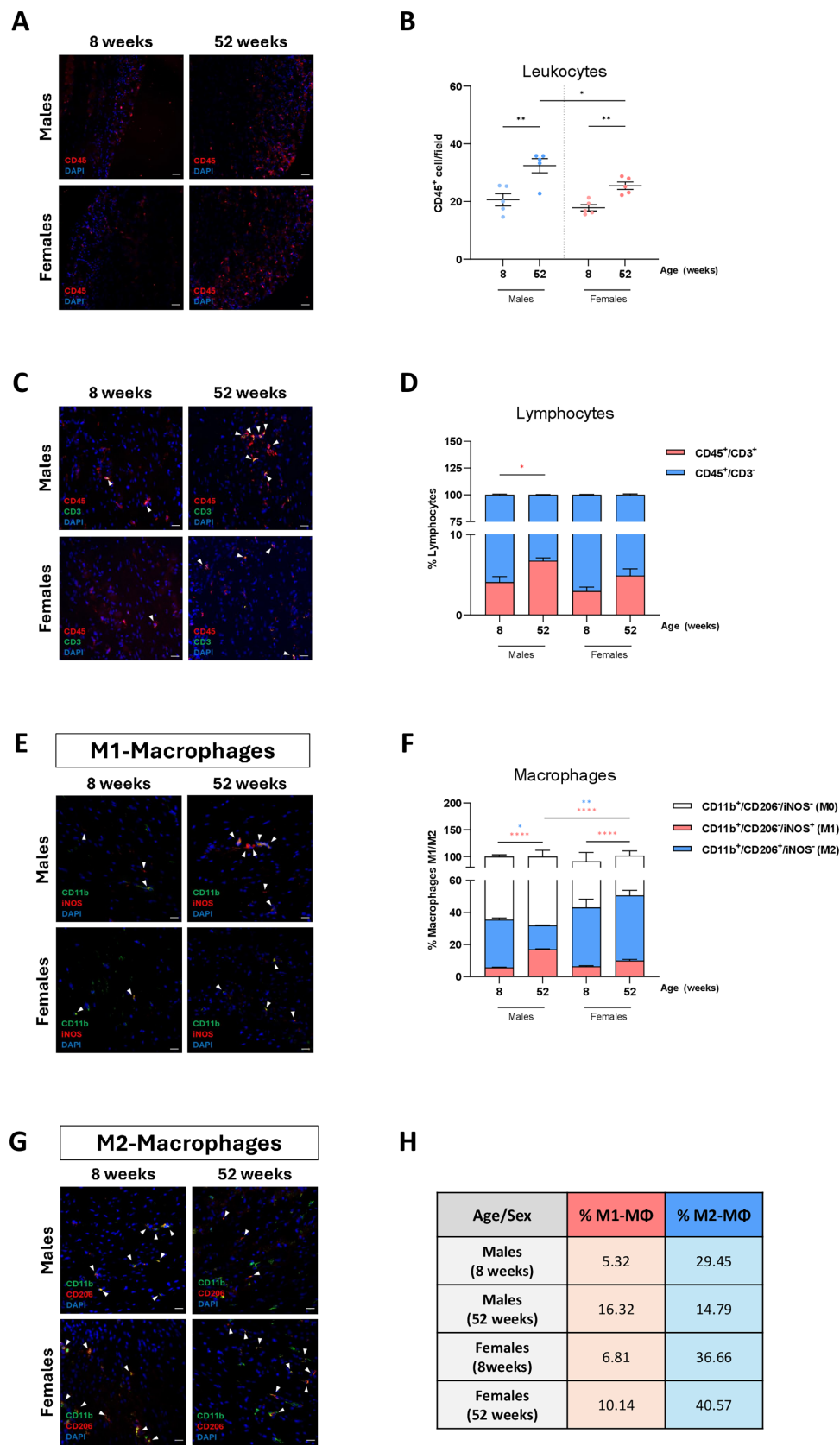


FIGURE 7. Aging induces inflammation in the TG differently in male and female mice. (A) Representative pictures showing CD45 staining in the ophthalmic branch of the TG (V1) of male and female mice at 8 and 52 weeks old ($n = 5$ TG/group). Scale bar: 75 μ m. (B) Graph depicting the number of CD45⁺ cells in the TG (V1) of male and female mice during aging. Statistical analysis was performed using unpaired Student's *t*-tests. * $P < 0.05$, ** $P < 0.01$. (C) TG (V1) stained for CD45 (red) and CD3 (green) in the central cornea; arrowheads indicate CD45⁺/CD3⁺ cells. (D) Graphs showing the percentages of CD45⁺/CD3⁺ cells out of the total number of CD45⁺ cells in the V1 area of the TG ($n = 4$ or 5 TG/group). (E–G) Representative images of CD11b (green) and iNOS (E) or CD206 (G) (red) staining in the TG (V1);

arrowheads indicate CD11b⁺/iNOS⁺ or CD206⁺ cells. The graph (F) and representative table (H) show the percentages of CD11b⁺/iNOS⁺ and CD206⁺ cells out of the total number of CD11b⁺ cells ($n = 4$ or 5 TG/group). Scale bar: 50 μ m. The graphs represent mean values $\pm$ SEM. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

infiltrating both the cornea (Figs. 6I, 6J) and the limbus (Figs. 6K, 6L).

Aging Induces Inflammation in the Ophthalmic Branch of the TG in a Sex-Dependent Way

Next, we performed CD45 staining on the TG of male and female mice at 8 and 52 weeks to study the effect of aging and sex in modulating leukocyte infiltration. We quantified the phenotype of immune cells infiltrating the V1 area of the TG performing CD11b plus iNOS or CD206 staining for M Φ , and CD45 plus CD3 staining for lymphocytes. Specifically, we focused our analysis on the ophthalmic branch of the TG (V1 area).

We found a significant age-dependent increase in the number of leukocytes infiltrating the V1 area of the TG in male and female mice (males, from 20.6 ± 2.14 to 32.42 ± 2.46 CD45⁺ cells, +57.4%, $P = 0.0068$; females, from 17.82 ± 1.09 to 25.46 ± 1.29 CD45⁺ cells, +41.3%, $P = 0.002$) (Figs. 7A, 7B). Interestingly, older female mice showed a lower number of leukocytes when compared to age-matched males ($P = 0.0368$).

Among these, we observed an age-dependent increase of lymphocytes (CD45⁺/CD3⁺) in male mice (from 4.1% to 6.8%, +65%; $P = 0.0256$), but no statistically significant differences were found in females (Figs. 7C, 7D). Regarding the quantification of macrophages in the TG (V1), we found an age-dependent increase in the population of pro-inflammatory M Φ (M1; CD11b⁺/iNOS⁺) in both male and female mice (males, from 5.72% to 17.05%, $P < 0.0001$; females, from 6.44% to 10.04%, $P < 0.0001$). However, the percentage of M1-M Φ in 52-week-old female mice was significantly lower than in males ($P < 0.0001$) (Figs. 7E, 7F). Conversely, our findings revealed that the population of anti-inflammatory M Φ (M2; CD11b⁺/CD206⁺) was reduced in male mice during aging, whereas it was stable in females (males, from 29.86% to 14.79%, $P = 0.0463$; females, from 36.66% to 40.57%, $P = 0.4159$) (Fig. 7G). Of note, older females showed a higher percentage of M2-M Φ compared to older males ($P = 0.0008$). Figure 7H shows the percentages of M1-M Φ and M2-M Φ quantified in 8- and 52-week-old mice of both sexes.

DISCUSSION

According to the World Health Organization, the number of people who are 60 years old or older is expected to increase significantly over the next 30 years, reaching 2.1 billion worldwide.¹⁸ The rapid aging of a large part of the population will certainly impact the ophthalmology field, as a number of ocular disorders show increased prevalence with aging. Corneal nerves are deeply affected by aging, in terms of both morphology and function,^{8,10,19,20} and it is known that this can impact ocular surface integrity, such as in DED.⁵ However, the specific role of sex on corneal nerve aging and the potential mechanisms underlying such alterations are still largely unknown. A better understanding of these aspects could result in more effective and gender-tailored treatments, especially in those diseases (such as DED) for

which the prevalence is substantially higher in females and with increasing age.^{14,15,21,22} In this study, we identified age- and sex-related differences in corneal nerve morphology and function. Moreover, we examined the TG for potential molecular and cellular mechanisms that could be responsible for these observations.

Biological Mechanisms Underlying Age-Dependent Changes in Corneal Innervation

Our findings reveal that aging impacts corneal nerve morphology and function in both male and female mice. In terms of nerve morphology, we found a significant age-dependent reduction of nerve density in corneal epithelium, in line with the literature.^{5,7-11} Mechanistic investigation revealed that a lower density of corneal innervation was associated with a reduced number of SP-positive neurons in the ophthalmic branch of the TG. RT-PCR analysis in the TG revealed age-dependent downregulation of the neuropeptide SP (TAC1) and the receptors NK1R (TACR1) and RAMP1, which are well-known markers of sensory neurons in the TG. This reduction in sensory neuropeptide signaling may be a key feature of aged trigeminal neurons. These data corroborate previous findings, which demonstrated an age-dependent decrease in corneal nerve density associated with lower SP and CGRP levels in the cornea and tear fluid.^{6,9,12,23} Molecular analysis revealed an age-dependent reduction of BDNF and GDNF expression in the TG, both of which are pivotal neurotrophic factors for neuronal survival and maintenance.^{24,25} Our findings are consistent with the existing literature, which reports reduction of BDNF and GDNF in aged neurons and links this reduction to neuronal impairment and degeneration.^{24,26} Specifically, GDNF is an age-specific survival factor for sensory neurons,^{24,26,27} and its decrease may be linked to the age-specific loss of SP⁺ neurons in the TG (V1). Surprisingly, we observed an age-dependent upregulation of ATF3 and NGF genes in the TG. This finding suggests that trigeminal neurons undergo a chronic, low-grade state of injury during aging. Indeed, we observed upregulation of ATF3, which is a strong indicator of axonal damage.²⁸ Similarly, the increased NGF expression strongly suggests activation of a compensatory axonal regenerative program after injury.²⁹⁻³¹ However, when looking at the master transcriptional regulators of axonal regeneration, SOX11 and GAP43,^{32,33} we did not observe any age-dependent gene expression change. These findings together suggest that aged trigeminal sensory neurons have an inefficient regenerative response.

We also investigated whether aging is associated with a differential response to chemical or mechanical stimuli. Indeed, older mice showed a lower response to chemical (hyperosmotic) and mechanical stimuli. To test whether the reduced mechanical/chemical sensitivity we observed could be subtended by specific alterations in the expression of certain sensory receptors, we quantified their expression in the TG. Surprisingly, we did not detect any age- or sex-associated changes in the expression of mechanical, polymodal, and cold receptors in the TG. Although we cannot exclude that other receptors may be involved, our data may

be the result of a diffuse reduction in the density of trigeminal SP⁺ neurons and the downregulation of SP, NK1R, and RAMP1 genes in the TG. Indeed, the SP and CGRP pathways are pivotal for transmitting sensory stimuli in the cornea.^{34–36} On the other hand, the functional impairment of corneal nerves could be a consequence of molecular, biomechanical, and physiological changes in the sensory receptors per se during aging.^{15,37,38}

Subsequently, we investigated the hypothesis that low-grade chronic neuroinflammation, also referred to as inflammaging, may be involved in age-induced corneal neurodegeneration, as previously reported.^{6,39,40} We initially hypothesized that leukocyte infiltration was increased in the aging cornea; however, this was not the case. In contrast, our data clearly show that the primary site of neurodegeneration (and neuroinflammation) is the TG. Specifically, we observed increased leukocyte infiltration in TG (V1) of older mice, specifically lymphocytes, which are involved in corneal neuropathy.⁴¹ Moreover, we found that aging promotes the switch of MΦ from the M2 to the M1 phenotype, suggesting the establishment of an inflammatory milieu, as reported in extra-corneal tissues.⁴² Although it is known that systemic inflammation tends to increase with aging,^{5,6,8,40} the observation that aging induces inflammation in the ophthalmic branch of the TG has not been reported before.

Role of Sex in Modulating Age-Induced Corneal Neuropathy

In the present study, we investigated the impact of sex on corneal innervation during aging. The neuroprotective activity of sexual hormones and their role in controlling neuroinflammation are widely reported in literature.^{43–45} It is well known that aging induces significant changes in the serum level of sexual hormones in human subjects. Specifically, it has been reported that males experience an age-dependent reduction of testosterone and other androgens, whereas females have a marked reduction of estradiol and androgens after menopause.⁴⁶ Conversely, mice have a different pattern of sex hormone secretion with aging. Notably, male mice show a reduction of testosterone (similarly to humans) but females maintain a stable expression of estradiol.^{47,48} Such a different hormonal profile should be taken into account when comparing animal models with human subjects, especially females, who show an increased incidence after menopause.^{14,15,21,22}

Surprisingly, our data also show that aging affects corneal nerves in a sex-dependent manner, with males suffering more severe corneal neurodegeneration (males, −47.4%, females, −33.7%). These findings are consistent with a more severe loss of SP⁺ neurons in the TG (V1) (males, 53.95%; females, 17.88%). The molecular analysis on the TG revealed that the preserved neuronal phenotype observed in females may be the consequence of increased expression of SP, CGRP, NK1R, and RAMP1 during aging. The apparent preservation of corneal nerve fibers in older multiparous female mice corroborates findings by Stepp et al.,⁴⁴ who demonstrated that parity attenuates age-related corneal nerve loss in female mice. These morphological differences were paralleled by age- and sex-dependent differences in corneal nerve function. For example, corneal sensitivity was preserved in female mice as opposed to males, with reduced response to mechanical stimuli. This finding could perhaps be explained

by our observation that Piezo2 expression is (although non-significantly) higher in the TG of old female mice compared to age-matched males.

The female healthier corneal nerve phenotype is further corroborated by RT-PCR analysis on the TG. Specifically, we found that the expression of neurotrophic factors BDNF and GDNF remained stable during aging in females, whereas in males it was decreased. This finding is relevant because BDNF expression is primarily modulated by estradiol, which is maintained in older female mice.^{47,49} Moreover, the expression of ATF3 was found to be significantly upregulated in female mice compared to males during aging. This finding is particularly interesting because ATF3 is known to be an estrogen-responsive marker that promotes neurite outgrowth following estradiol stimulation.⁵⁰ Consistent with this observation, the enhanced regenerative response indicated by elevated ATF3 was further supported by the significantly higher gene expression of SOX11 and GAP43 in aged female mice, collectively suggesting a more robust neuroregenerative pattern compared to males.

Additional evidence supporting a “protected” neuronal phenotype in female mice comes from the analysis of immune cells infiltrating the ophthalmic branch of the TG. We found a lower uptake of leukocytes during aging in females. Similarly, the relative number of lymphocytes was stable in females, as opposed to males, where it increased with aging and could subtend the more severe neuropathy, as reported before by Vereerbrugghen et al.⁴¹ Finally, we analyzed the impact of sex in modulating MΦ phenotype during aging, which is an indicator of the neuroinflammatory state, and it is pivotal for driving axonal regeneration.^{51,52} We found that MΦ-M1 cell numbers were lower in older females compared to males; conversely, MΦ-M2 cell numbers were preserved in female mice. The specific role of estrogens in promoting the anti-inflammatory M2 phenotype has been extensively described in the literature.^{53,54}

Taken together, our findings have significant implications for the clinical understanding and definition of DED. The Tear Film and Ocular Surface Society (TFOS) Dry Eye Workshop (DEWS) III definition reports that “ocular surface inflammation and damage and neurosensory abnormalities are etiological factors” in DED.⁵⁵ Although clinical focus often remains on the ocular surface, our results suggest that, at least in the aging population, these pathological changes may be rooted in primary neurodegeneration/neuroinflammation within the TG. The identification of TG as a primary site of inflammation and neuropeptide dysregulation provides a cellular/molecular substrate for DED definition. Moreover, our finding of a substantial difference in females versus males should prompt clinical research and, if confirmed in humans, should also be acknowledged in the definition of DED.

CONCLUSIONS

Our findings show that age-induced trigeminal neuron degeneration is associated with abnormalities in corneal nerve morphology and function, as well as trigeminal sensory neuron loss, with females being protected from these detrimental changes when compared with males. The biological mechanism driving these differences certainly involves sex hormones. The role of estrogens in controlling chronic neuroinflammation and promoting the expression of neurotrophic factors could explain why corneal nerves are protected in females (Fig. 8). Although differ-

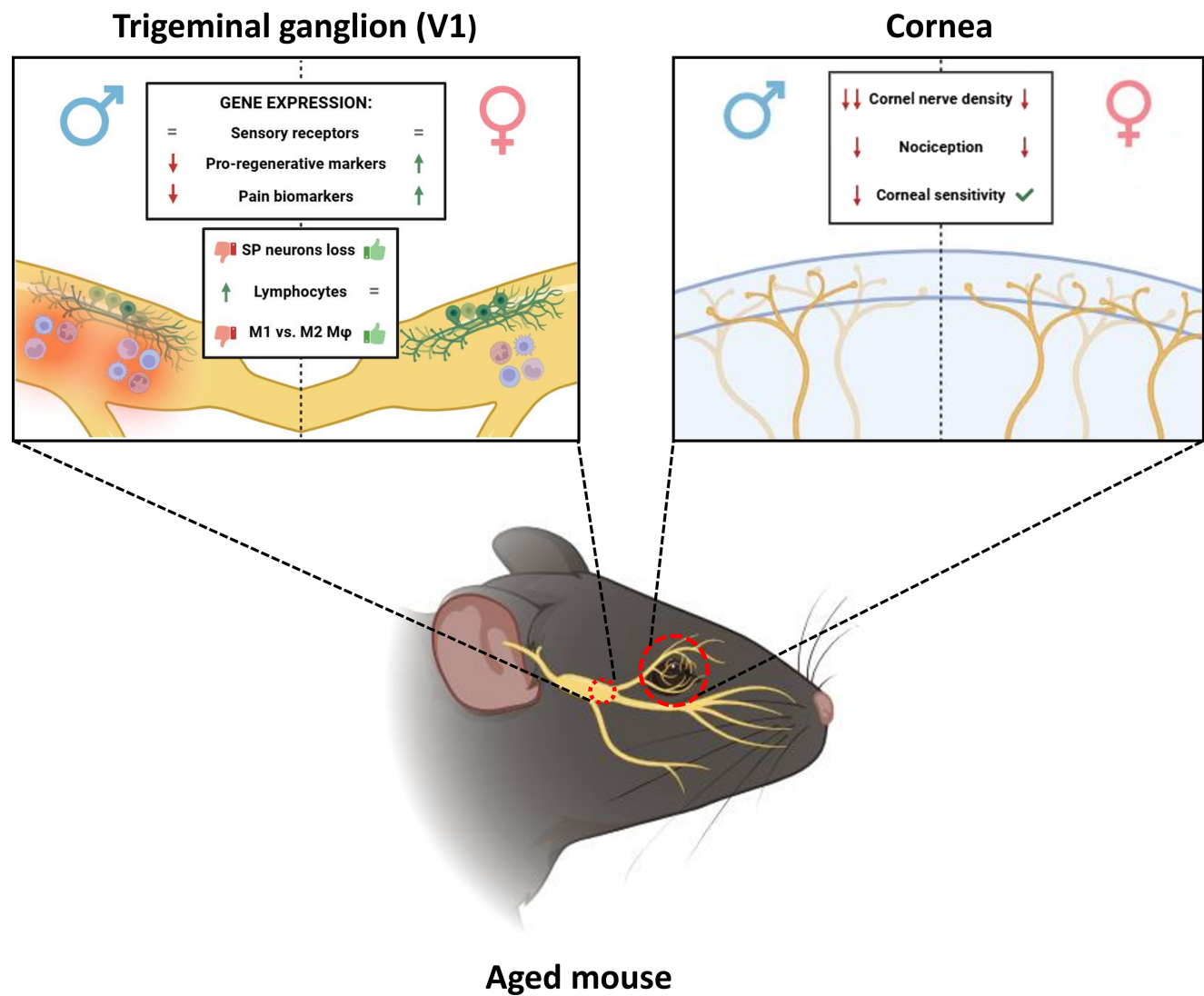


FIGURE 8. Hypothesis of the biological mechanisms underlying the differences in corneal nerve morphology and function between male and female mice.

ences in hormonal settings in mice and humans differ and should be taken into account, these findings have relevant clinical implications and call for the development of novel sex hormone- and age-specific treatment protocols in neuroregenerative treatments. Although sexual hormone-based therapies have been explored for DED,^{56–58} their controversial efficacy underscores the critical need for sex- and age-specific formulations.

Moreover, our findings call for a paradigm shift in the current management of ocular neuropathic pain. First is the need to develop more targeted therapies to treat TG as a primary site of inflammaging and neurodegeneration, secondarily affecting corneal nerves. Second is recognition of the importance of tailoring therapy based on patient gender and age. Gender- and age-guided therapy modulation is routinely performed in many medical specialties. Our data suggest that this should also be the case in ophthalmology. In addition, several lines of evidence indicate that estrogens significantly influence pain perception in the TG and that the synthesis and signaling of these sex hormones are altered by aging.^{59,60} We believe that the onset of a chronic

neuroinflammatory state, combined with estrogen dysregulation in the TG during aging, may induce central sensitization and exacerbate corneal neuropathic pain in older patients with DED. Consequently, it is reasonable to suggest that neuroprotective strategies targeting TG microenvironment and neuroinflammation may improve (neuropathic) ocular pain in older patients with DED.

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