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Protein kinase C expression in the rabbit retina after laser photocoagulation

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Abstract

Background: Laser photocoagulation is a well established treatment for diabetic retinopathy but the mechanism behind its effectiveness has not been elucidated. The protein kinase C (PKC) family is a group of enzymes which has been the subject of extensive interest in clinically related research since the advent of its role in the pathogenesis of diabetic retinopathy. With this study we wanted to explore if PKC expression is altered in the retina after laser photocoagulation.

Methods: Normal rabbit eyes were treated with laser photocoagulation of varying intensity and examined after 30 mins.-7 weeks. Treated and untreated regions of the retina were investigated histologically with the MC5 monoclonal antibody against PKC. Labeling for glial fibrillary acidic protein (GFAP), as well as hematoxylin and eosin staining was also performed to assess the laser-induced trauma.

Results: In the normal retina, the MC5 antibody labeled rod bipolar cells and photoreceptor outer segments corresponding to PKC alpha. A translocated PKC expression with labeling concentrated to the rod bipolar terminals was seen in specimens examined 30 mins. after laser treatment, and after 1 week, no expression was seen in any part of the retina. After 2 weeks, PKC expression again indicated a translocated labeling pattern. After 5 weeks, labeling was only found in rod bipolar terminals in the peripheral retina. When comparing high versus low intensity laser treatment 7 weeks postoperatively, no labeling was found in the high intensity treated retinas, whereas low intensity treated eyes displayed a near normal labeling pattern. Hematoxylin and eosin staining revealed focal neuroretinal edema immediately after laser treatment, also in untreated areas. At later stages, destruction of the outer nuclear layer and migration of pigment epithelial cells in laser lesioned areas was seen.

GFAP labeled Müller cells were seen 1 week postoperatively in the entire retina. Labeling after this time decreased, but was still present in laser spots after 5 and 7 weeks.

Conclusions: Laser photocoagulation alters the expression of PKC in the entire normal rabbit retina. The response follows a temporal pattern, and is also related to laser intensity. The results may help to explain the high efficacy of laser treatment in diabetic retinopathy.

Introduction

The protein kinase C (PKC) family consists of at least 12 different isoforms, many of which are present in the mammalian retina, in various neuronal cell types [7, 9, 23]. Among the different isoforms, PKC alpha is the most abundant [23]. PKC enzymes act by transferring a phosphate group from adenosine triphosphate (ATP) to other molecules such as hormones, other enzymes or membrane receptors, thereby activating them. PKC has been linked to various normal retinal functions such as regulation of phototransduction, transmitters, and cytoskeletal interactions, as well as to important roles in pathological conditions [23]. The family of PKC enzymes has been the subject of extensive interest in clinically related research recently since the advent of its role in the pathogenesis of diabetic retinopathy [1, 4]. PKC is necessary for many of the various effects of vascular endothelial growth factor (VEGF) [20], one of the key mediators of diabetic retinopathy [3]. Interestingly, elevated levels of all isoforms of retinal PKC have been found in model systems of the disease [16], and clinical trials of oral PKC inhibitors are under way [1].

Retinal laser photocoagulation is a well established treatment procedure for various retinal and choroidal disorders. In the treatment of diabetic retinopathy, laser photocoagulation has proven highly efficient [5, 18], even though the underlying mechanisms have not been fully understood. A number of studies have reported alterations of growth factor levels as well gene expression after laser therapy [6, 22, 24, 25]. Given the important role of PKC in the orchestration of protein expression and activation in the retina, and its documented relationship with diabetic retinopathy, we in this paper have chosen to examine retinal PKC expression after laser therapy.

photocoagulation. To monitor regional changes in PKC expression as well as intracellular distribution of PKC after laser photocoagulation, we chose to use immunohistochemistry with the well documented MC5 anti-PKC antibody.

Materials and Methods

Animals and surgical procedure

A total of 18 pigmented rabbits of mixed strain, aged 4-5 months, were used for surgery, and 2 age-matched animals for controls. Laser photocoagulation was applied to the right eye only, and spots were placed so that the distance between them was approximately one spot diameter. Laser burns were applied using an indirect ophthalmoscope delivery system and a 25 diopter condensing lens, and were placed in an area extending inferiorly from the inferior border of the myelinated streak (Fig. 1).

In order to test the temporal as well as the intensity effect of laser photocoagulation on PKC expression, the research material was divided into 2 groups. In the first group of 12 animals, the retina was treated in an area covering approximately 3 disc diameters (68-75 spots) using moderately intense (0,2s, 0,12W), grayish burns (Fig. 1A). An EyeLite® 532 nm frequency-doubled YAG laser photocoagulator (Alcon Laboratories Inc., Fort Worth, USA) was used for these 12 eyes. Animals were euthanized at 30 mins.-2hrs. (n=3), 1 week (n=3), 2 weeks (n=3), and 5 weeks (n=3) after laser photocoagulation.

In the second group of 6 animals, the quantitative effect of laser photocoagulation on PKC expression was studied. An Ultima SE argon laser (Coherent, Santa Clara, CA) was used for these experiments. Three eyes were treated with low intensity laser burns in an area corresponding to 1 disc diameter (0,2 s, 0,3W, 14-18 spots), producing slightly gray lesions (Fig. 1B). The remaining 3 eyes were treated with a higher energy level producing distinctly white burns in an area corresponding to approximately 10 disc diameters (309-432 spots, 0,2s 0,5-0,7W) (Fig. 1C). Animals in this group were euthanized 7 weeks after laser photocoagulation.

Anaesthesia and euthanasia

The right eye of the rabbit was instilled with cyclopentolate (1%) and phenylephrine (10%) 30 minutes prior to surgery. General anaesthesia was provided with Hypnorm 1.5 ml. (phentanyl citrate 0.315 mg/ml. and Fluanisone 10 mg/ml, Janssen pharmaceutica Beerse, Belgium). Topical tetracaine (0.5%) was applied just before surgery. After the surgery, Naloxonhydrochloride 0.5ml (0.02mg/ml., Apoteksbolaget, Umeå, Sweden) was administered intramuscularly. Animals were euthanized with an overdose of sodium pentobarbital intravenously.

All proceedings and animal treatment were in accordance with the guidelines and requirements of the Government Committee on Animal Experimentation at Lund University and the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised 1986) and the U.S. Animal Welfare Act, as amended, were followed.

Tissue preparation

Eyes were enucleated and fixed for 30 min. in formaldehyde (4%, generated from paraformaldehyde) at pH 7.4 in a 0.1 M Sørensen's phosphate buffer. The anterior segment was then removed and the posterior eyecup was postfixed in the same solution for 4 hours. All eyecups were examined in an operating microscope (Carl Zeiss, Oberkochen, Germany), and photographed using an attached digital camera (Sony, Tokyo, Japan). Tissue specimens were obtained as approximately 5-6 mm. wide pieces, including the laser treated area together with parts of the myelinated fibers and optic nerve. After fixation, the specimens were washed with SøSørensen's phosphate buffer (0.1 M, pH 7.4) and then washed again using the same solution with added sucrose of rising concentrations (5, 10, 15, 20%) before serial sectioning in the vertical plane at 12 μ m on a cryostat.

For immunohistochemistry, sections were washed in 0.1 M sodium phosphate buffered saline pH 7.2 (PBS) (Merck, Darmstadt, Germany) with 0.25% Triton X-100 (PBS/Triton, ICN Biomedicals Inc. Aurora, Ohio, USA), and then incubated with the primary antibody overnight at +4°C. A monoclonal antibody directed against human protein kinase C alpha, beta I and beta II (PKC, clone MC5, Nordic BioSite, Täby, Sweden) was used. The antibody was diluted to 1:200 with PBS/Triton with 1% bovine serum albumin (BSA). After incubation, the slides were rinsed in PBS/Triton, incubated with Texas Red (TR)-conjugated antibodies (1:200) for 45 mins., rinsed again and finally mounted in custom-made antifading mounting media.

To assess the trauma imposed by the laser photocoagulation on the neuroretina, sections from all eyes were also labeled with an antibody raised against glial fibrillary acidic protein (GFAP, clone G-A-5, Boehringer Mannheim Scandinavia AB, Bromma, Sweden), which is expressed in activated Müller cells in response to retinal injury [2, 17]. This antibody was used at a dilution of 1:4 and fluorescein isothiocyanate (FITC) was used as secondary antibody. After examination in the fluorescence microscope, the GFAP labeled slides were also prepared for routine microscopy. The coverslip was removed and slides were rinsed in distilled water to remove the anti-fading mounting media. The sections were then stained with hematoxylin and eosin, dipped in distilled water, dipped in 70% ethanol, 96% ethanol and then placed in 99% ethanol for 2 cycles of 2 minutes. Finally the slides were put in Xylol for 2 cycles of 2 minutes and coverslipped. There was no apparent shrinkage of the tissue after this procedure.

For negative controls, the complete labeling procedure without primary antibodies was performed on both normal and on transplanted specimens. No labeling was found in these specimens.

Analysis

Vertical sections including the optic nerve head and the laser treated area were examined at 4 specific locations: superior and inferior peripheral retina, treated area, and the area adjacent to and superior to the optic nerve. In addition, vertical sections lateral to the optic nerve head, and outside the treated area were examined. Laser treated eyes were compared with eyes from 2 untreated aged-matched control animals. Photographs of all specimens were obtained with a digital camera system (Olympus, Tokyo, Japan). When comparing immunolabeled sections, specimens were always derived from the same labeling batch, and were photographed in one session using a fixed exposure time at ISO 200. No digital image manipulation was performed on photographs from immunolabeled sections. Photographs of hematoxylin and eosin stained sectioned were adjusted for brightness.

Results

Macroscopic findings

Specimens examined 30 mins.-2 hrs. after treatment displayed focal neuroretinal edema corresponding to laser burns in the treated area (Fig. 2A). Smaller round lesions consisting of neuroretinal edema were also seen on the nasal and temporal side of the treated area corresponding to the visual streak located slightly inferior to the myelinated streak. This phenomenon was also seen in the untreated area superior to the optic nerve. In older specimens, lesions within the treated area were pigmented (Fig. 2B), and no other visible signs of laser treatment was present in the retina.

Histology

Normal rabbit retina

Well labeled cells, corresponding to rod bipolar cells [7] were seen in all untreated retinal sections using the PKC antibody (Fig. 3A). These cells displayed perikarya in the outer part of the inner nuclear layer (INL), and extended axons with terminal bulbs to the inner part of the inner plexiform layer. In addition, labeling was also seen in some of the photoreceptor outer segments. The well labeled rod bipolar cells and photoreceptor outer segments seen in our untreated eyes is concurrent with earlier reports [7, 13], and indicates that the MC5 antibody in the rabbit retina recognizes predominantly the PKC alpha isoform.

GFAP labeled sections displayed well labeled astrocytes in the optic nerve area, but no Müller cells were seen.

Laser treated eyes

In the 3 eyes examined 30 mins.-2 hrs. after laser application, rod bipolar cells as well as well labeled structures in the outer segment (OS) region were seen in the entire retina using

the PKC antibody (Fig. 3B). The number and labeling intensity of rod bipolar terminals was equal to the one seen in the normal retina, but fewer of the perikarya and axons were labeled. In sections examined 1 week postoperatively, PKC labeled structures could not be identified in any part of the retina in any of the 3 specimens (Fig. 3C). In specimens examined after 2 weeks, intensely labeled rod bipolar cell terminals were seen in most parts of the retina as well as structures in the OS (Fig. 3D). Rod bipolar axons were weakly labeled and almost no labeling of perikarya was seen. At 5 weeks postoperatively, no PKC labeling was present in any of the specimens examined in the central part of the retina (Fig. 3E). In these specimens, weakly labeled rod bipolar cell terminals with no corresponding perikarya or axons were seen in the far superior and inferior part of the retina (Fig. 3F). In these areas, weakly labeled OS could also be seen. In the 3 eyes treated with mild laser burns, PKC labeled rod bipolar cells were seen in the entire retina 7 weeks after treatment (Fig. 3G). Labeled rod bipolar cell perikarya and axons were somewhat fewer than in the normal retina. The labeling intensity in these specimens was weaker in the treated area, but comparable to the normal retina in all other areas, and labeled structures in the OS were seen throughout the retina. In the 3 eyes treated with high energy level laser burns, no PKC labeled rod bipolar cells or structures in the OS were seen after 7 weeks (Fig 3H).

Hematoxylin and eosin labeled sections examined at 30 mins - 2 hrs after laser, revealed focal neuroretinal edema corresponding to the laser treated area (Fig 4A), but also focal spots with neuroretinal swelling in untreated areas both superior to the optic nerve (Fig. 4B) as well as in the visual streak lateral to the treated area. From 1 week postoperatively, focal destruction of the outer nuclear layer (ONL) as well as migration of retinal pigment epithelial (RPE) cells were seen (Fig. 4 C and D). The neuroretina between spots appeared intact even in the 3 animals receiving high energy level laser burns.

GFAP labeling in specimens examined 30 mins.-2 hrs. after treatment was comparable to the normal retina with well labeled astrocytes, but no labeled Müller cells. The labeling pattern and intensity in specimens examined after 1 and 2 weeks revealed activated Müller cells in all specimens in the entire retina. Labeling intensity was strongest in the treated area, and declined towards the periphery. At 5 and 7 weeks after treatment, GFAP labeling was confined to the laser treated area with labeling concentrated to spots (Fig. 4E and F).

Discussion

PKC expression in laser treated eyes

Cellular events following laser photocoagulation clearly involves PKC alpha expression in the entire rabbit neuroretina. Leibu and colleagues found a reduction of ERG responses (dark-adapted b-wave amplitude) 1-2 hrs. after intense laser application (225 spots 700 μ m in size, 0.1 s, 0.5 W) in the rabbit eye [10]. We found that also a less intense laser photocoagulation induces a profound macroscopic and microscopic neuroretinal response not only in the lesioned area, but also in untreated regions, already at 30 mins. after treatment.

The labeling pattern in the immediate postoperative period and after 2 and 5 weeks, indicates translocation of PKC from the rod bipolar perikarya towards the axon terminal. This pattern has been well described previously, and is associated with enzyme activation, i.e. a change of conformation in order to phosphorylate target proteins [23]. One possible mechanism behind the phenomenon is laser-induced glutamate excitotoxicity [14], causing an

increase of intracellular calcium, a well known prerequisite for PKC activation.

The complete absence of PKC in rod bipolar cells in eyes at 1 week postop. in moderately treated eyes, and at 7 weeks in eyes treated with high intensity, strongly indicates that laser treatment also affects the intracellular level of the enzyme. The pattern of PKC activation followed by depletion of the enzyme has been reported previously in phorbol ester stimulated cells, and has been explained by PKC degradation via the ubiquitin-proteasome pathway [11]. This is in accordance with the temporal pattern of PKC expression seen in moderately treated eyes. Whether high-intensity treated eyes actually went through a phase of PKC activation before total depletion was not studied, but future experiments may confirm the hypothesis that kinetics of PKC expression after laser treatment depends on laser intensity.

To what extent the reduced expression of PKC alpha affects retinal function is at this point difficult to estimate. It is unlikely that a radical change of expression of such a prominent enzyme would be beneficial in the normal eye. However, existing pathological conditions involving elevated levels of PKC, makes the previously unknown relationship between laser treatment and PKC alpha expression intriguing.

PKC and diabetic retinopathy

The molecular and cellular mechanisms behind diabetic retinopathy (DRP) have not yet been fully elucidated, but studies implicate activated PKC as one of its key molecules. Most attention has been directed against PKC beta II, but elevated levels of all isoforms have been found in model systems of diabetic retinopathy [15]. Biochemically, the chronic hyperglycemia of diabetic patients leads to intracellular accumulation of diacylglycerols (DAGs), derivatives of glycerol, which in turn are known activators of PKC. The activation of PKC is necessary for many of the various effects of vascular endothelial growth factor (VEGF) [20], which has been identified as one of the key mediators of diabetic retinopathy

[3]. Further, elevated levels of activated PKC appears to induce VEGF expression in some tissues, but this has not yet been fully explored in the retina [21].

The present study is based entirely on normal rabbit eyes, but the profound effect of laser photocoagulation on PKC expression may still help to explain the effects of treatment also in eyes with DRP. The highly activated state of PKC in the earlier phases after laser application in the present study correlates well with common clinical findings such as transient macular edema and visual disturbances after laser photocoagulation [8, 12, 16]. It is of particular interest to note that PKC downregulation persists for at least 7 weeks after intensive laser photocoagulation which may explain the long-term beneficial effects seen in patients after treatment [5, 18].

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Figure legends

Figure 1. Cartoon of the rabbit retina showing the intensity and extension of laser burns. Eyes were treated with burns of either: **A.** moderate intensity covering an area of approximately 3 disc diameters, **B.** low intensity covering 1 disc diameter, or **C.** high intensity covering 10 disc diameters.

Figure 2. **A.** Posterior eyecup 30 mins. after laser photocoagulation. The laser treated area is seen as gray spots descending in rows from the inferior part of the myelinated streak. Smaller focal grayish lesions are visible on both sides of the treated area (white arrows). **B.** Posterior eyecup 7 weeks after laser photocoagulation. Focal pigmented lesions are visible in the treated area.

Figure 3. PKC labeling. Cryostat sections. All sections have been processed for PKC immunohistochemistry in the same batch. All images except **F** are from an area not treated with laser photocoagulation, approximately 1 disc diameter superior to the edge of the optic nerve. All images have been photographed in one session using the same exposure time (1/1.5s) at ISO 200. No digital image processing has been applied. Scale bar = 50 μ m.

A. Normal rabbit retina. Labeling is present in rod bipolar cells with perikarya in the inner nuclear layer (INL), extending axons which terminate in the inner part of the inner plexiform layer (IPL). Labeling is also present in focal spots in the outer segment (OS) region. ONL = outer nuclear layer.

B. Thirty mins. after laser photocoagulation. Rod bipolar terminals are well labeled and as numerous as in the normal retina. Fewer of the rod bipolar perikarya and axons are labeled. Some of the outer segments are labeled.

C. One week after laser photocoagulation. No labeled structures are seen.

D. Two weeks after laser photocoagulation. Rod bipolar terminals in the IPL are intensely labeled. Some axons are labeled, but no perikarya can be seen in the INL. Numerous outer segments are labeled.

E and F. Five weeks after laser photocoagulation. The same section photographed in different areas. In the area superior to the optic nerve (**E**), some auto fluorescence is present in the OS, but no labeling can be found in this or in other retinal layers. In the far peripheral inferior area (**F**), weakly labeled rod bipolar cell terminals in the IPL and outer segments are seen.

G. Seven weeks after low intensity laser photocoagulation. Well labeled rod bipolar cell terminals are seen in the IPL. Labeled rod bipolar cell perikarya and axons appear to be fewer than in the normal retina. Some labeled outer segments are seen.

H. Seven weeks after high intensity laser photocoagulation. Some auto fluorescence is present in the OS, but no labeled structures can be seen in this or in other retinal layers.

Figure 4. Hematoxylin and eosin staining (**A-D**). GFAP labeling (**E and F**). Cryostat sections. Scale bar = 100 μ m.

A. Rabbit retina 1 hour after laser photocoagulation. Focal neuroretinal edema is seen in the area of laser treatment inferior to the optic nerve.

B. The same section as in **A**, but from the untreated area superior to the optic nerve. Edema of the neuroretina can also be seen in this area.

C and D. Seven weeks after low (**C**) and high (**D**) intensity laser photocoagulation. Local tissue destruction of the outer nuclear layer (ONL) is evident, but the ONL bordering the lesion appears intact. Some pigmentation within the neuroretina is present especially in **D**.

E and **F**. The same sections as in **C** and **D**. GFAP labeling of Müller cells is concentrated to the laser lesioned spot.

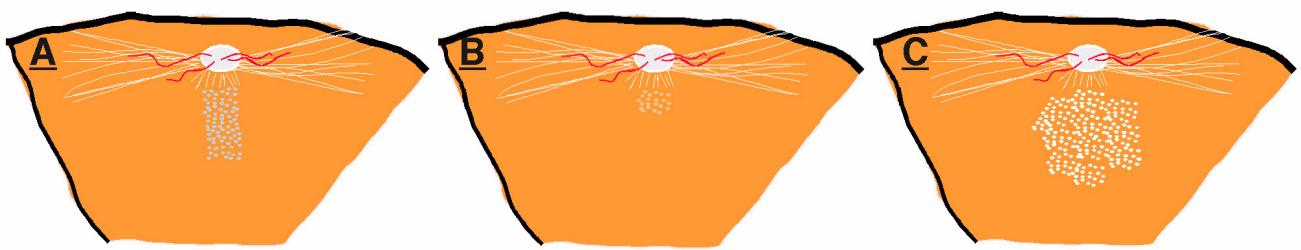


Figure 1. Ghosh and Gjörloff

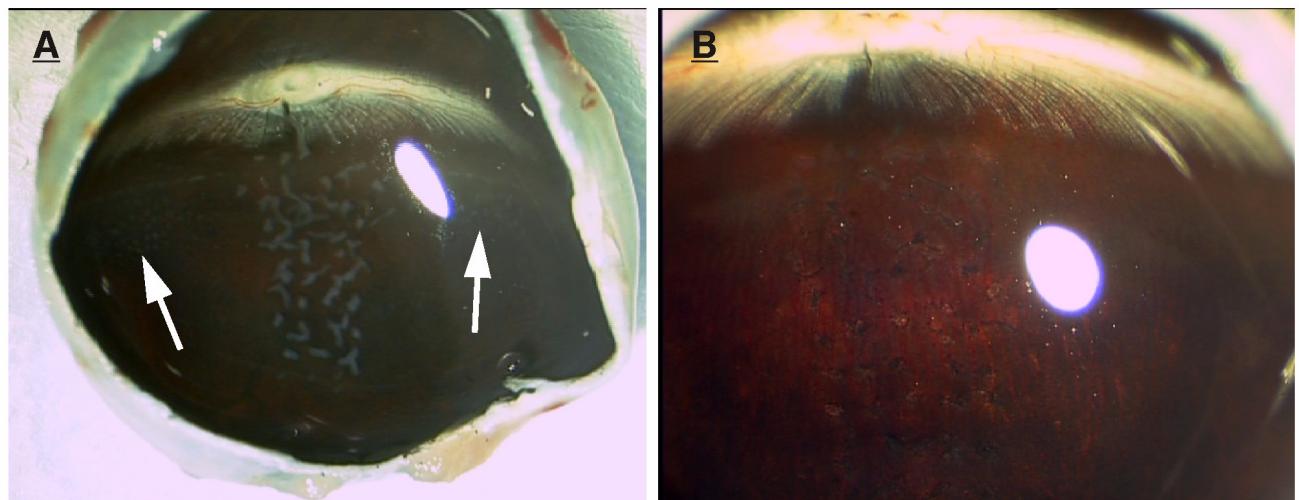


Figure 2. Ghosh and Gjörloff

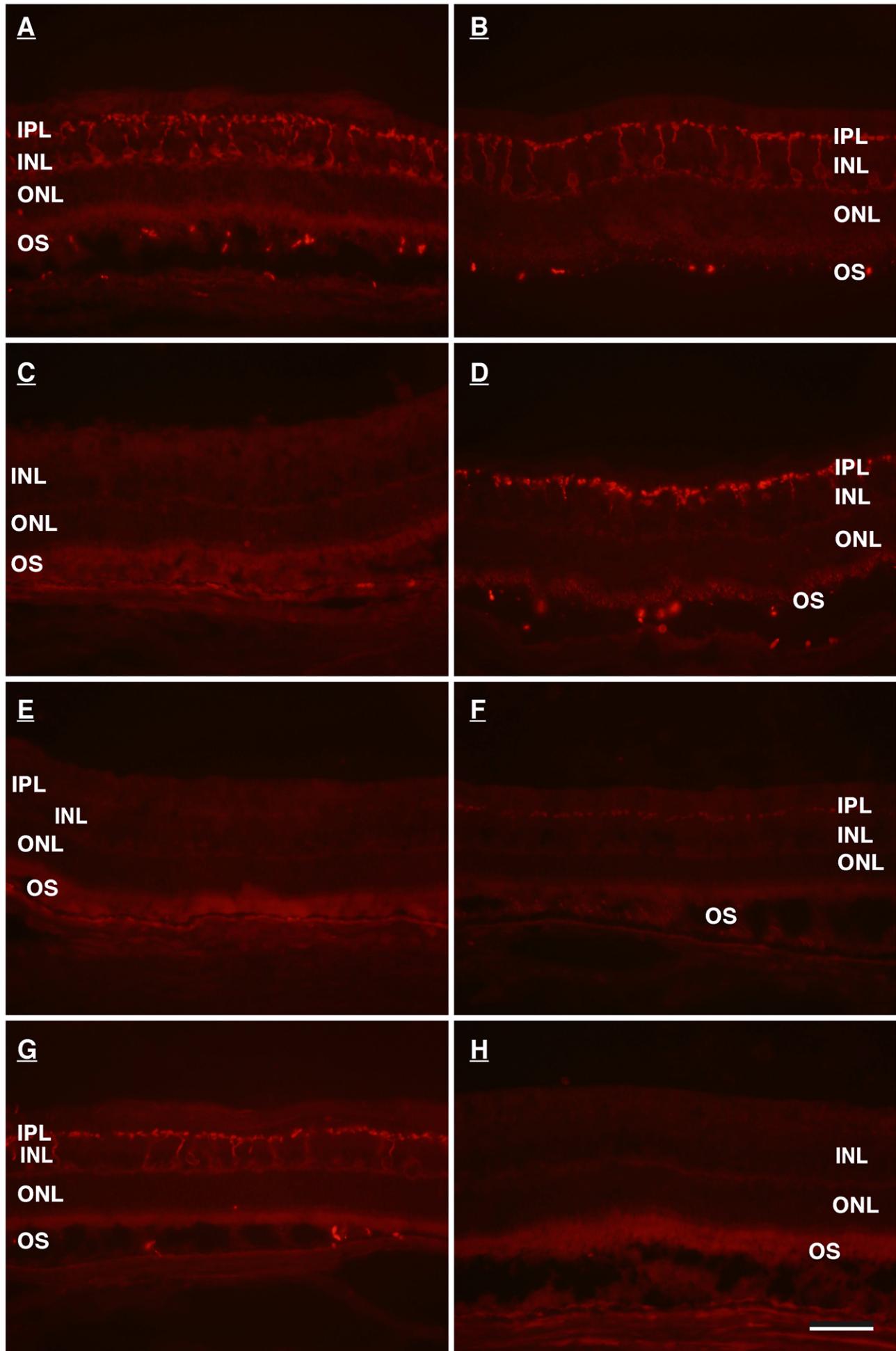


Figure 3. Ghosh and Gjörloff

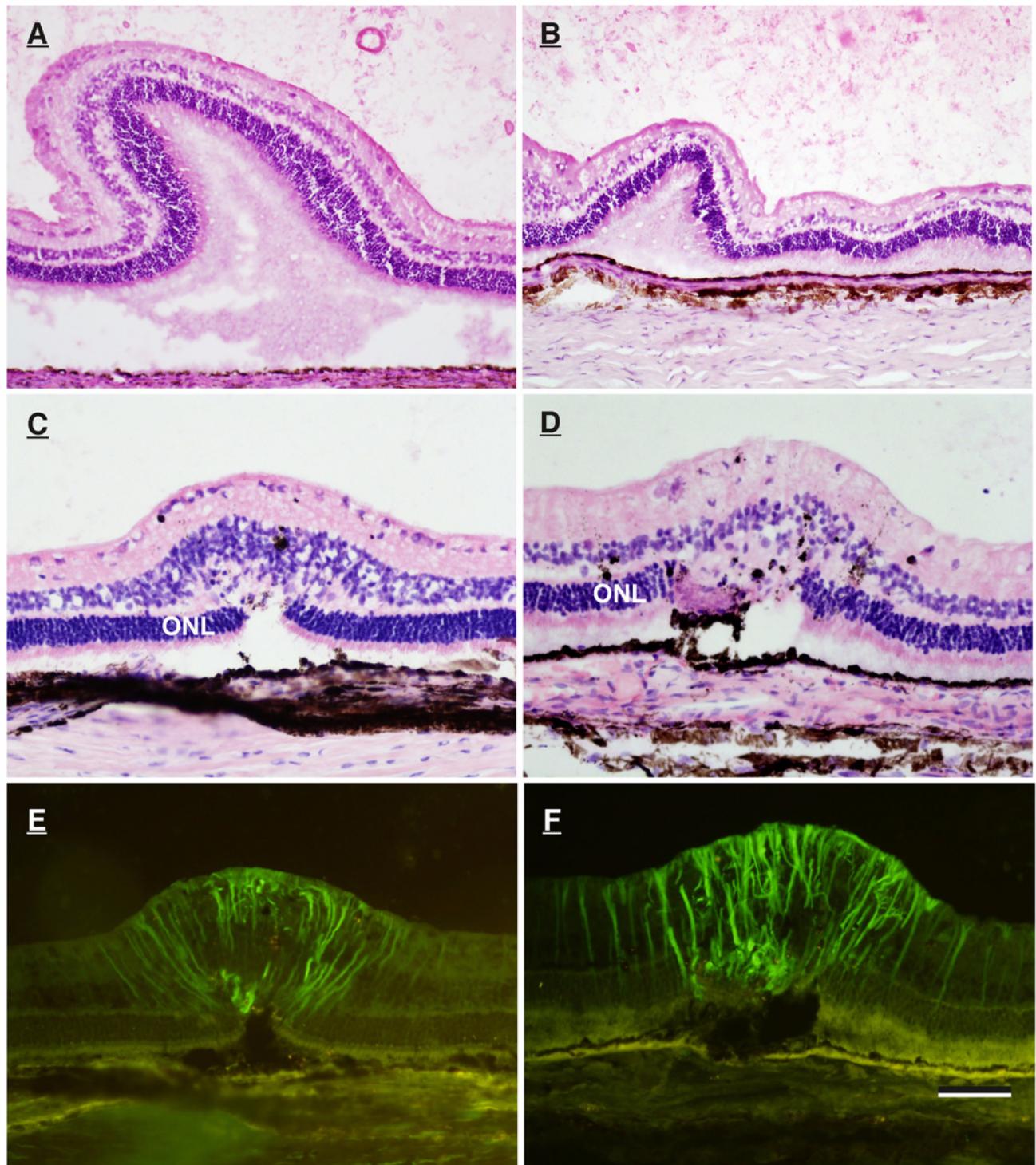


Figure 4. Ghosh and Gjörloff