Topical Treatment With Bromfenac Reduces Retinal Gliosis and Inflammation After Optic Nerve Crush

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Purpose. To study the effect of topical administration of bromfenac, a nonsteroidal anti-inflammatory drug (NSAID), on retinal gliosis and levels of prostaglandin E_2 (PGE_2) after complete optic nerve crush (ONC).

Methods. Adult albino rats were divided into the following groups ($n = 8$ retinas/group): (1) intact, (2) intact and bromfenac treatment (twice a day during 7 days), (3) ONC (7 days), and (4) ONC (7 days) + bromfenac treatment (twice a day during 7 days). Animals from groups 3 and 4 were imaged in vivo with spectral-domain optical coherence tomography (SD-OCT) before the procedure and 15 minutes, 3, 5, or 7 days later. Retinas from all groups were analyzed by immunodetection, Western blotting, or enzyme-linked immunoabsorbent assay (ELISA).

Results. Quantification of Brn3a (brain-specific homeobox/POU domain protein 3A) $^+$RGCs (retinal ganglion cells) in cross sections showed that bromfenac treatment does not accelerate ONC-induced degeneration. Cellular retinaldehyde binding protein 1 regulation indicated that bromfenac improves retinal homeostasis in injured retinas. Spectral-domain OCT showed that the thickness of the retina and the retinal nerve fiber layer at 7 days post ONC was significantly reduced in bromfenac-treated animals when compared to untreated animals. In agreement with these data, hypertrophy of astrocytes and Müller cells and expression of glial fibrillary acidic protein and vimentin were greatly diminished by bromfenac treatment. While no changes in cyclooxygenase (COX) enzyme COX1 and COX2 expression were observed, there was a significant increase of PGE_2 after ONC that was controlled by bromfenac treatment.

Conclusions. Topical administration of bromfenac is an efficient and noninvasive treatment to control the retinal gliosis and release of proinflammatory mediators that follow a massive insult to the RGC population.

Keywords: nonsteroidal anti-inflammatory drug, NSAID, astrocyte, Müller cell, prostaglandin, cyclooxygenease, optical coherence tomography

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most common drugs used to treat ophthalmic pain, photophobia, inflammation, and edema (reviewed in Refs. 1, 2). Nonsteroidal anti-inflammatory drugs inhibit cyclooxygenases, and this inhibition in turns results in a reduced level of proinflammatory prostaglandins. Although corticosteroids have a stronger anti-inflammatory effect than NSAIDs, they also have various side effects and may trigger neuronal apoptosis and axonal loss.[1–6] Therefore, NSAID treatment is preferred, and these compounds are being extensively studied in patients and animal models to assess their toxicity, pharmacokinetics, neuroprotective properties, and anti-inflammatory and anti-edema effects, among others.[4,7–16]

One such NSAID, bromfenac, is an inhibitor of the cyclooxygenase (COX) enzymes COX1 and COX2, and is more selective for COX2 than for COX1.[17] In rabbits, topical bromfenac reaches the vitreous and retinochoriodal tissues, and after a single topical administration is detectable in the retinochoroid up to 24 hours.[8,17,18] Kida et al.[19] showed that compared to nepafenac and diclofenac, bromfenac concentration after topical application on rabbit eyes was continuously higher in the retinochoroidal tissues. Thus, bromfenac may have a better therapeutic effect than these two other NSAIDs in retinochoroidal inflammatory diseases.

In patients, bromfenac treatment has been shown to be more efficient and safer than dexamethasone and fluorometholone to control postoperative inflammation.[16] Moreover, it has been recently demonstrated that bromfenac as well as two other NSAIDs, indomethacin and nepafenac, significantly reduced, in the same fashion, the levels of prostaglandin E_2 (PGE_2) in the vitreous of patients undergoing vitrectomy for macular pucker.[14] Finally, in patients, as in rabbits,[18] bromfenac shows good penetration, and its concentration remains stable in the aqueous humor up to approximately 12 hours after instillation.[20]

Regarding the therapeutic effect of bromfenac eye drops in animal models, it has been shown that they reduce retinal edema triggered by lipopolysaccharide systemic injection in

Investigative Ophthalmology & Visual Science

rats and rabbits[9,17] and prevent reactivation of herpes virus 1 in infected mice.[21]

Inflammation and edema are, as well, common responses to tissue damage or disease, as has been shown for the retina in animal models of glaucoma or optic nerve injury.[22–28] Macroglial retinal cells, astrocytes, and Müller cells readily respond to intravitreral injections,[29] injury, or disease.[30–33] A very well characterized model of retinal damage and degeneration is optic nerve axotomy in rodents.[34–39] Complete intraorbital optic nerve crush (ONC) is an acute insult that kills the vast majority of retinal ganglion cells (RGCs) within the first 2 weeks after injury.[34,38,40,41] Moreover, this injury also triggers an important inflammatory response within the injured and contralateral fellow eye.[27,28,42] Optic nerve crush is a model that has been widely used to test neuroprotective therapies[36,37,41,43–47] and to investigate the molecular events underlying central nervous system neuronal death upon axonal trauma.[25,48,49] Optic nerve crush is as well often used as a simplified model of glaucomatous injury. To the best of our knowledge there are no previous studies investigating the effects of topical administration of a NSAID on the retinal inflammation after complete optic nerve axotomy. Thus, in the present study we investigated the effect of topical bromfenac on retinal degeneration, gliosis, and release of PGE_2 in the rat retina after ONC.

Materials and Methods

Animal Handling

This study was approved by the Committee of Animal Care of the University of Murcia (Murcia, Spain). All experimental procedures were performed in accordance with the European Union Directive 2010/63/EU for animal experiments and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult female Sprague-Dawley rats (200–250 g body weight) were fed ad libitum and kept in an environmentally controlled room with an alternating 12-hour/12-hour light/dark cycle. Surgery and spectral-domain optical coherence tomography (SD-OCT) analysis were carried out under deep anesthesia induced by an intraperitoneal (IP) injection of a mixture of xylazine (10 mg/kg, Rompun; Bayer, Kiel, Germany) and ketamine (70 mg/kg, Imalgene; Merial laboratorios S.A., Barcelona, Spain). Analgesia was provided by subcutaneous administration of buprenorphine (0.1 mg/kg). All efforts were made to minimize animal suffering. For euthanasia, rats were killed with an IP overdose of sodium pentobarbital diluted 1:1 in saline (Dolethal, Vetoquinol; Especialidades Veterinarias, S.A., Madrid, Spain).

Several groups were formed ($n = 8$ retinas/group): (1) intact, (2) intact + bromfenac treatment (7 days), (3) ONC (7 days), and (4) ONC + bromfenac treatment (7 days). Animals from groups 3 and 4 were imaged in vivo with SD-OCT. Retinas from all groups were analyzed by immunodetection, Western blotting, or ELISA (see below).

Surgery

The left optic nerve was intraorbitally crushed at approximately 2 mm from the optic disc following previously reported methods.[34–36,50]

Topical Administration of Bromfenac

On the left eye of experimental animals and both eyes of intact animals, two drops of bromfenac (0.9 mg/mL 0.09% Yellox; Bausch & Lomb (S.A., Alcobendas, Madrid, Spain) were instilled topically right after the injury and then every 12 hours for 7 days. This dosage was selected following the prescribed treatment for human patients.[51] When the administration of the drug did not coincide with the SD-OCT analysis, animals were anesthetized with inhalational anesthesia (2% isoflurane, IsoFLo; Laboratorios Veterinarios Esteve, City, Spain), and they were kept anesthetized for 15 further minutes after applying the drops.

Spectral-Domain Optical Coherence Tomography

The left retinas from the ONC and ONC + bromfenac groups were analyzed using SD-OCT before the lesion (baseline), after the injury (15 minutes) and 3, 5 and 7 days after the injury. At the end of the study (7 days) animals were euthanized and the retinas dissected for further analysis.

Briefly, from the second measurement onward, animals were anesthetized with intraperitoneal anesthesia, and a drop of tropicamide (Tropicamida 1%; Alcon-Cusi, S.A, Barcelona, Spain) was instilled in both eyes to induce mydriasis. Eyes were kept hydrated with artificial tears, and a custom-made contact permeable lens (3.5-mm posterior radius of curvature, 5.0-mm optical zone diameter, +5.0-diopter [D] back vertex power) was placed on the cornea to maintain corneal hydration and clarity. Then, the retinas were imaged according to manufacturer instructions (Spectralis; Heidelberg Engineering, Heidelberg, Germany). To adapt for the rat's eye, a commercially available 78-D double aspheric fundus lens (Volk Optical, Inc., Mentor, OH, USA) was mounted in front of the camera unit. Imaging was performed with a proprietary software package (Eye Explorer, version 3.2.1.0; Heidelberg Engineering) as described.[22]

Retinas were scanned with a raster pattern of 31 equally spaced horizontal B-scans spanning the central retina (3000-μm length). In four central sections/animal, the retinal nerve fiber layer (RNFL) and retinal thickness were manually measured at 600 (center) and 1200 (periphery) μm from the optic nerve.

Retinal Cross Sections and Immunodetection

Animals were perfused transcardially with 4% paraformaldehyde (PFA) in phosphate buffer 0.1 M after a saline rinse. Eyes were dissected and cryoprotected in increasing gradients of sucrose (15% to 30%; Sigma-Aldrich, Madrid, Spain). Tissue was embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA), frozen in liquid nitrogen, and kept at –80°C. Retinas ($n = 4$/group) were sectioned at 15 μm in a cryostat. For immunodetection, sections were blocked in 2% donkey serum in phosphate-buffered saline (PBS) with 0.1% Triton (PBST) and incubated overnight at 4°C with a mixture of rabbit antiglial fibrillary acidic protein (GFAP; 1:500 Sigma-Aldrich) and goat anti-vimentin (1:500 sc-7557; Santa Cruz Biotechnologies, Heidelberg, Germany) or a mixture of mouse anticellular retinaldehyde binding protein 1 (CRALBP1 [B2], 1:200, ab15051; Abcam, Cambridge, UK) and goat anti-brain-specific homeobox/POU domain protein 3A (Brn3a, 1:750, sc-sc-31984; Santa Cruz Biotechnologies). Secondary detection was done with donkey anti-goat Alexa 488 or 594, donkey anti-mouse Alexa 488, and donkey anti-rabbit Alexa 594 (1:500; Molecular Probes, ThermoFisher, Madrid, Spain). Finally, sections were counterstained with DAPI (4′,6-diamidino-2-phenylindole) (Vectashield mounting medium with DAPI; Vector Laboratories, Palex Medical, Barcelona, Spain). Images were acquired with a ×20 objective under an epifluorescence microscope (Axioscop 2 Plus; Zeiss Mikroskopie, Jena, Germany).

Brn3a+RGCs were manually counted, in a masked fashion, in sections spanning the optic nerve (3 sections/animal).

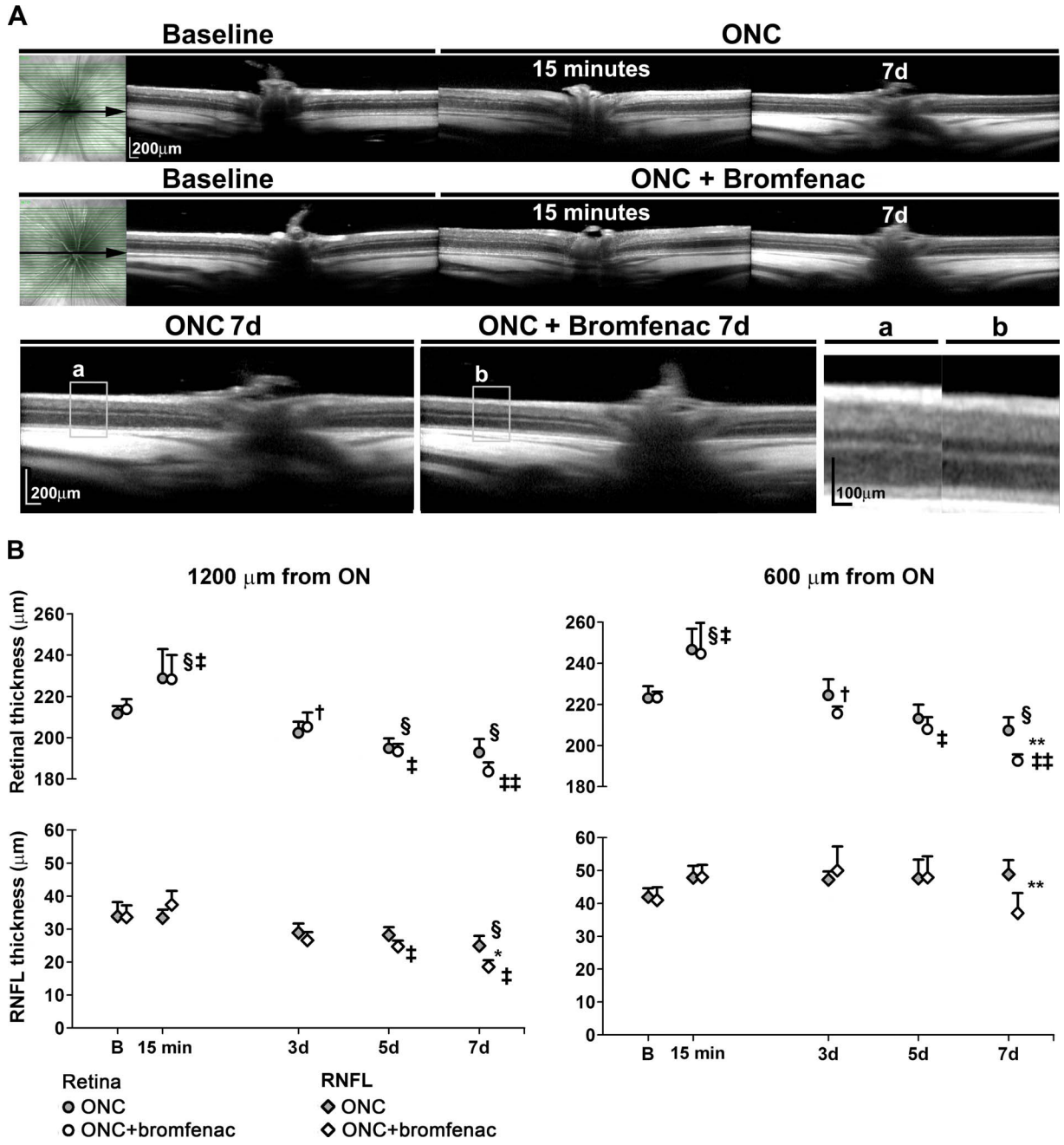

Figure 1. Bromfenac treatment diminishes retinal swelling after ONC. (**A**) OCT sections spanning the optic disc acquired before (baseline) and 15 minutes or 7 days after ONC or ONC + bromfenac. (**a**, **b**) Magnifications from the *squared areas*. (**B**) Graphs showing the course of the retinal (*top*) and RFNL (*bottom*) thickness after ONC and ONC + bromfenac. These data were measured in the OCT images at 1200 and 600 μm from the optic disc. §‡Significant compared to baseline values (§ONC, ‡ONC + bromfenac; Kruskal-Wallis, Dunn's post hoc §$P < 0.05$, ‡$P < 0.05$, ‡‡$P < 0.01$). †Significant compared to 15 minutes for both groups (Kruskal-Wallis, Dunn's post hoc, $P < 0.05$) *Significant difference between both groups (Kruskal-Wallis, Dunn's post hoc, *$P < 0.05$, **$P < 0.01$). B, baseline; Imm, immediately after the lesion; d, days; ON, optic nerve; ONC, optic nerve crush.

Numbers are given as the mean number ± standard difference of RGCs per section.

Western Blotting

Retinas were fresh dissected and immediately frozen in dry ice ($n = 4$/group). Then, retinas were homogenized in lysis buffer (Pro-prep protein extraction solution; Intron Biotechnologies, Sevilla, Spain). Lysates were incubated 1 hour on ice and centrifuged to remove particulate matter. Protein concentration was determined using SimpliNano spectrophotometer (Biochrom Ltd, Cambridge, UK). A total of 20 to 60 μg protein was resolved in 4% to 20% SDS-PAGE gels (Bio-Rad laboratories, SA, Madrid, Spain) and transferred to nitrocellulose membranes (GE Healthcare, Barcelona, Spain) by electroblotting. Blots were blocked for 1 hour with 5% skim milk in PBS containing 0.5% Tween-20 (PBS-T, pH 7.4) and then were incubated overnight at 4°C with rabbit anti-cyclooxygenase 2 (COX2, 1:1000), mouse anti-cyclooxygenase 1 (COX1, 1:2000), rabbit anti-vimentin (1:1000), mouse anti-CRALBP1 (1:1000), all from

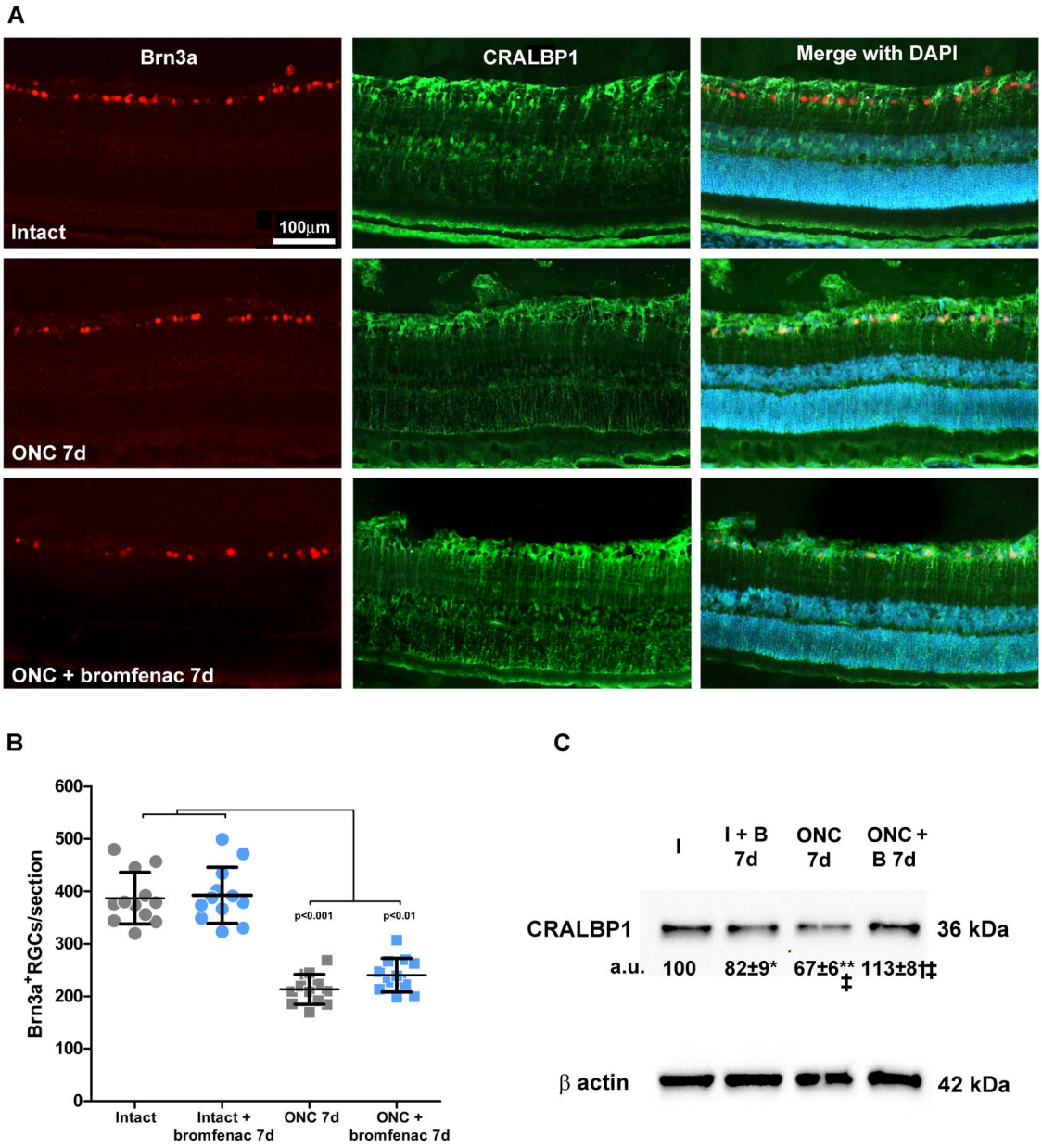

FIGURE 2. Bromfenac treatment does not accelerate ONC-induced RGC degeneration. (**A**) Brn3a (RGCs) and CRALBP1 (Müller cells) immunodetection in retinal sections. (**B**) Scatter plot showing the number of Brn3a$^+$RGCs per section in retinal cross sections from intact retinas, intact retinas treated during 7 days with bromfenac, retinas dissected 7 days after axotomy, or 7 days after ONC and bromfenac treatment. The number of RGCs is not significantly different between intact and intact + bromfenac, or ONC and ONC + bromfenac. Compared to intact or intact + bromfenac retinas, the loss of RGCs is significant in both injured groups (Kruskal-Wallis, Dunn's post hoc, $P < 0.001$ for ONC and 0.01 for ONC + bromfenac). (**C**) Western blotting showing the expression levels of CRABLP in retinal extracts from the same groups as above. a.u., arbitrary units taking intact retinas as 100% and normalized versus β-actin signal and total protein staining (see Methods). *Significant compared to intact retinas (*t*-test, $^*P < 0.05$, $^{**}P < 0.01$). †Significant compared to ONC (*t*-test, $P < 0.005$). ‡Significant compared to intact + bromfenac (*t*-test, $P < 0.05$ for ONC and $P < 0.001$ for ONC+bromfenac).

Abcam, or rabbit anti-GFAP (1:500, Sigma-Aldrich). Detection was carried out with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit or anti-mouse, Santa Cruz Biotechnologies) at 1:5000 dilution.

Membranes were developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare). As loading control, total protein/lane in the membranes was visualized using reversible protein staining (R-PROB, Sigma-Aldrich) followed by β-actin identification using anti-β-actin–HRP mouse monoclonal antibody (Sigma-Aldrich). β-actin signal was equivalent to total protein staining in all lanes, and thus its intensity was used for normalization. The density of the protein bands was quantified using image analyzer Chemidoc XRS+ (Bio-Rad, Hercules, CA, USA) and the software (ImageLab 5.2.1).

Prostaglandin E_2 Detection

Prostaglandin E_2 levels in retinal homogenates from the different experimental groups ($n = 4$/group) were assayed by PARAMETER PGE_2 enzyme-linked immunoabsorbent assay

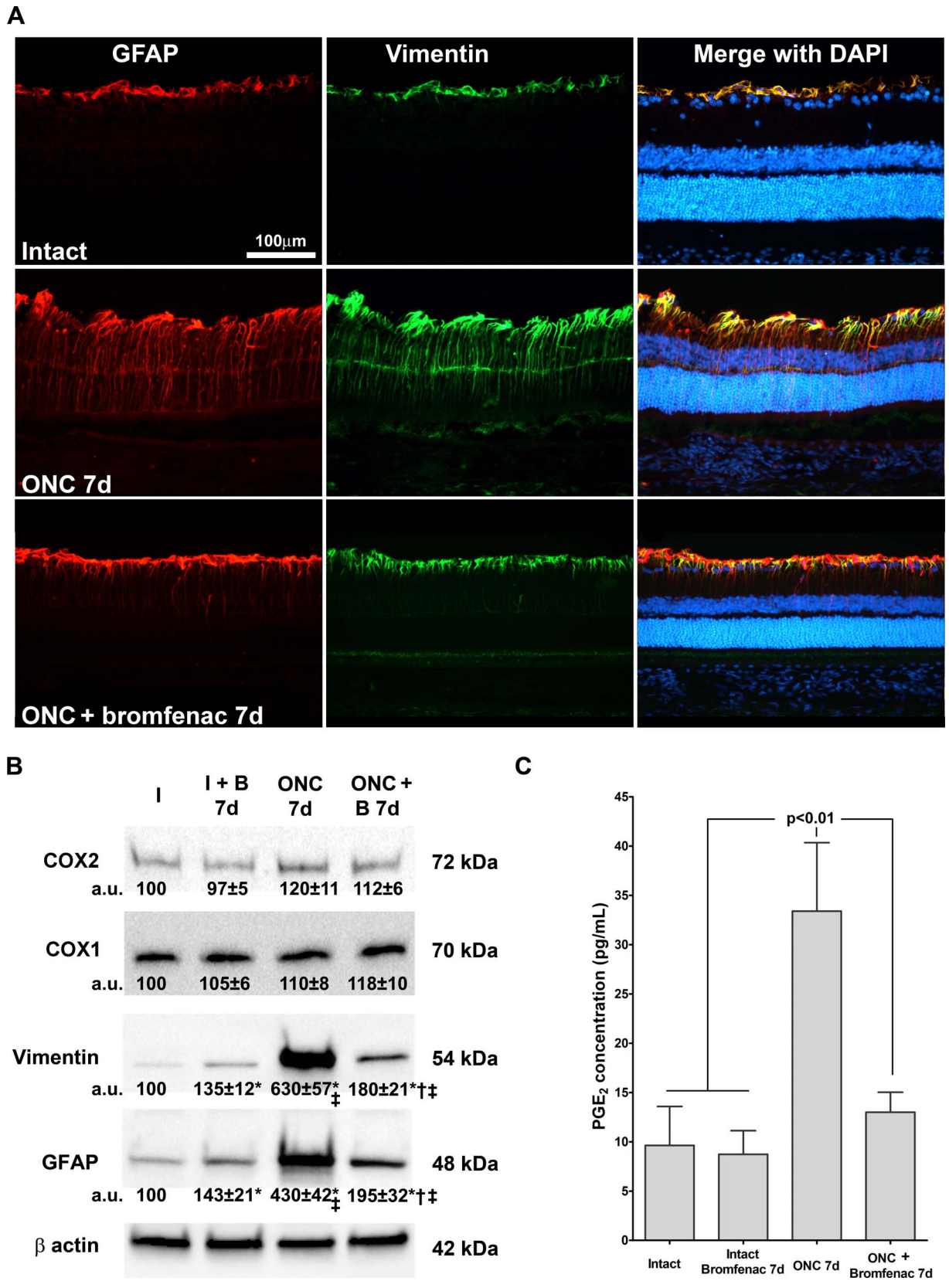

FIGURE 3. Bromfenac treatment decreases reactive gliosis and PGE_2 production in axotomized retinas. (**A**) GFAP (astrocytes and Müller cells) and vimentin (Müller cells) immunodetection in retinal sections. ONC causes hypertrophy of astrocytes and Müller cells (gliosis). This gliotic response is greatly reduced by bromfenac treatment. (**B**) Western blotting showing the expression levels of COX2, COX1, vimentin, and GFAP in extracts from intact retinas (I), intact retinas treated during 7 days with bromfenac (I+B 7 d), retinas dissected 7 days after axotomy (ONC 7 d), or 7 days after ONC and bromfenac treatment (ONC7d+B7d). a.u., arbitrary units taking intact retinas as 100% and normalized versus β-actin signal and total protein

Investigative Ophthalmology & Visual Science

staining (see Methods). *Significant compared to intact retinas (*t*-test, $P < 0.05$ for I+B, $P < 0.01$ ONC+B, $P < 0.0001$ ONC). †Significant compared to ONC (*t*-test, $P < 0.001$). ‡Significant compared to intact + bromfenac (*t*-test, $P < 0.001$ for ONC and $P < 0.05$ for ONC+B). (**C**) PGE_2 concentration (pg/mL) in extracts from the same groups as above. ONC causes a significant increase of PGE_2 that is abolished by bromfenac treatment (Kruskal-Wallis, Dunn's post hoc, $P < 0.001$).

(ELISA) (R&D Systems, Minneapolis, MN, USA). Tests were performed according to the manufacturer's instructions.

Graphs and Statistical Analysis

Graphs and pairwise multiple comparisons (Kruskal-Wallis ANOVA, Dunn's post hoc) were done with GraphPad Prism v. 6 software (GraphPad, San Diego, CA, USA). Data are presented as mean ± standard deviation (SD), and differences were considered significant when $P < 0.05$.

Results

Retinal Thickness

In both groups, ONC and ONC + bromfenac, 15 minutes after the lesion the retina had swollen compared to baseline values, although this swelling was transient and at day 3 the retina was back to normal (Fig. 1). Thereafter, the retinal thickness decreased gradually, reaching statistical significance for both groups at day 5 in the periphery, and closer to the optic disc in the bromfenac-treated group. Seven days after the injury, the retina was significantly thinner in both groups and at both distances. Interestingly, comparing both groups, the retina was thinner at day 7 in the bromfenac-treated animals, reaching this difference statistical significance at 600 μm from the optic disc (for detailed values see Supplementary Table S1).

At 1200 μm from the optic disc, the thickness of RNFL significantly decreased by day 5 in the bromfenac-treated group and at day 7 in both groups. At this latter time point, the RNFL was significantly thinner in the treated versus untreated group. At 600 μm from the optic disc, there were not significant changes in either group compared to baseline. However, 7 days after the lesion, the RNFL thickness was significantly reduced in the bromfenac-treated retinas compared to untreated ones.

Bromfenac Treatment Does Not Accelerate Retinal Degeneration

In view of the above data, the question arises: Does the reduced RNFL thickness in treated animals reflect a reduction in edema/gliosis, or does it reflect increased neurodegeneration? To answer it, we quantified $Brn3a^+$RGCs in retinal cross sections (Figs. 2A, 2B) and analyzed the expression of CRALBP1 (Figs. 2A, 2C), a protein expressed by Müller cells that is a marker of retinal homeostasis.[52] Bromfenac treatment did not alter the number of $Brn3a^+$RGCs, either in intact or in ONC-injured retinas. Regarding CRALBP1, compared to intact retinas, its expression declined after ONC but was maintained in ONC-treated samples. In intact but treated retinas, CRABLP levels declined as well, although to a lesser extent than after ONC.

Retinal Gliosis

Next, we assessed astrocyte and Müller cell hypertrophy using the classical gliosis markers GFAP and vimentin. Optic nerve crush triggers a strong glial activation, which, at 7 days, almost reverted with bromfenac treatment, as observed by immunodetection and Western blotting (Figs. 3A–B). In retinal extracts from intact animals treated during 7 days with bromfenac, Western blotting analysis showed that GFAP and vimentin are upregulated compared to intact retinas, but their levels are still lower than those found in ONC + bromfenac retinas.

Cyclooxygenases and Prostaglandin E_2

Bromfenac mechanism of action is due to its ability to block prostaglandin synthesis by inhibiting COX1 and COX2. Thus, in retinal extracts we studied the regulation of both enzymes and measured the levels of PGE_2 (Figs. 3B, 3C). Neither COX1 nor COX2 showed a significant regulation at 7 days post lesion and/or treatment (Fig. 1B). Regarding PGE_2, its concentration in intact or intact and treated retinas was similar (9.6 ± 4 or 8.7 ± 2 pg/mL, respectively), and significantly increased ~4 fold after ONC (33.4 ± 7 pg/mL). Interestingly, in the ONC + bromfenac group, PGE_2 levels were reduced almost to the values found in intact retinas (13 ± 2 pg/mL) (Fig. 3C).

Discussion

The main findings of this work are that topical instillation of bromfenac (0.09%) reduces retinal thickness, retinal gliosis, and the release of PGE_2 after a complete intraorbital ONC, a drastic injury to the retina that results in acute and massive RGC death. Importantly, bromfenac treatment does not affect the number of RGCs in intact retinas or accelerate ONC-induced degeneration. In fact, current experiments in our lab indicate that bromfenac treatment increases RGC survival after ONC, although this protection is not immediate but is delayed to 9 days onward (Rovere G, Nadal-Nicolás FM, Vidal-Sanz M, Agudo-Barriuso, M. unpublished data, 2016).

Macroglial cells respond quickly to retinal stress or injury.[33] Indeed, it has been recently shown that macroglial cells hypertrophy across the whole retina after an apparently innocuous manipulation such as intravitreal injection of phosphate-buffered saline.[29] It was interesting to find that they also responded to topical administration of bromfenac, as evidenced by the significant increase of GFAP and vimentin and the decrease of CRALBP1 in extracts from intact retinas treated during 7 days with the NSAID. However, these changes reverted in ONC + bromfenac retinas when compared to ONC alone, indicating, firstly, that the gliosis triggered by ONC is very strong and almost but not completely abolished by bromfenac, and, secondly, that treatment with this NSAID maintains retinal homeostasis after ONC. In line with this, it is worth highlighting that gliotic and homeostatic markers seem to be regulated in opposite ways here, after ONC, and in isolated retinal explants.[52]

Retinal gliosis is reported to have both neuroprotective as well as neurodegenerative effects (reviewed in Ref. 53). Data in this work do not support neuroprotection, but they indicate that, at least in this model and at this time point, the reduced gliosis is neutral in terms of RGC degeneration. But, because as mentioned above we have preliminary data indicating that indeed bromfenac treatment protects RGCs from 9 days onward, it would seem that a reduction in gliosis is neuroprotective. However, this might well occur because the reduced gliosis is accompanied by maintenance of the retinal homeostasis or by other mechanism(s) not related to the gliotic response.

Bromfenac mechanism of action goes through the nonselective inhibition of COX1 and COX2, although recent work indicates that bromfenac is more selective for COX2.[17] Expression of COX2 is inducible in response to injury and the release of cytokines and proinflammatory molecules,[54] both of which occur in the retina after ONC.[25] In mild brain injuries, COX2 expression elevates shortly after the lesion, but this upregulation is extended ($\geq$3 days) if the injury is severe,[55] and this may explain why we did not find upregulation of COX2 in our experiments, since we analyzed the retinas 7 days after ONC. Nevertheless, we found that at this time point there was approximately a 4-fold increase of PGE_2 concentration in ONC retinas compared to intact ones, indicating that even though COX2 is not upregulated, it is activated. Furthermore, bromfenac instillation almost suppressed completely the increment of PGE_2, suggesting that bromfenac inhibits COX2 increased activity. Interestingly, in spite of the increase of macroglial markers in intact + bromfenac retinas compared to intact ones, the concentration of PGE_2 was similar in both groups, suggesting that perhaps the cytokines/inflammatory mediators that activate COX are released by microglial cells.[56,57]

Finally, we found that bromfenac treatment significantly reduced in injured retinas the retinal and RNFL thickness 7 days after the insult, without this thinning being due to an increased RGC death. This is of special importance in the central retina because in clinical practice, RFNL measurement is employed as an index of RGC loss. Our group and others[22,58–61] have shown in animal models that following axotomy of the optic nerve or ocular hypertension there is a 9-day time lapse mismatch between the onset of RGC disappearance and the slower, more protracted, degeneration of the intraretinal axons, which form the RFNL.[22] It is tempting to suggest that such a time delay could be influenced by a RFNL swollen by the astrocyte and Müller cell hypertrophy that occurs upon lesion.

Besides reducing gliosis, bromfenac abolishes the release of PGE_2, which in turn may effectively reduce the retinal edema, since prostaglandins in the eye disrupt the blood–ocular barrier, increase vasodilation, and facilitate leukocyte migration causing edema and inflammation.[2] If this hypothesis is sustained, it would be of interest to instill NSAIDs in patients with retinal edema when diagnosed by in vivo measurement of the retina.

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References

1. Rodrigues EB, Farah ME, Bottos JM, Bom AF. Nonsteroidal anti-inflammatory drugs in the treatment of retinal diseases. *Dev Ophthalmol*. 2016;55:212–220.
2. Schoenberger SD, Kim SJ. Nonsteroidal anti-inflammatory drugs for retinal disease. *Int J Inflam*. 2013;2013:281981.
3. Sheppard JD, Comstock TL, Cavet ME. Impact of the topical ophthalmic corticosteroid loteprednol etabonate on intraocular pressure. *Adv Ther*. 2016;33:532–552.
4. Chastain JE, Sanders ME, Curtis MA, et al. Distribution of topical ocular nepafenac and its active metabolite amfenac to the posterior segment of the eye. *Exp Eye Res*. 2016;145:58–67.
5. Prodanov D, Mantchev G, Iliev A, et al. Effects of dexamethasone in rat neonatal model of axotomy-induced motoneuronal cell death. *Arch Physiol Biochem*. 1998;106:355–361.
6. Steinsapir KD, Goldberg RA, Sinha S, Hovda DA. Methylprednisolone exacerbates axonal loss following optic nerve trauma in rats. *Restor Neurol Neurosci*. 2000;17:157–163.
7. Afrashi F, Hashas AS, Shahbazov C, et al. Reliability of intravitreal nepafenac in rabbits. *J Ocul Pharmacol Ther*. 2015;31:43–50.
8. Baklayan GA, Patterson HM, Song CK, Gow JA, McNamara TR. 24-hour evaluation of the ocular distribution of (14) C-labeled bromfenac following topical instillation into the eyes of New Zealand White rabbits. *J Ocul Pharmacol Ther*. 2008;24:392–398.
9. Bucolo C, Marrazzo G, Platania CB, Romano GL, Drago F, Salomone S. Effects of topical indomethacin, bromfenac and nepafenac on lipopolysaccharide-induced ocular inflammation. *J Pharm Pharmacol*. 2014;66:954–960.
10. Chien JY, Sheu JH, Wen ZH, Tsai RK, Huang SP. Neuroprotective effect of 4-(Phenylsulfanyl) butan-2-one on optic nerve crush model in rats. *Exp Eye Res*. 2016;143:148–157.
11. Gamache DA, Graff G, Brady MT, Spellman JM, Yanni JM. Nepafenac a unique nonsteroidal prodrug with potential utility in the treatment of trauma-induced ocular inflammation: I. Assessment of anti-inflammatory efficacy. *Inflammation*. 2000;24:357–370.
12. Kapin MA, Yanni JM, Brady MT, et al. Inflammation-mediated retinal edema in the rabbit is inhibited by topical nepafenac. *Inflammation*. 2003;27:281–291.
13. Nadal-Nicolas FM, Rodriguez-Villagra E, Bravo-Osuna I, et al. Ketorolac administration attenuates retinal ganglion cell death after axonal injury. *Invest Ophthalmol Vis Sci*. 2016;57:1183–1192.
14. Russo A, Morescalchi F, Vezzoli S, et al. Reduction of vitreous prostaglandin E2 levels after topical administration of indomethacin 0.5%, bromfenac 0.09%, and nepafenac 0.1. *Retina*. 2016;36:1227–1231.
15. Strauss KI. Antiinflammatory and neuroprotective actions of COX2 inhibitors in the injured brain. *Brain Behav Immun*. 2008;22:285–298.
16. Wang QW, Yao K, Xu W, et al. Bromfenac sodium 0.1% fluorometholone 0.1% and dexamethasone 0.1% for control of ocular inflammation and prevention of cystoid macular edema after phacoemulsification. *Ophthalmologica*. 2013;229:187–194.
17. Waterbury LD, Silliman D, Jolas T. Comparison of cyclooxygenase inhibitory activity and ocular anti-inflammatory effects of ketorolac tromethamine and bromfenac sodium. *Curr Med Res Opin*. 2006;22:1133–1140.
18. Baklayan GA, Munoz M. The ocular distribution of (14) C-labeled bromfenac ophthalmic solution 0.07% in a rabbit model. *Clin Ophthalmol*. 2014;8:1717–1724.
19. Kida T, Kozai S, Takahashi H, Isaka M, Tokushige H, Sakamoto T. Pharmacokinetics and efficacy of topically applied nonsteroidal anti-inflammatory drugs in retinochoroidal tissues in rabbits. *PLoS One*. 2014;9:e96481.
20. Macri A, Vagge A, Salis A, et al. Bromfenac ophthalmic solution 0.09 %: human aqueous humor concentration detected by high-performance liquid chromatography [published online ahead of print June 20, 2016]. *Int Ophthalmol*. doi:10.1007/s10792-016-0279-3.

21. Higaki S, Watanabe K, Itahashi M, Shimomura Y. Cyclooxygenase (COX)-inhibiting drug reduces HSV-1 reactivation in the mouse eye model. *Curr Eye Res*. 2009;34:171–176.
22. Rovere G, Nadal-Nicolas FM, Agudo-Barriuso M, et al. Comparison of retinal nerve fiber layer thinning and retinal ganglion cell loss after optic nerve transection in adult albino rats. *Invest Ophthalmol Vis Sci*. 2015;56:4487–4498.
23. Mac Nair CE, Nickells RW. Neuroinflammation in glaucoma and optic nerve damage. *Prog Mol Biol Transl Sci*. 2015;134:343–363.
24. Vohra R, Tsai JC, Kolko M. The role of inflammation in the pathogenesis of glaucoma. *Surv Ophthalmol*. 2013;58:311–320.
25. Agudo M, Perez-Marin MC, Lonngren U, et al. Time course profiling of the retinal transcriptome after optic nerve transection and optic nerve crush. *Mol Vis*. 2008;14:1050–1063.
26. Salvador-Silva M, Vidal-Sanz M, Villegas-Pérez MP. Microglial cells in the retina of Carassius auratus: effects of optic nerve crush. *J Comp Neurol*. 2000;417:431–447.
27. Sobrado-Calvo P, Vidal-Sanz M, Villegas-Pérez MP. Rat retinal microglial cells under normal conditions, after optic nerve section and after optic nerve section and intravitreal injection of trophic factors or macrophage inhibitory factor. *J Comp Neurol*. 2007;501:866–878.
28. Lönngren U, Näpänkangas U, Lafuente M, et al. The growth factor response in ischemic rat retina and superior colliculus after brimonidine pre-treatment. *Brain Res Bull*. 2006;71:208–218.
29. Di Pierdomenico J, García-Ayuso D, Jimenez-Lopez M, Agudo-Barriuso M, Vidal-Sanz M, Villegas-Perez MP. Different ipsi- and contralateral glial responses to anti-VEGF and triamcinolone intravitreal injections in rats. *Invest Ophthalmol Vis Sci*. 2016;57:3533–3544.
30. Ramírez AI, Salazar JJ, de Hoz R, et al. Quantification of the effect of different levels of IOP in the astroglia of the rat retina ipsilateral and contralateral to experimental glaucoma. *Invest Ophthalmol Vis Sci*. 2010;51:5690–5696.
31. Gallego BI, Salazar JJ, de Hoz R, et al. IOP induces upregulation of GFAP and MHC-II and microglia reactivity in mice retina contralateral to experimental glaucoma. *J Neuroinflammation*. 2012;9:92.
32. Rojas B, Gallego BI, Ramírez AI, et al. Microglia in mouse retina contralateral to experimental glaucoma exhibit multiple signs of activation in all retinal layers. *J Neuroinflammation*. 2014;11:133.
33. de Hoz R, Rojas B, Ramirez AI, et al. Retinal macroglial responses in health and disease. *Biomed Res Int*. 2016;2016:2954721.
34. Nadal-Nicolas FM, Sobrado-Calvo P, Jimenez-Lopez M, Vidal-Sanz M, Agudo-Barriuso M. Long-term effect of optic nerve axotomy on the retinal ganglion cell layer. *Invest Ophthalmol Vis Sci*. 2015;56:6095–6112.
35. Parrilla-Reverter G, Agudo M, Nadal-Nicolas F, et al. Time-course of the retinal nerve fibre layer degeneration after complete intra-orbital optic nerve transection or crush: a comparative study. *Vision Res*. 2009;49:2808–2825.
36. Parrilla-Reverter G, Agudo M, Sobrado-Calvo P, Salinas-Navarro M, Villegas-Perez MP, Vidal-Sanz M. Effects of different neurotrophic factors on the survival of retinal ganglion cells after a complete intraorbital nerve crush injury: a quantitative in vivo study. *Exp Eye Res*. 2009;89:32–41.
37. Jehle T, Dimitriu C, Auer S, et al. The neuropeptide NAP provides neuroprotection against retinal ganglion cell damage after retinal ischemia and optic nerve crush. *Graefes Arch Clin Exp Ophthalmol*. 2008;246:1255–1263.
38. Villegas-Pérez MP, Vidal-Sanz M, Rasminsky M, Bray GM, Aguayo AJ. Rapid and protracted phases of retinal ganglion cell loss follow axotomy in the optic nerve of adult rats. *J Neurobiol*. 1993;24:23–36.
39. Aguayo AJ, Vidal-Sanz M, Villegas-Pérez MP, Bray GM. Growth and connectivity of axotomized retinal neurons in adult rats with optic nerves substituted by PNS grafts linking the eye and the midbrain. *Ann N Y Acad Sci*. 1987;495:1–9.
40. Berkelaar M, Clarke DB, Wang YC, Bray GM, Aguayo AJ. Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. *J Neurosci*. 1994;14:4368–4374.
41. Peinado-Ramón P, Salvador M, Villegas-Pérez MP, Vidal-Sanz M. Effects of axotomy and intraocular administration of NT-4, NT-3 and brain-derived neurotrophic factor on the survival of adult rat retinal ganglion cells. A quantitative in vivo study. *Invest Ophthalmol Vis Sci*. 1996;37:489–500.
42. Bodeutsch N, Siebert H, Dermon C, Thanos S. Unilateral injury to the adult rat optic nerve causes multiple cellular responses in the contralateral site. *J Neurobiol*. 1999;38:116–128.
43. Mansour-Robaey S, Clarke DB, Wang YC, Bray GM, Aguayo AJ. Effects of ocular injury and administration of brain-derived neurotrophic factor on survival and regrowth of axotomized retinal ganglion cells. *Proc Natl Acad Sci U S A*. 1994;91:1632–1636.
44. Vidal-Sanz M, Lafuente M, Sobrado-Calvo P, et al. Death and neuroprotection of retinal ganglion cells after different types of injury. *Neurotox Res*. 2000;2:215–227.
45. Sanchez-Migallon MC, Valiente-Soriano FJ, Nadal-Nicolas FM, Vidal-Sanz M, Agudo-Barriuso M. Apoptotic retinal ganglion cell death after optic nerve transection or crush in mice: delayed RGC loss with BDNF or a caspase 3 inhibitor. *Invest Ophthalmol Vis Sci*. 2016;57:81–93.
46. Galindo-Romero C, Valiente-Soriano FJ, Jimenez-Lopez M, et al. Effect of brain-derived neurotrophic factor on mouse axotomized retinal ganglion cells and phagocytic microglia. *Invest Ophthalmol Vis Sci*. 2013;54:974–985.
47. Lindqvist N, Peinado-Ramónn P, Vidal-Sanz M, Hallböök F. GDNF, Ret, GFRalpha1 and 2 in the adult rat retino-tectal system after optic nerve transection. *Exp Neurol*. 2004;187:487–499.
48. Agudo M, Perez-Marin MC, Sobrado-Calvo P, et al. Immediate upregulation of proteins belonging to different branches of the apoptotic cascade in the retina after optic nerve transection and optic nerve crush. *Invest Ophthalmol Vis Sci*. 2009;50:424–431.
49. Agudo-Barriuso M, Lahoz A, Nadal-Nicolas FM, et al. Metabolomic changes in the rat retina after optic nerve crush. *Invest Ophthalmol Vis Sci*. 2013;54:4249–4259.
50. Bray GM, Villegas-Pérez MP, Vidal-Sanz M, Carter DA, Aguayo AJ. Neuronal and nonneuronal influences on retinal ganglion cell survival, axonal regrowth and connectivity after axotomy. *Ann N Y Acad Sci*. 1991;633:214–228.
51. Jones J, Francis P. Ophthalmic utility of topical bromfenac a twice-daily nonsteroidal anti-inflammatory agent. *Expert Opin Pharmacother*. 2009;10:2379–2385.
52. Taylor L, Arner K, Ghosh F. First responders: dynamics of pregliotic Müller cell responses in the isolated adult rat retina. *Curr Eye Res*. 2015;40:1245–1260.
53. Bringmann A, Iandiev I, Pannicke T, et al. Cellular signaling and factors involved in Müller cell gliosis: neuroprotective and detrimental effects. *Prog Retin Eye Res*. 2009;28:423–451.
54. Minghetti L. Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. *J Neuropathol Exp Neurol*. 2004;63:901–910.
55. Strauss KI. Antiinflammatory and neuroprotective actions of COX2 inhibitors in the injured brain. *Brain Behav Immun*. 2008;22:285–298.
56. Karlstetter M, Scholz R, Rutar M, Wong WT, Provis JM, Langmann T. Retinal microglia: just bystander or target for therapy? *Prog Retin Eye Res*. 2015;45:30–57.

Investigative Ophthalmology & Visual Science

57. Madeira MH, Boia R, Santos PF, Ambrosio AF, Santiago AR. Contribution of microglia-mediated neuroinflammation to retinal degenerative diseases. *Mediators Inflamm*. 2015; 2015:673090.
58. Chauhan BC, Stevens KT, Levesque JM, et al. Longitudinal in vivo imaging of retinal ganglion cells and retinal thickness changes following optic nerve injury in mice. *PLoS One*. 2012; 7:e40352.
59. Choe TE, Abbott CJ, Piper C, Wang L, Fortune B. Comparison of longitudinal in vivo measurements of retinal nerve fiber layer thickness and retinal ganglion cell density after optic nerve transection in rat. *PLoS One*. 2014;9:e113011.
60. Nagata A, Higashide T, Ohkubo S, Takeda H, Sugiyama K. In vivo quantitative evaluation of the rat retinal nerve fiber layer with optical coherence tomography. *Invest Ophthalmol Vis Sci*. 2009;50:2809–2815.
61. Guo L, Normando EM, Nizari S, Lara D, Cordeiro MF. Tracking longitudinal retinal changes in experimental ocular hypertension using the cSLO and spectral domain-OCT. *Invest Ophthalmol Vis Sci*. 2010;51:6504–6513.

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