

# Neuroprotective Effects of FGF2 and Minocycline in Two Animal Models of Inherited Retinal Degeneration

Johnny Di Pierdomenico,<sup>1</sup> Rebecca Scholz,<sup>2</sup> F. Javier Valiente-Soriano,<sup>1</sup> María C. Sánchez-Migallón,<sup>1</sup> Manuel Vidal-Sanz,<sup>1</sup> Thomas Langmann,<sup>2</sup> Marta Agudo-Barriuso,<sup>1</sup> Diego García-Ayuso,<sup>1</sup> and María Paz Villegas-Pérez<sup>1</sup>

<sup>1</sup>Departamento de Oftalmología, Facultad de Medicina, Universidad de Murcia and Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca (IMIB-Arrixaca), Murcia, Spain

<sup>2</sup>Laboratory for Experimental Immunology of the Eye, Department of Ophthalmology, University of Cologne, Cologne, Germany

Correspondence: Diego García-Ayuso, Laboratorio de Oftalmología Experimental, Universidad de Murcia e Instituto Murciano de Investigación Biosanitaria (IMIB-Arrixaca), Edificio Departamental, Carretera Buenavista, El Palmar, Murcia 30120, Spain; diegogarcia@um.es.

María Paz Villegas-Pérez, Laboratorio de Oftalmología Experimental, Universidad de Murcia e Instituto Murciano de Investigación Biosanitaria (IMIB-Arrixaca), Edificio Departamental, Carretera Buenavista, El Palmar, Murcia 30120, Spain; mpville@um.es.

Submitted: April 19, 2018

Accepted: July 28, 2018

Citation: Di Pierdomenico J, Scholz R, Valiente-Soriano FJ, et al. Neuroprotective effects of FGF2 and minocycline in two animal models of inherited retinal degeneration. *Invest Ophthalmol Vis Sci.* 2018;59:4392-4403. <https://doi.org/10.1167/iovs.18-24621>

**PURPOSE.** The purpose of this study was to study the effect of minocycline and several neurotrophic factors, alone or in combination, on photoreceptor survival and macro/microglial reactivity in two rat models of retinal degeneration.

**METHODS.** P23H-1 (rhodopsin mutation), Royal College of Surgeon (RCS, pigment epithelium malfunction), and age-matched control rats (Sprague-Dawley and Pievald Viro Glaxo, respectively) were divided into three groups that received at P10 for P23H-1 rats or P33 for RCS rats: (1) one intravitreal injection (IVI) of one of the following neurotrophic factors: ciliary neurotrophic factor (CNTF), pigment epithelium-derived factor (PEDF), or basic fibroblast growth factor (FGF2); (2) daily intraperitoneal administration of minocycline; or (3) a combination of IVI of FGF2 and intraperitoneal minocycline. All animals were processed 12 days after treatment initiation. Retinal microglial cells and cone photoreceptors were immunodetected and analyzed qualitatively in cross sections. The numbers of microglial cells in the different retinal layers and number of nuclei rows in the outer nuclear layer (ONL) were quantified.

**RESULTS.** IVI of CNTF, PEDF, or FGF2 improved the morphology of the photoreceptors outer segment, but only FGF2 rescued a significant number of photoreceptors. None of the trophic factors had qualitative or quantitative effects on microglial cells. Minocycline treatment reduced activation and migration of microglia and produced a significant rescue of photoreceptors. Combined treatment with minocycline and FGF2 had higher neuroprotective effects than each of the treatments alone.

**CONCLUSIONS.** In two animal models of photoreceptor degeneration with different etiologies, minocycline reduces microglial activation and migration, and FGF2 and minocycline increase photoreceptor survival. The combination of FGF2 and minocycline show greater neuroprotective effects than their isolated effects.

Keywords: royal college of surgeons, P23H-1, microglia, minocycline, FGF2

Retinal photoreceptor degenerations are the most frequent cause of irreversible blindness in industrialized countries. Inherited photoreceptor degenerations<sup>1-3</sup> are rare diseases that may cause blindness, and their most frequent form is retinitis pigmentosa (RP).

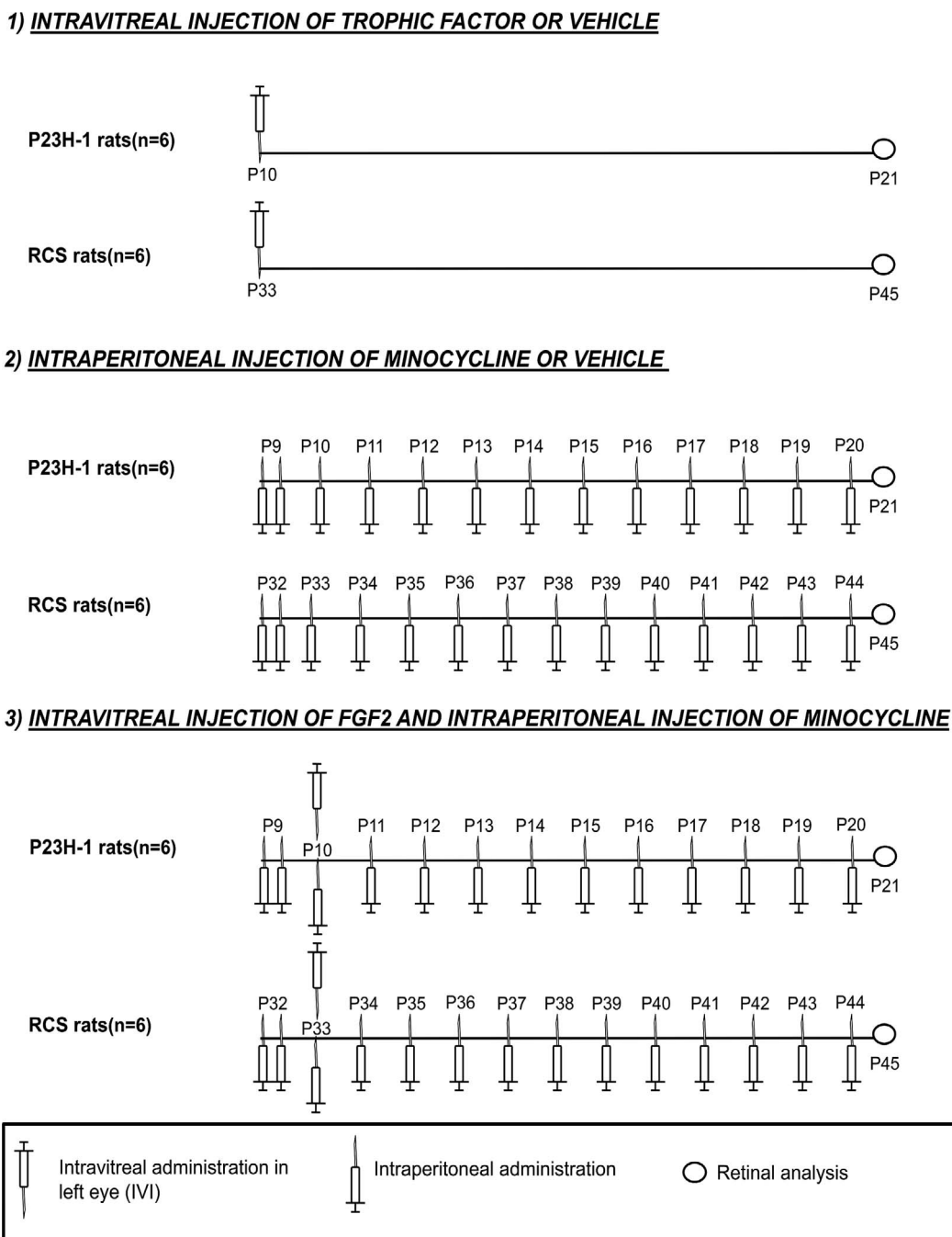
There are several animal models to study the inherited photoreceptor degenerations, and some of them such as the P23H rat and the Royal College of Surgeons (RCS) rat bear monogenic diseases with the same genetic defects observed in human RP patients.<sup>4-6</sup> The P23H rat strain bears an autosomal dominant mutation in the rhodopsin gene<sup>7-10</sup> that causes, first, rod and, second, cone degeneration.<sup>11</sup> The RCS rats suffer a mutation of the *MERKT* gene, a tyrosinase kinase receptor necessary for normal retinal pigment epithelium phagocytosis<sup>4,6,12,13</sup> that triggers the simultaneous degeneration of rods and cones.

Microglial cells are the resident immune cells of the central nervous system, including the retina. In healthy retinas, they are located in the nerve fiber layer, the ganglion cell layer

(GCL), and both plexiform layers.<sup>14-19</sup> After injury or infection, microglial cells became active.<sup>20-23</sup> Furthermore, activated microglial cells are believed to contribute to the degenerative events observed in photoreceptor degenerations.<sup>24-26</sup> Indeed, we recently described that microglial activation starts simultaneously with the beginning of photoreceptor death in the RCS and P23H-1 rat strains.<sup>27</sup>

In various animal models of retinal degeneration, morphologic and/or functional rescue of photoreceptors has been documented after treatment with different trophic factors such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), pigment epithelium-derived factor (PEDF), and basic fibroblast growth factor (FGF2)<sup>28-32</sup> or substances that inhibit microglial cells, like minocycline.<sup>26,33</sup> The therapeutic doses of these substances have been previously established.<sup>28,32-38</sup> The question arises whether the combination of trophic factors with minocycline, substances that increase survival presumably through different mechanisms,





**FIGURE 1.** Experimental design. P23H-1 and RCS rats were divided in three groups that received (1) one intravitreal injection of trophic factor or vehicle; (2) daily intraperitoneal injections of minocycline or vehicle; and (3) one intravitreal injection of bFGF (FGF2) and daily intraperitoneal injections of minocycline. Two intraperitoneal injections were administered the first day. *Black line:* Treatment period. P, postnatal days when treatments (*syringes*) or processing (*open circle*) were performed. *n* = number of animals in each group.

could further increase photoreceptor survival and therefore be used for the treatment of retinal degenerations.

In this study, we analyze in the P23H line 1 (P23H-1) and RCS rat strains the effects of (1) different neurotrophic factors, (2) minocycline, or (3) a combination of the neurotrophic factor that gave best results (FGF2) and minocycline on photoreceptor survival and microglial cell activation during the period of early photoreceptor degeneration.

**MATERIALS AND METHODS**

**Animals and Experimental Groups**

Homozygous albino female P23H-1, homozygous pigmented RCS rats, and their healthy controls albino Sprague-Dawley (SD) and pigmented Piebald Virol Glaxo (PVG) rats were used in this work. Transgenic homozygous P23H-1 animals were obtained from M. LaVail (University of California at San

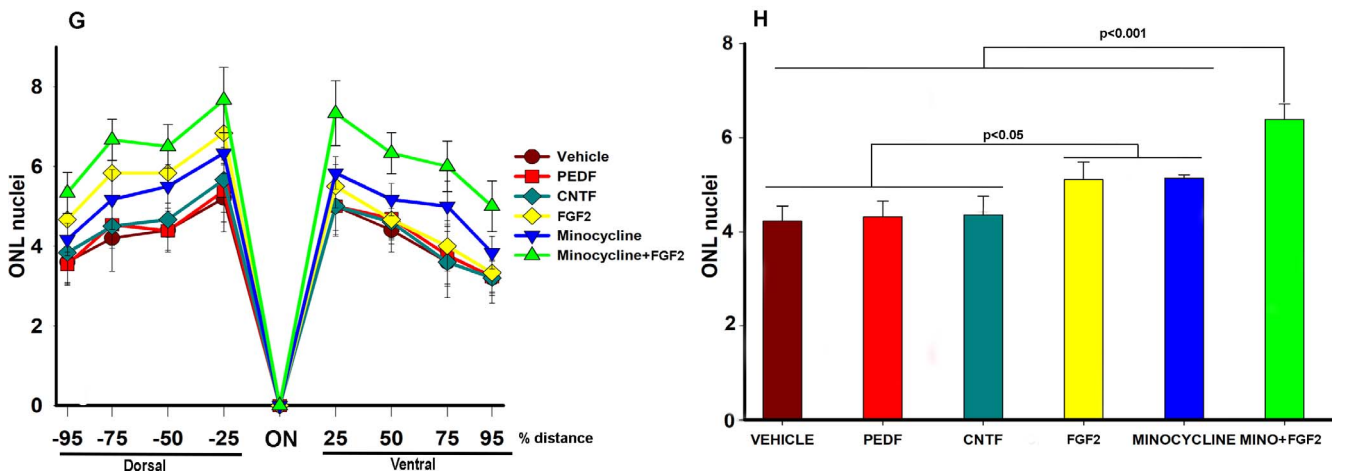
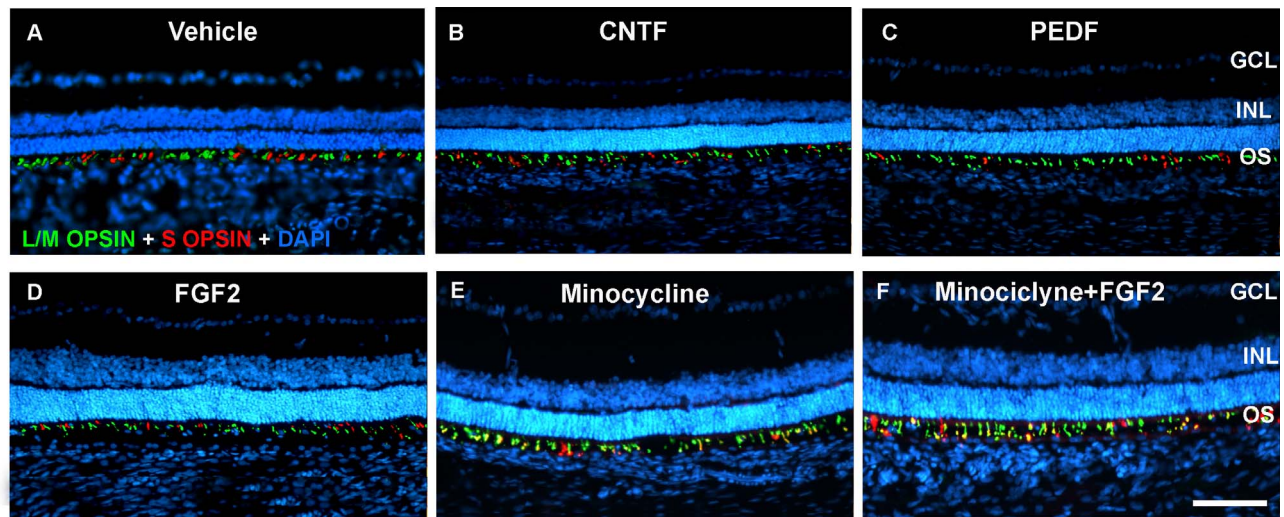


FIGURE 2. S- and L-cone outer segment morphology and nuclei quantification in P21 P23H-1 animals. Photomicrographs of representative retinal cross sections taken from dystrophic animals treated with vehicle (A) CNTF (B), PEDF (C), FGF2 (D), minocycline (E), or the combination of minocycline and FGF2 (F) showing S- (red) and L/M- (green) opsin immunoreactivity and DAPI counterstaining (blue). (G, H) Graphs showing mean numbers  $\pm$  SD of nuclei rows in the ONL of dystrophic animals treated with vehicle, different neurotrophic factors, minocycline, or combination of minocycline and FGF2. Retinas treated with FGF2, minocycline, or its combination showed a higher number of nuclei rows in the ONL. Scale bar: 100  $\mu$ m. One-way ANOVA:  $F(6,41) = 222.843$ ;  $P < 0.001$ .

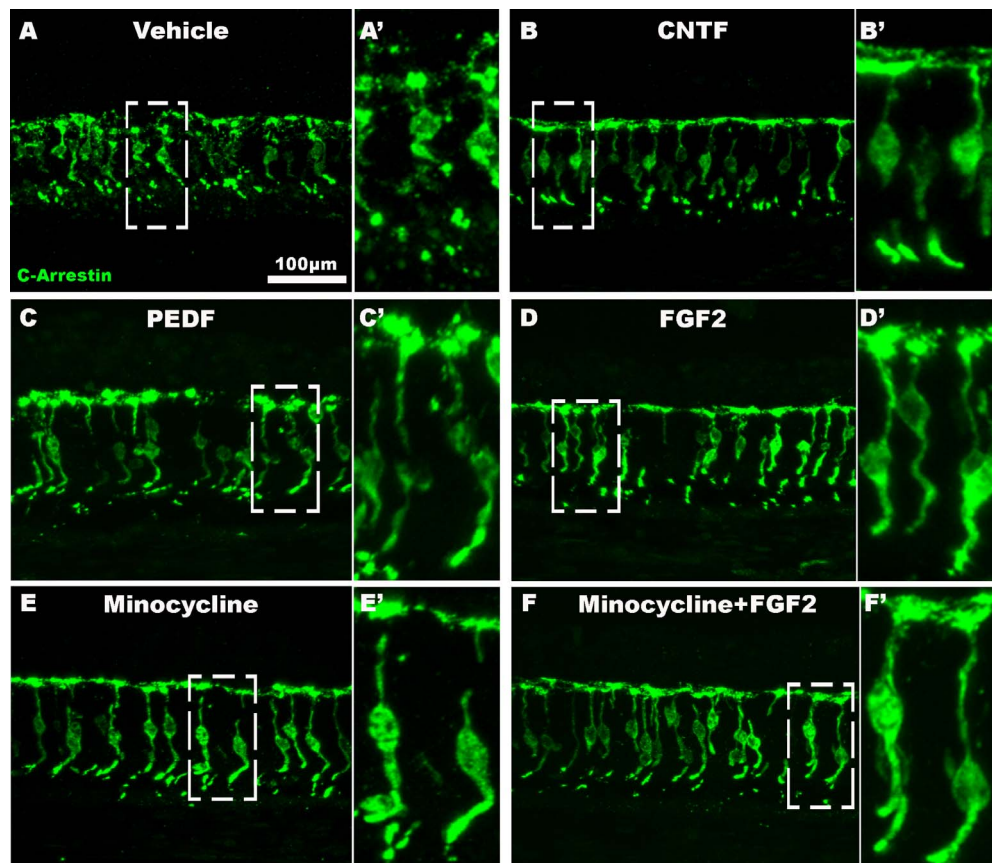
Francisco School of Medicine; [http://www.ucsfeye.net/mla\\_vailRDratmodels.shtml](http://www.ucsfeye.net/mla_vailRDratmodels.shtml); Steinberg RH, et al. *IOVS* 1996;37:AR-VO Abstract 3190), and bred at the University of Murcia; RCS, SD, and PVG rats were obtained from the breeding colony of the University of Murcia, Murcia, Spain. Rats were housed in temperature- and light-controlled rooms with a 12-hour light/dark cycle (light from 8 AM to 8 PM) and had food and water ad libitum. Animal manipulations were carried out following the Spanish and European Union regulations for the use of animals in research (Council Directive 86/609/EEC), the indication of the University of Murcia ethical committee, and the ARVO statement for the use of animals in ophthalmic and vision research.

P23H and RCS rats were divided into different experimental groups (Fig. 1). In the first experimental group, the animals received an intravitreal injection (IVI) of one of the following neurotrophic factors: CNTF, PEDF, or FGF2 ( $n = 6$ /neurotrophic factor) at postnatal day (P)10 or P33 for P23H-1 or RCS rats, respectively. These time points were chosen because in a previous work<sup>27</sup> we showed that photoreceptor death starts at

P15 and is maximal between P15 and P21 in P23H rats and starts at P33 and is maximal between P33 and P45 in RCS rats. Another experimental group received daily intraperitoneal injections of minocycline hydrochloride (M9511; Sigma-Aldrich, Alcobendas, Madrid, Spain), from P9 or P32 for P23H-1 or RCS rats, respectively. Finally, another group of animals was treated with a combination of both treatments: daily intraperitoneal injections of minocycline and one IVI of FGF2. P23H-1 animals were processed at P21, and RCS animals were processed at P45. Age-matched groups of P23H-1 and RCS rats treated with intravitreal or intraperitoneal injections of vehicle were used as controls. Additional controls were untreated SD and PVG rats.

### IVIs

IVIs were performed only in the left eye following previously described methods that are standard in our laboratory.<sup>39</sup> Briefly, the injections were made through the supratemporal sclera using a Hamilton microsyringe (26G; Hamilton 701 N,



**FIGURE 3.** Cone morphology in P23H-1 animals. Representative retinal cross sections taken from dystrophic animals treated with vehicle (A), CNTF (B), PEDF (C), FGF2 (D), minocycline (E), or the combination of minocycline and FGF2 (F) showing cones labeled with arrestin (green). Insets show magnified images of the boxed areas in each image.

Es slab, Benfleet, UK). Once the needle was visualized retrolenticularly, the liquid was injected and the needle was removed.

The following quantities of neurotrophic factors were injected: (1) 0.4 µg CNTF (R&D Systems, Vitro S.A. Madrid, Spain), 2 µg PEDF (Preprotech, London, UK), both diluted in PBS, or 1 µg FGF2 (Preprotech) diluted in Tris-Cl 2 mM, pH 7.6. Matched groups of P23H-1 and RCS rats were treated with the corresponding vehicle solutions. These doses were used following previously published works.<sup>28,32,36-38</sup> The injection volume was always 5 µL.

### Intraperitoneal Minocycline

Minocycline (45 mg/kg) or vehicle (saline, NaCl 0.9%) intraperitoneal injections were administered twice the first day and daily for the remaining days. This dose was used following previously published results.<sup>33-35</sup> For the group of animals that received the combined treatment, the first dose of minocycline was given 1 day before the IVI.

### Tissue Processing

Rats were first sedated by an intraperitoneal injection of diluted sodium pentobarbital (1:1 in saline; Doletal Vetoquinol, S.A., Lure, France) and then killed with a lethal dose of sodium pentobarbital. Then, rats were perfused transcardially through the ascending aorta, first with saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Finally,

all the retinas were prepared for cryostat cross sections following previously described methods.<sup>27,40,41</sup>

### Immunohistochemistry

Immunohistochemistry staining was performed on retinal whole mounts following standard procedures in our laboratory.<sup>27</sup> Briefly, cross sections were washed in PBS containing 0.1% Triton X-100 (Tx; Sigma-Aldrich) and incubated overnight at 4°C with a mixture of the primary antibodies (see next paragraph) diluted in blocking buffer (PBS containing 2% Tx and 2% normal donkey serum). After washing with PBS-0.1%Tx, the sections were incubated for 1 hour at room temperature with a mixture of the secondary antibodies (see next paragraph) diluted in PBS-2%Tx. Sections were washed again with PBS-0.1%Tx and, after a rinse in PBS, mounted with a mounting media containing DAPI (4',6-diamidino-2-phenylindole; Vectashield Mounting Medium con DAPI; Vector Atom, Alicante, Spain) to counterstain all retinal nuclei.

### Antibodies

**Primary Antibodies.** Microglial cells were detected with rabbit anti-Iba1 antibody (1:1000, 019-19741; Wako Chemicals, Neuss, Germany). L- and S-cone outer segments were detected with rabbit anti-red/green opsin (1:1200, AB5405; Chemicon-Millipore Iberica, Madrid, Spain) and goat anti-blue opsin (1:1000, N-20; OPN1SW; Santa Cruz Biotechnology, Heidelberg, Germany), respectively. Cones outer and inner segments

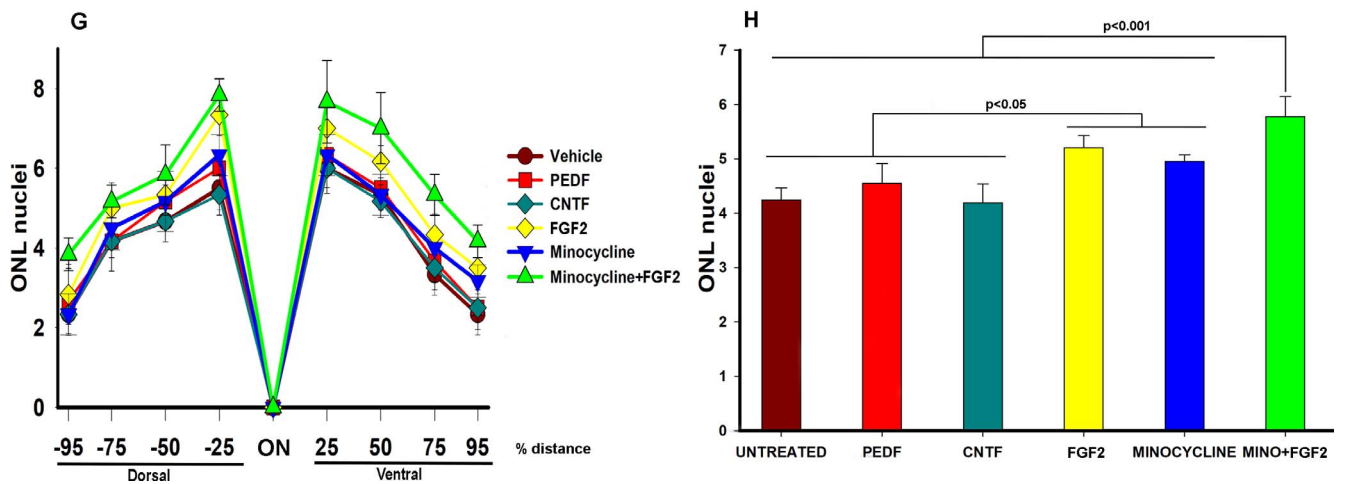
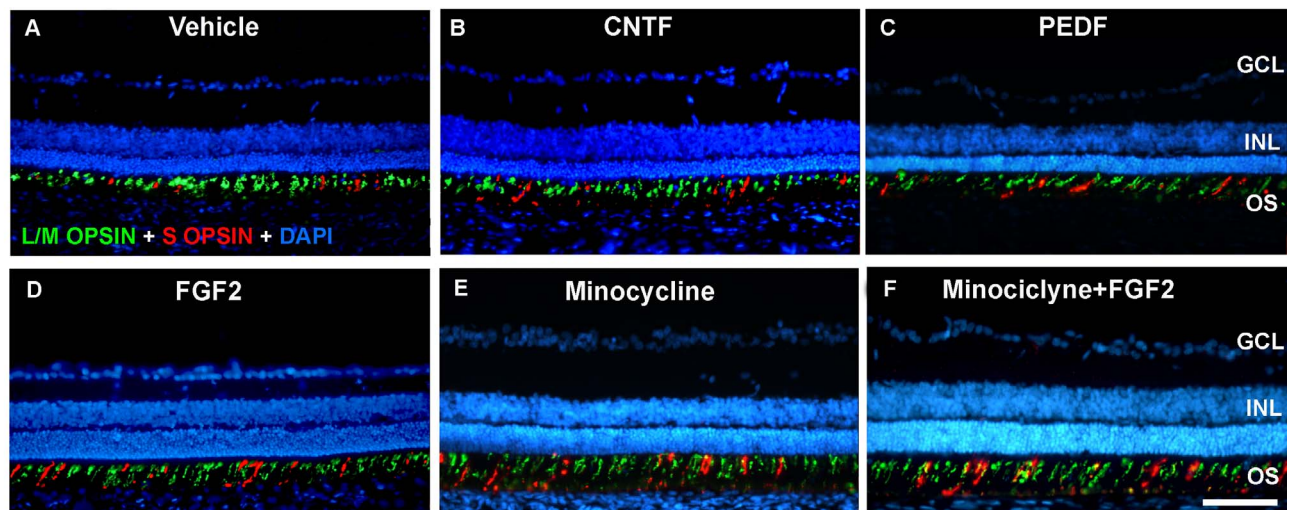


FIGURE 4. S- and L-cone outer segment morphology and nuclei quantification in P45 RCS animals. Photomicrographs of representative retinal cross sections taken from dystrophic animals treated with vehicle (A), CNTF (B), PEDF (C), FGF2 (D), minocycline (E), or combination of minocycline and FGF2 (F) showing S- (red) and L/M- (green) opsin immunoreactivity and DAPI counterstaining (blue). (G, H) Graphs showing mean numbers  $\pm$  SD of nuclei rows in the ONL of dystrophic animals treated with vehicle, different neurotrophic factors, minocycline, or combination of minocycline and FGF2. Retinas treated with FGF2, minocycline, or its combination showed a higher number of nuclei rows in the ONL. Scale bar: 100  $\mu$ m. One-way ANOVA:  $F(6,41) = 364.792$ ,  $P < 0.001$ .

were immunodetected with rabbit anti-cone arrestin (1:1000, AB15282; Chemicon-Millipore Iberica).

**Secondary Antibodies.** For secondary detection, two antibodies were used: Donkey anti-goat Alexa Fluor 594 and donkey anti-rabbit Alexa Fluor 488, both purchased from Molecular Probes (Invitrogen, ThermoFisher, Madrid, Spain) and diluted at 1:500.

**Image Analysis**

Image analysis was performed following previously described methods.<sup>27</sup> Briefly, retinal cross sections spanning the optic disk were selected (three cross sections/retina and antibody combination;  $n = 6$  retinas/group). Samples were examined and photographed under a fluorescence microscope (Axioscop 2 Plus; Zeiss Mikroskopie, Jena, Germany) equipped with various filters and a digital high-resolution camera (ProgRes C10; Jenoptik, Jena, Germany).

Eight images (530  $\times$  390  $\mu$ m) were taken per retinal section: four from the dorsal (superior) retina and four from the ventral

(inferior) retina. Images were acquired at equidistant distances from the optic disk to the retinal periphery (25%, 50%, 75%, and 95%, considering 100% the length of each hemi-retina). Images were further processed with Adobe Photoshop CS 6 (Adobe Systems, Inc., San Jose, CA, USA) when needed.

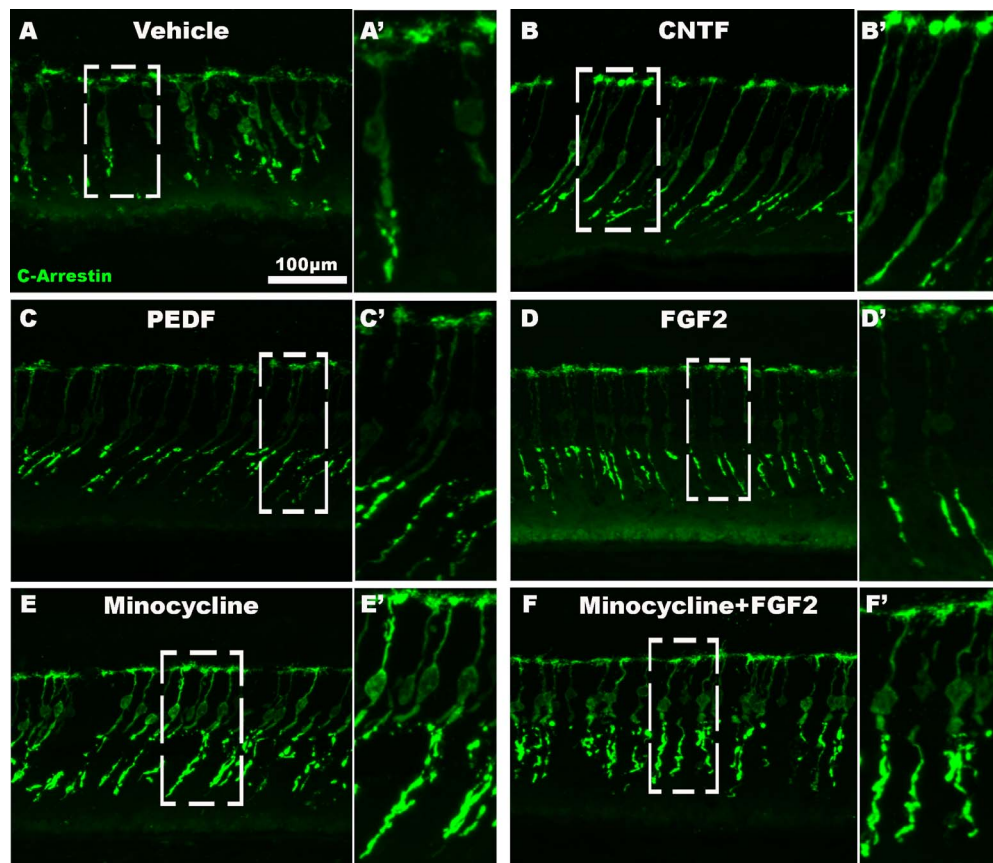
Some photographs of the cross sections were also taken with confocal microscope Leica SP8 (Leica Microsystems, Wetzlar, Germany).

**Nuclei Rows and Microglial Cell Quantification**

The number of nuclei rows in the outer nuclear layer (ONL) and the number of microglial cells in each retinal layer were manually quantified in each image<sup>27</sup> ( $n = 3$  retinal sections from six different retinas per time point and experimental group).

**Statistics**

The number of nuclei rows in the ONL and the number of microglial cells were compared among retinal regions and



**FIGURE 5.** Cone morphology in RCS animals. Representative retinal cross sections taken from dystrophic animals treated with vehicle (A), CNTF (B), PEDF (C), FGF2 (D), minocycline (E), or the combination of minocycline and FGF2 (F) showing cones labeled with arrestin (green). Insets show magnified images of the boxed areas in each image.

groups. Statistical analysis was carried out using SigmaStat 3.11 for Windows (Systat Software, Inc., Richmond, CA, USA). The Student's *t*-test was used to compare between two different groups (i.e., different strains following the same treatments) and 1-way ANOVA (followed by Tukey's post hoc test) was used to compare several groups (i.e., different treatments on the same strain). Differences were considered significant when  $P \leq 0.05$ .

## RESULTS

### Untreated P23H-1 and RCS Retinas

P23H-1 and RCS animals were processed at P22 and P45, respectively (Fig. 1). At this age, there were no significant differences in photoreceptor loss between the untreated or vehicle-treated animals. Photoreceptor degeneration had started in both groups; cones showed shortened outer segments and the mean number of nuclei rows in the ONL were significantly reduced to four to six or two to four rows in the P23H-1 (Figs. 2, 3) or RCS (Figs. 4, 5) compared with 12 to 14 rows in controls (SD, PVG rats; data not shown). In addition, we could observe activated retinal microglial cells, with amoeboid morphology and migration of these cells from their normal location to the photoreceptors layers: ONL and outer segment layer (Figs. 6, 7), in accordance with previously published data.<sup>27</sup>

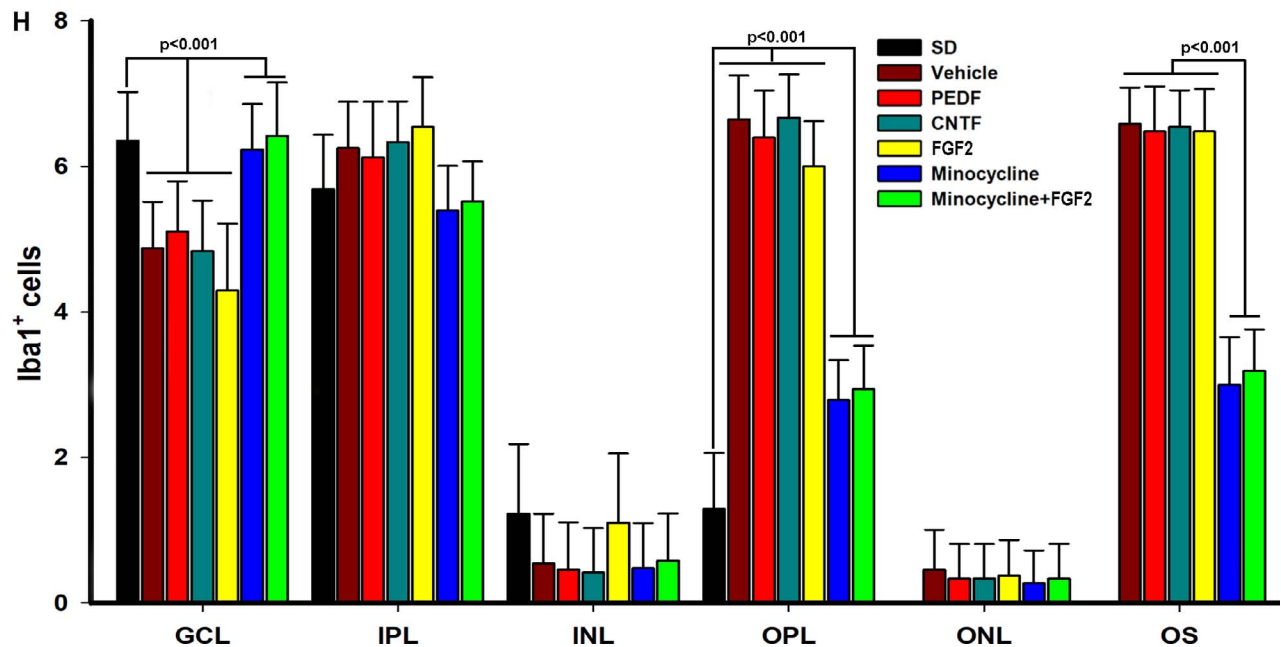
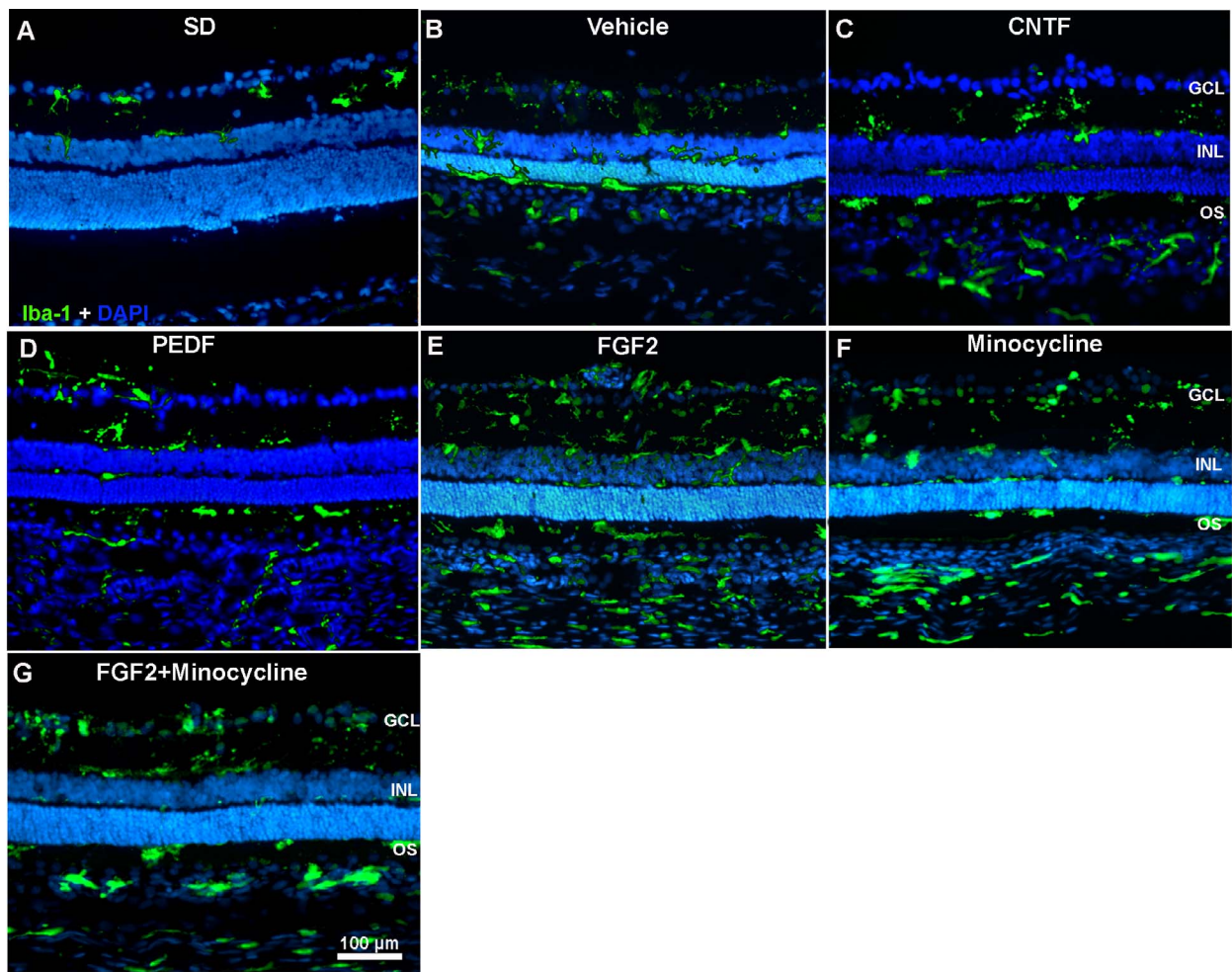
### Efficacy of CNTF, PEDF, or FGF2

CNTF, PEDF, or FGF2 was administered intravitreally at P10 (P23H) or P33 (RCS), and the retinas were examined 12 days later. These three factors improved the morphology of the photoreceptors outer segment compared with the vehicle-treated retinas (Figs. 2A–2D, 4A–4D). The outer segment of the photoreceptors appeared straightened, longer, and more strongly labeled with arrestin in the trophic factor-treated retinas, both in P23H-1 rats and RCS rats (Figs. 3, 5). However, despite the observed morphologic rescue, only FGF2 produced a significant increase in the mean number of nuclei rows in the ONL (Figs. 2G, 2H, 4G, 4H, 6E) in the dystrophic animals, and this effect was more pronounced in the dorsal retina in the P23H strain (Fig. 2G). When expressed as percentages of increased survival in relation to vehicle-treated animals, FGF2 increased +21% or +23% the number of nuclei rows in P23H-1 or RCS rats (Fig. 8), respectively.

None of the trophic factors had an effect on the numbers of microglial cells or on their migration, as their numbers in the different retinal layers were similar to those observed in vehicle-treated P23H and RCS rats (Figs. 6H, 7H).

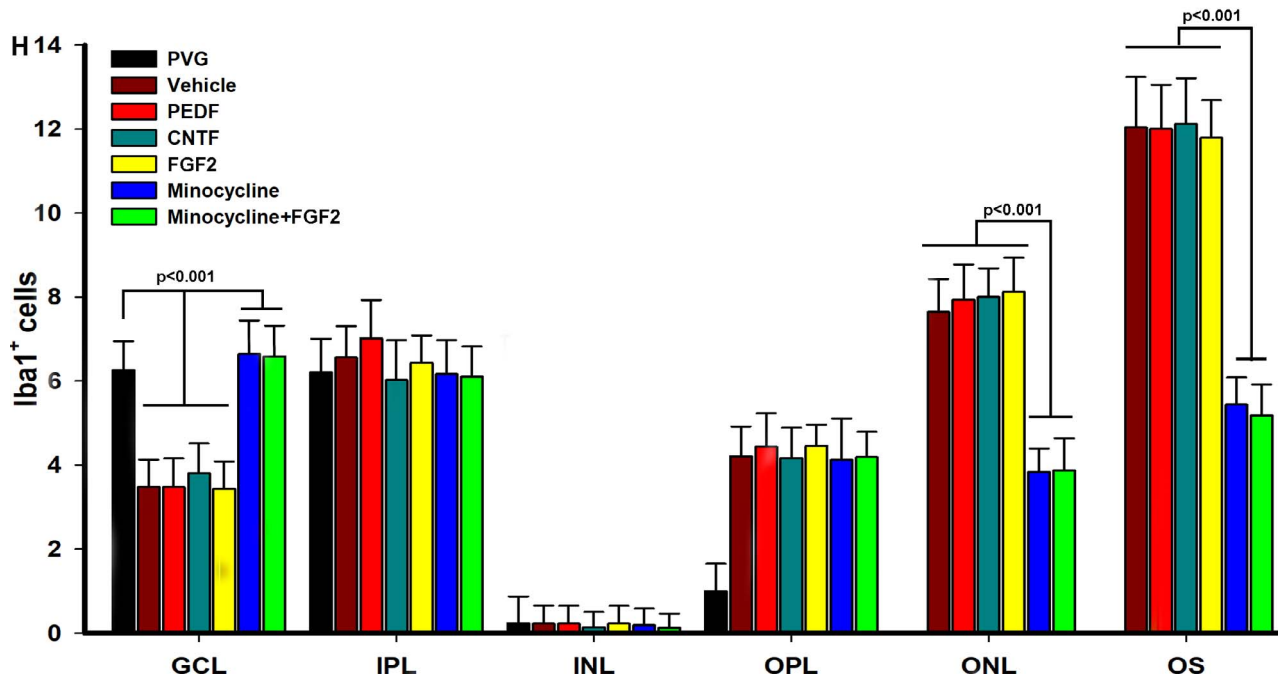
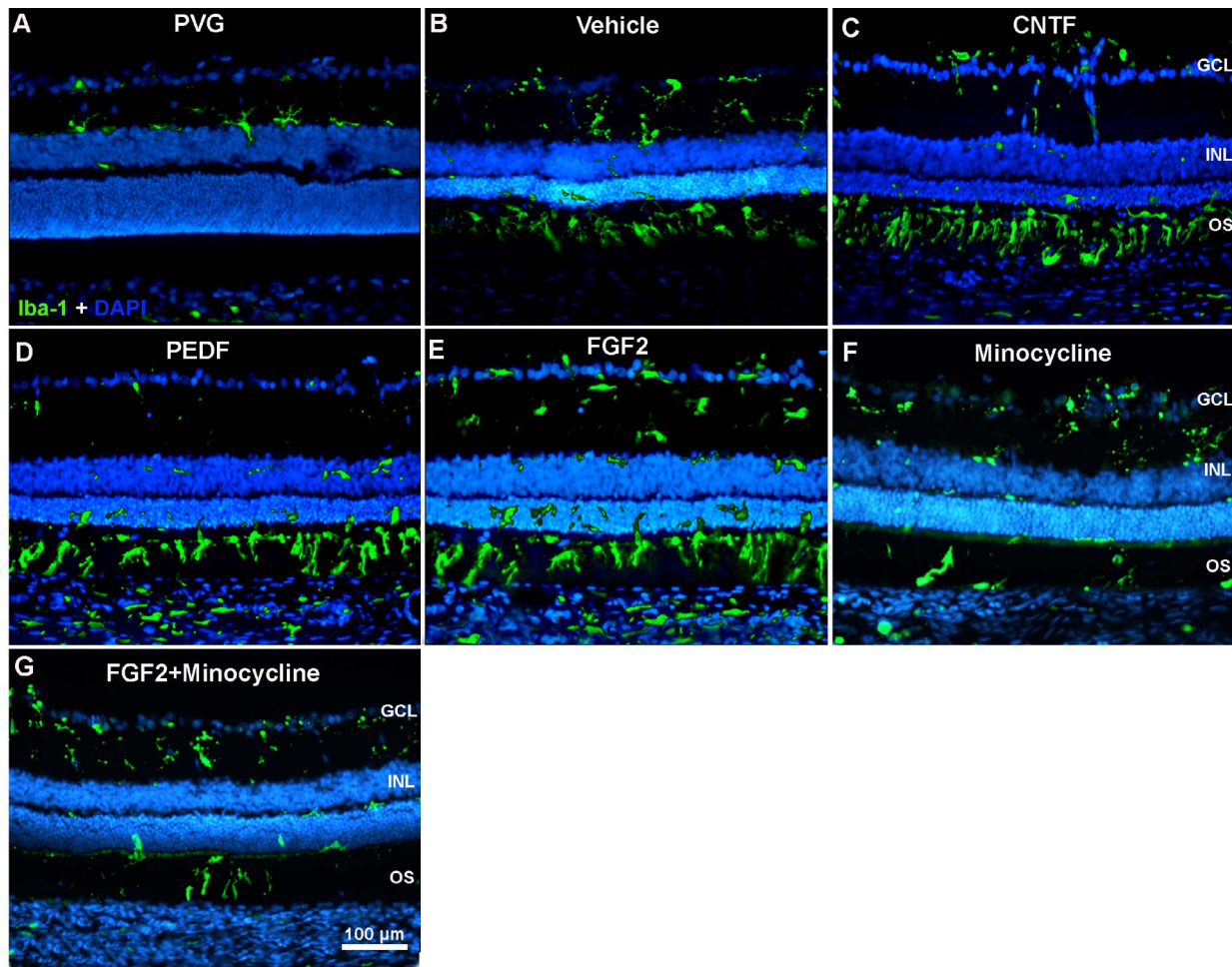
### Efficacy of Minocycline

In both dystrophic strains, intraperitoneal treatment with minocycline induced an important reduction of the microglial cell activation and migration (Figs. 6F, 6H, 7E, 7H). Indeed, compared with vehicle- or trophic factor-treated retinas, the



**FIGURE 6.** Microglial cell expression and quantification in P23H-1 rats. Photomicrographs of representative retinal cross sections taken from SD rats (A) and P23H-1 rats treated with vehicle (B) CNTF; (C), PEDF (D), FGF2 (E), minocycline (F), or combination of minocycline and FGF2 (G) showing microglial cells (green) and DAPI counterstaining (blue). (H) Graph showing mean number  $\pm$  SD of microglial cells counted in each retinal layer in control naïve Sprague-Dawley (SD) rats and in dystrophic animals treated with vehicle, different neurotrophic factors, minocycline, or combination of both. In the animals treated with minocycline (alone or in combination with FGF2), the mean number of microglial cells observed in the outer retinal layers was significantly lower than that observed in the animals treated with vehicle, CNTF, PEDF or FGF2. However, for the retinal ganglion

cell layer, the mean number of microglial cells observed was higher in the animals treated with minocycline (alone or in combination with FGF2). One-way ANOVA: for the retinal ganglion cell layer,  $F(4,29) = 29.698, P < 0.001$ ; for the ONL,  $F(4,29) = 10.809, P < 0.001$ ; for the outer segment layer,  $F(4,29) = 1834.715, P < 0.001$ .





**FIGURE 7.** Microglial cell expression and quantification in RCS rats. Photomicrographs of representative retinal cross sections taken from PVG rats (A) and RCS rats treated with vehicle (B), CNTF (C), PEDF (D), FGF2 (E), minocycline (F), or combination of minocycline and FGF2 (G) showing microglial cells (green) and DAPI counterstaining (blue). (H) Graph showing mean number  $\pm$  SD of microglial cells counted in each retinal layer in control naïve Sprague-Dawley (SD) rats and in dystrophic animals treated with vehicle, different neurotrophic factors, minocycline, or combination of both. In the animals treated with minocycline (alone or in combination with FGF2), the mean number of microglial cells observed in the outer retinal layers was significantly lower than that observed in the animals treated with vehicle, CNTF, PEDF, or FGF2. However, for the retinal ganglion cell layer, the mean number of microglial cells observed was higher for the animals treated with minocycline (alone or in combination with FGF2). One-way ANOVA: for the retinal ganglion cell layer,  $F(4,29) = 103.856, P < 0.001$ ; for the ONL,  $F(4,29) = 518.717, P < 0.001$ ; for the outer segment layer,  $F(4,29) = 883.111, P < 0.001$ .

mean numbers of microglial cells in the minocycline group were significantly higher in the GCL and significantly lower in the ONL and outer segment layer (Figs. 6H, 7H), indicating less microglial cell migration from the inner to the outer retina.

In addition to its effect on microglial dynamics, minocycline treatment protected photoreceptors, with a significant rescue of the nuclei rows of +22% in P23H-1 and +17% in RCS rats compared with vehicle-treated animals (Fig. 8). This rescue was similar to that observed when the animals were treated with FGF2 (see above).

**Efficacy of FGF2 and Minocycline Combined Treatment**

Finally, we analyzed the animals treated with FGF2 and minocycline to assess whether their combined neuroprotective effect was additive. As expected, the effect of the combination of treatments on the photoreceptors outer segment morphology and microglial modulation was similar to the corresponding single treatments, FGF2 and minocycline, respectively (compare Figs. 3D, 3E, 5D, 5E, 6E, 6F, 7E, 7F with Figs. 3F, 5E, 6G, 7G, respectively). However, both treatments together induced a significantly higher photoreceptor neuroprotection than each treatment alone; for instance, the increase of nuclei rows was +30% in P23H-1 and +15% in RCS rats compared with FGF2 alone, and +29% in P23H-1 and +12% in RCS rats compared with minocycline alone (Fig. 8). Compared with

vehicle-treated animals, this increase reached +51% in P23H-1 rats and +38% in RCS rats, an increased neuroprotection that was greater than that obtained by each treatment alone (Fig. 8).

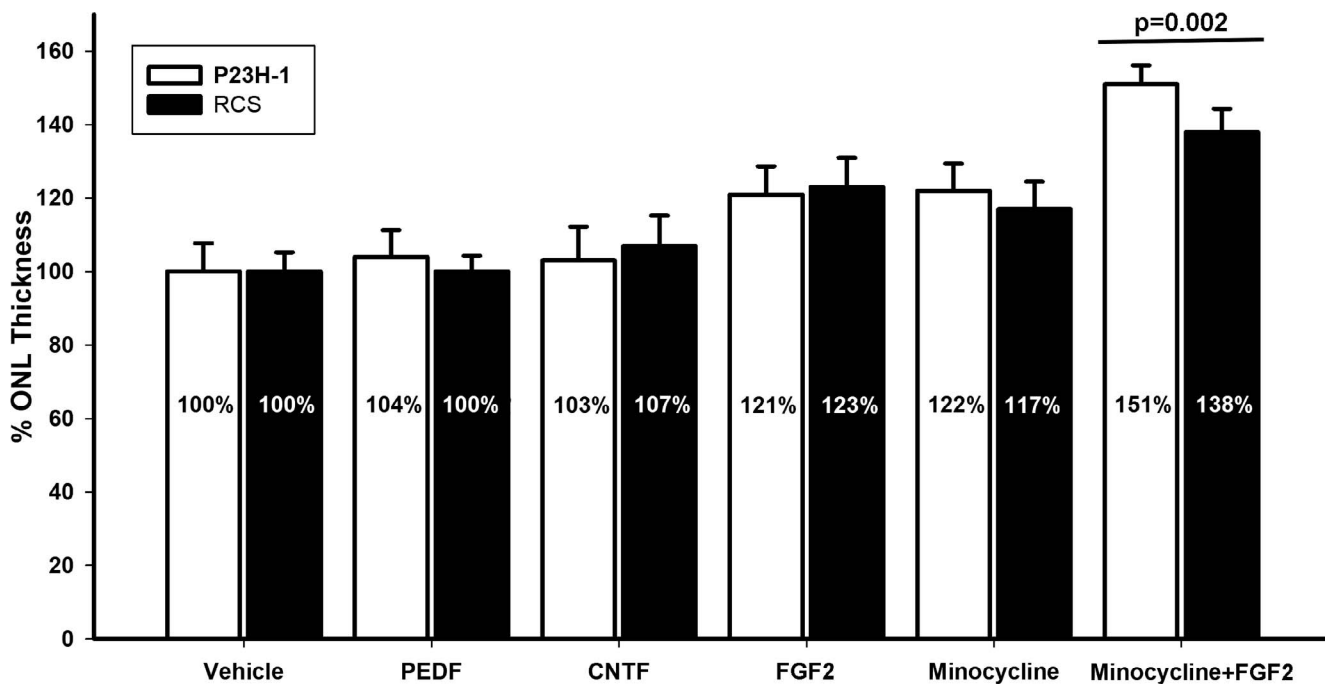
**Rescue Effects in P23H-1 and RCS Rats**

As stated in the previous paragraphs, the different neurotrophic factors, minocycline or the combination of FGF2 and minocycline, increased photoreceptor survival both in P23H-1 rats and RCS rats. However, the combination of FGF2 and minocycline produced significantly more photoreceptor survival in P23H-1 rats than in RCS rats (Fig. 8).

**DISCUSSION**

In this work, we studied the effect on photoreceptor survival and microglial behavior of different neurotrophic factors and minocycline either alone or in combination during the early period of photoreceptor degeneration in two different animal models of inherited retinal degeneration.

Because the loss of photoreceptors is irrecoverable, strategies to rescue them depend on the number of remaining photoreceptors,<sup>42</sup> and therefore, patients should be treated in the early stages of these progressive diseases. Early therapies may delay the progression of photoreceptor loss and in doing so prevent the retinal remodeling that occurs following photoreceptor degeneration.<sup>40,43-45</sup> This is of great impor-



**FIGURE 8.** Percentage of effectiveness of each treatment studied. Graph showing in percentage the increase of nuclei in ONL and, consequently, photoreceptor survival as a measure to evaluate the effectiveness of each treatment. Combination of minocycline and FGF2 produces an additive neuroprotective effect of +51% in P23H-1 and +38% RCS rats. Student's *t*-test,  $P < 0.05$ .

tance, because most therapeutic strategies for RP (i.e., retinal prosthesis or cell transplantation) are based on the assumption that the retina remains viable after photoreceptor degeneration.

Trophic factors are small proteins with essential functions such as promoting proliferation, growth, regeneration, maturation, or neuronal survival.<sup>46</sup> Several studies have shown their role in the prevention of photoreceptor degeneration.<sup>14,28–32,47</sup> Our results showed that IVI of neurotrophic factors (CNTF, PEDF, or FGF2) produced a morphologic rescue of the photoreceptors outer segment, both in the P23H-1 and in the RCS rats, indicating that neurotrophic factors have a neuroprotective effect that is independent of the etiology of the degeneration (rhodopsin mutation or ERP malfunction). However, only FGF2 treatment produced a significant rescue in the mean number of nuclei rows in the ONL. This is in accordance with previous studies showing that treatment with CNTF or PEDF rescues photoreceptor morphology but does not improve the electroretinographic responses.<sup>29–31,48</sup> On the other hand, several authors have shown that FGF2 not only rescues photoreceptor morphology, but also increases photoreceptor survival, reducing apoptosis, and ameliorates the ERG responses<sup>28,49–51</sup> in accordance with our results.

Microglial activation is an early event of retinal degenerations,<sup>26,27,52</sup> and thus modulating these cells could be a potential treatment to provide photoreceptor neuroprotection. Conversely, some studies have suggested that microglia is required to promote photoreceptor survival.<sup>52,53</sup> Indeed, different roles (neuroprotective and neurodestructive) have been proposed for resident, activated, and migrating microglia.<sup>54,55</sup> Nevertheless, it is thought that the neuroinflammation triggered by the primary loss of rods plays a major role in the secondary cone loss, the most severe consequence of RP.<sup>53,55</sup>

Minocycline (a microglial inhibitor) is widely used to study neuroprotection in central nervous system diseases.<sup>33,56–59</sup> Minocycline is a broad-spectrum tetracycline antibiotic, and previous studies have demonstrated that minocycline has antimicrobial, anti-inflammatory, antiapoptotic, and neuroprotective properties.<sup>33,52,60</sup> The neuroprotective effect of minocycline against photoreceptor apoptosis in retinal degenerations is explained by mainly two different mechanisms: (1) a direct antiapoptotic effect on photoreceptors<sup>61</sup> and/or (2) an effect through its anti-inflammatory properties.<sup>26,33,62,63</sup> Here we show that minocycline administration significantly reduces microglial activation and migration, preserves retinal structure by maintaining the morphology of the photoreceptors outer segments, and neuroprotects these neurons. Thus, our results support the abovementioned hypothesis that microglia have a role in the course of retinal degenerations, and thus substances that modulate microglial reactivity could be potential candidates for the treatment of these diseases.

The therapeutic doses of FGF2 and minocycline used in this study have been shown to increase photoreceptor<sup>28,32,33,35</sup> or neuronal<sup>34</sup> survival in previous studies. In these studies, increasing the dose of the substances did not significantly augment photoreceptor<sup>28,36</sup> or neuronal<sup>34</sup> survival. Also, these doses did not cause significant side effects.<sup>28,32–36,49–51</sup> One previous article, however, documented that the dose FGF2 used in this study may cause cataract formation and increased retinal vascularization,<sup>36</sup> but we have not observed any side effects.

In this work, we show that the combined treatment with FGF2 and minocycline elicited a photoreceptor rescue (morphology and number of nuclei in the ONL) that was higher than that observed with the individual treatments. Because previous studies have shown that increasing the dose of the substances does not increase survival,<sup>28,34,36</sup> our results suggest that the observed improvement is due to the combined effect of the two treatments acting through different mecha-

nisms. Both treatments have an effect on the apoptotic pathway,<sup>28,33,37,61</sup> but only minocycline has a direct effect in microglial cells.<sup>33,60</sup>

In summary, our study provides strong support to the concepts that, in retinal degenerations, (1) CNTF, PEDF, and FGF2 rescue the photoreceptor outer segment morphology and FGF2 increases photoreceptor survival; (2) minocycline reduces microglial activation and migration and promotes photoreceptor survival; and finally, (3) the combination of FGF2 and minocycline treatments has an additive effect. Thus, here we propose that anti-inflammatory agents (i.e., minocycline) as an adjuvant therapy in combination with neurotrophic factors could be the best choice as potential treatments in the earlier stages of retinal degenerations.

### Acknowledgments

Supported by grants from Fundación Séneca, Agencia de Ciencia y Tecnología Región de Murcia (19881/GERM/15), Spanish Ministry of Economy and Competitiveness, Instituto de Salud Carlos III, Fondo Europeo de Desarrollo Regional “Una Manera de Hacer Europa” (SAF2015-67643-P, PI16/00380, RD16/0008/0026, PI16/00031).

Disclosure: **J. Di Pierdomenico**, None; **R. Scholz**, None; **F.J. Valiente-Soriano**, None; **M.C. Sánchez-Migallón**, None; **M. Vidal-Sanz**, None; **T. Langmann**, None; **M. Agudo-Barriuso**, None; **D. García-Ayuso**, None; **M.P. Villegas-Pérez**, None

### References

- Hartong DT, Berson EL, Dryja TP. Retinitis pigmentosa. *Lancet*. 2006;368:1795–1809.
- Swaroop A, Sieving PA. The golden era of ocular disease gene discovery: race to the finish. *Clin Genet*. 2013;84:99–101.
- van Soest S, Westerveld A, de Jong PT, Bleeker-Wagemakers EM, Bergen AA. Retinitis pigmentosa: defined from a molecular point of view. *Surv Ophthalmol*. 1999;43:321–334.
- LaVail MM. Analysis of neurological mutants with inherited retinal degeneration. Friedenwald lecture. *Invest Ophthalmol Vis Sci*. 1981;21:638–657.
- Slijkerman RW, Song F, Astuti GD, et al. The pros and cons of vertebrate animal models for functional and therapeutic research on inherited retinal dystrophies. *Prog Retin Eye Res*. 2015;48:137–159.
- Veleri S, Lazar CH, Chang B, et al. Biology and therapy of inherited retinal degenerative disease: insights from mouse models. *Dis Model Mech*. 2015;8:109–129.
- LaVail MM, Yasumura D, Matthes MT, et al. Ribozyme rescue of photoreceptor cells in P23H transgenic rats: long-term survival and late-stage therapy. *Proc Natl Acad Sci U S A*. 2000;97:11488–11493.
- Lewin AS, Drenser KA, Hauswirth WW, et al. Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa. *Nat Med*. 1998;4:967–971.
- Machida S, Kondo M, Jamison JA, et al. P23H rhodopsin transgenic rat: correlation of retinal function with histopathology. *Invest Ophthalmol Vis Sci*. 2000;41:3200–3209.
- Steinberg RH. Survival factors in retinal degenerations. *Curr Opin Neurobiol*. 1994;4:515–524.
- García-Ayuso D, Ortin-Martínez A, Jiménez-López M, et al. Changes in the photoreceptor mosaic of P23H-1 rats during retinal degeneration: implications for rod-cone dependent survival. *Invest Ophthalmol Vis Sci*. 2013;54:5888–5900.
- Duncan JL, LaVail MM, Yasumura D, et al. An RCS-like retinal dystrophy phenotype in mer knockout mice. *Invest Ophthalmol Vis Sci*. 2003;44:826–838.

13. Parinot C, Nandrot EF. A comprehensive review of mutations in the MERTK proto-oncogene. *Adv Exp Med Biol.* 2016;854:259-265.
14. Terubayashi H, Murabe Y, Fujisawa H, Itoi M, Iyata Y. Enzymohistochemical identification of microglial cells in the rat retina: light and electron microscopic studies. *Exp Eye Res.* 1984;39:595-603.
15. Boya J, Calvo J, Carbonell AL. Appearance of microglial cells in the postnatal rat retina. *Arch Histol Jpn.* 1987;50:223-228.
16. Ashwell KW, Hollander H, Streit W, Stone J. The appearance and distribution of microglia in the developing retina of the rat. *Vis Neurosci.* 1989;2:437-448.
17. Thanos S. Sick photoreceptors attract activated microglia from the ganglion cell layer: a model to study the inflammatory cascades in rats with inherited retinal dystrophy. *Brain Res.* 1992;588:21-28.
18. Salvador-Silva M, Vidal-Sanz M, Villegas-Perez MP. Microglial cells in the retina of *Carassius auratus*: effects of optic nerve crush. *J Comp Neurol.* 2000;417:431-447.
19. Sobrado-Calvo P, Vidal-Sanz M, Villegas-Perez 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.
20. de Hoz R, Gallego BI, Ramirez AI, et al. Rod-like microglia are restricted to eyes with laser-induced ocular hypertension but absent from the microglial changes in the contralateral untreated eye. *PLoS One.* 2013;8:e83733.
21. de Hoz R, Ramirez AI, Gonzalez-Martin R, et al. Bilateral early activation of retinal microglial cells in a mouse model of unilateral laser-induced experimental ocular hypertension. *Exp Eye Res.* 2018;171:12-29.
22. Rojas B, Gallego BI, Ramirez 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.
23. 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.
24. Gupta N, Brown KE, Milam AH. Activated microglia in human retinitis pigmentosa, late-onset retinal degeneration, and age-related macular degeneration. *Exp Eye Res.* 2003;76:463-471.
25. Langmann T. Microglia activation in retinal degeneration. *J Leukoc Biol.* 2007;81:1345-1351.
26. Zhao L, Zabel MK, Wang X, et al. Microglial phagocytosis of living photoreceptors contributes to inherited retinal degeneration. *EMBO Mol Med.* 2015;7:1179-1197.
27. Di Pierdomenico J, Garcia-Ayuso D, Pinilla I, et al. Early events in retinal degeneration caused by rhodopsin mutation or pigment epithelium malfunction: differences and similarities. *Front Neuroanat.* 2017;11:14.
28. Faktorovich EG, Steinberg RH, Yasumura D, Matthes MT, LaVail MM. Photoreceptor degeneration in inherited retinal dystrophy delayed by basic fibroblast growth factor. *Nature.* 1990;347:83-86.
29. Green ES, Rendahl KG, Zhou S, et al. Two animal models of retinal degeneration are rescued by recombinant adeno-associated virus-mediated production of FGF-5 and FGF-18. *Mol Ther.* 2001;3:507-515.
30. Miyazaki M, Ikeda Y, Yonemitsu Y, et al. Simian lentiviral vector-mediated retinal gene transfer of pigment epithelium-derived factor protects retinal degeneration and electrical defect in Royal College of Surgeons rats. *Gene Ther.* 2003;10:1503-1511.
31. Buch PK, MacLaren RE, Duran Y, et al. In contrast to AAV-mediated Cntf expression, AAV-mediated Gdnf expression enhances gene replacement therapy in rodent models of retinal degeneration. *Mol Ther.* 2006;14:700-709.
32. Ortin-Martinez A, Valiente-Soriano FJ, Garcia-Ayuso D, et al. A novel in vivo model of focal light emitting diode-induced cone-photoreceptor phototoxicity: neuroprotection afforded by brimonidine, BDNF, PEDF or bFGF. *PLoS One.* 2014;9:e113798.
33. Scholz R, Sobotka M, Caramoy A, et al. Minocycline counter-regulates pro-inflammatory microglia responses in the retina and protects from degeneration. *J Neuroinflammation.* 2015;12:209.
34. Wu DC, Jackson-Lewis V, Vila M, Tieu K, et al. Blockade of microglial activation is neuroprotective in the 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine mouse model of Parkinson disease. *J Neurosci.* 2002;22:1763-1771.
35. Peng B, Xiao J, Wang K, So KF, Tipoe GL, Lin B. Suppression of microglial activation is neuroprotective in a mouse model of human retinitis pigmentosa. *J Neurosci.* 2014;34:8139-8150.
36. Perry J, Du J, Kjeldbye H, Gouras P. The effects of bFGF on RCS rat eyes. *Curr Eye Res.* 1995;14:585-592.
37. LaVail MM, Unoki K, Yasumura D, Matthes MT, Yancopoulos GD, Steinberg RH. Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. *Proc Natl Acad Sci U S A.* 1992;89:11249-11253.
38. Cao W, Tombran-Tink J, Elias R, Sezate S, Mrazek D, McGinnis JE. In vivo protection of photoreceptors from light damage by pigment epithelium-derived factor. *Invest Ophthalmol Vis Sci.* 2001;42:1646-1652.
39. Di Pierdomenico J, Garcia-Ayuso D, Jimenez-Lopez M, et al. Different ipsi- and contralateral glial responses to anti-VEGF and triamcinolone intravitreal injections in rats. *Invest Ophthalmol Vis Sci.* 2016;57:3533-3544.
40. Garcia-Ayuso D, Salinas-Navarro M, Agudo M, et al. Retinal ganglion cell numbers and delayed retinal ganglion cell death in the P23H rat retina. *Exp Eye Res.* 2010;91:800-810.
41. Garcia-Ayuso D, Di Pierdomenico J, Hadj-Said W, et al. Taurine depletion causes ipRGC loss and increases light-induced photoreceptor degeneration. *Invest Ophthalmol Vis Sci.* 2018;59:1396-1409.
42. Sahni JN, Angi M, Irigoyen C, et al. Therapeutic challenges to retinitis pigmentosa: from neuroprotection to gene therapy. *Curr Genomics.* 2011;12:276-284.
43. Garcia-Ayuso D, Di Pierdomenico J, Esquivia G, et al. Inherited photoreceptor degeneration causes the death of melanopsin-positive retinal ganglion cells and increases their coexpression of Brn3a. *Invest Ophthalmol Vis Sci.* 2015;56:4592-4604.
44. Jones BW, Pfeiffer RL, Ferrell WD, et al. Retinal remodeling in human retinitis pigmentosa. *Exp Eye Res.* 2016;150:149-165.
45. Marc RE, Jones BW. Retinal remodeling in inherited photoreceptor degenerations. *Mol Neurobiol.* 2003;28:139-147.
46. Kolomeyer AM, Zarbin MA. Trophic factors in the pathogenesis and therapy for retinal degenerative diseases. *Surv Ophthalmol.* 2014;59:134-165.
47. Buch PK, MacLaren RE, Ali RR. Neuroprotective gene therapy for the treatment of inherited retinal degeneration. *Curr Gene Ther.* 2007;7:434-445.
48. Liang FQ, Dejneka NS, Cohen DR, et al. AAV-mediated delivery of ciliary neurotrophic factor prolongs photoreceptor survival in the rhodopsin knockout mouse. *Mol Ther.* 2001;3:241-248.
49. Akimoto M, Miyatake S, Kogishi J, et al. Adenovirally expressed basic fibroblast growth factor rescues photoreceptor cells in RCS rats. *Invest Ophthalmol Vis Sci.* 1999;40:273-279.

50. Neuner-Jehle M, Berghe LV, Bonnel S, et al. Ocular cell transfection with the human basic fibroblast growth factor gene delays photoreceptor cell degeneration in RCS rats. *Hum Gene Ther.* 2000;11:1875-1890.
51. Sakai T, Kuno N, Takamatsu F, et al. Prolonged protective effect of basic fibroblast growth factor-impregnated nanoparticles in royal college of surgeons rats. *Invest Ophthalmol Vis Sci.* 2007;48:3381-3387.
52. Arroba AI, Alvarez-Lindo N, van Rooijen N, de la Rosa EJ. Microglia-mediated IGF-I neuroprotection in the rd10 mouse model of retinitis pigmentosa. *Invest Ophthalmol Vis Sci.* 2011;52:9124-9130.
53. Guadagni V, Novelli E, Piano I, Gargini C, Strettoi E. Pharmacological approaches to retinitis pigmentosa: a laboratory perspective. *Prog Retin Eye Res.* 2015;48:62-81.
54. Lai AY, Todd KG. Differential regulation of trophic and proinflammatory microglial effectors is dependent on severity of neuronal injury. *Glia.* 2008;56:259-270.
55. Narayan DS, Wood JP, Chidlow G, Casson RJ. A review of the mechanisms of cone degeneration in retinitis pigmentosa. *Acta Ophthalmol.* 2016;94:748-754.
56. Cai Z, Yan Y, Wang Y. Minocycline alleviates beta-amyloid protein and tau pathology via restraining neuroinflammation induced by diabetic metabolic disorder. *Clin Interv Aging.* 2013;8:1089-1095.
57. Kumar A, Chaudhary T, Mishra J. Minocycline modulates neuroprotective effect of hesperidin against quinolinic acid induced Huntington's disease like symptoms in rats: behavioral, biochemical, cellular and histological evidences. *Eur J Pharmacol.* 2013;720:16-28.
58. Ferretti MT, Allard S, Partridge V, Ducatenzeiler A, Cuello AC. Minocycline corrects early, pre-plaque neuroinflammation and inhibits BACE-1 in a transgenic model of Alzheimer's disease-like amyloid pathology. *J Neuroinflammation.* 2012; 9:62.
59. Hashemi-Monfared A, Firouzi M, Bahrani Z, Zahednasab H, Harirchian MH. Minocycline decreases CD36 and increases CD44 in LPS-induced microglia. *J Neuroimmunol.* 2018;317: 95-99.
60. Wang AL, Yu AC, Lau LT, et al. Minocycline inhibits LPS-induced retinal microglia activation. *Neurochem Int.* 2005; 47:152-158.
61. Gargini C, Terzibasi E, Mazzoni F, Strettoi E. Retinal organization in the retinal degeneration 10 (rd10) mutant mouse: a morphological and ERG study. *J Comp Neurol.* 2007;500:222-238.
62. Yang LP, Li Y, Zhu XA, Tso MO. Minocycline delayed photoreceptor death in rds mice through iNOS-dependent mechanism. *Mol Vis.* 2007;13:1073-1082.
63. Bosco A, Inman DM, Steele MR, et al. Reduced retina microglial activation and improved optic nerve integrity with minocycline treatment in the DBA/2J mouse model of glaucoma. *Invest Ophthalmol Vis Sci.* 2008;49:1437-1446.