Ischemia Results 3 Months Later in Altered ERG, Degeneration of Inner Layers, and Deafferented Tectum: Neuroprotection with Brimonidine

Sergio Mayor-Torroglosa,1 Pedro De la Villa,2 María Elena Rodríguez,1 María P. Lafuente López-Herrera,1 Marcelino Avilés-Trigueros,1 Antonio García-Avilés,1 Jaime Miralles de Imperial,1 María P. Villegas-Pérez,1 and Manuel Vidal-Sanz1

PURPOSE. To investigate the long-term effects of transient ligation of the ophthalmic vessels (LOV) on the inner and outer retina as well as on retinotectal projection, and whether brimonidine (BMD) has protective effects.

METHODS. In adult rats, the left eye was subjected to 90 minutes of LOV. One hour before ischemia, 2 drops of saline alone (vehicle group) or saline containing 0.5% brimonidine (BMD group) were instilled in the left eye. The effects of LOV on the inner and outer retina were assessed with ERG recordings of a- and b-wave amplitudes at 1, 8, and 12 weeks after LOV and with analysis of layer thickness in paraffin sections. The retinotectal projection was orthogradely labeled with cholera toxin subunit B (CTB) injected in the left eye and measured in serial coronal sections of the superior colliculus.

RESULTS. There were significant reductions in the mean b-wave amplitudes of the ischemic eyes at 8 and 12 weeks after LOV in the vehicle-treated group of animals, but not in the BMD-treated group. The thickness of the inner nuclear and inner plexiform layers of the vehicle-treated group of retinas had decreased to approximately 71% of the thicknesses in the BMD-treated groups. Three months after LOV, the mean volume of the retinotectal projection in the vehicle- or BMD-treated group of animals had decreased to approximately 54% or 83%, respectively, of the mean values found in the control group of animals.

CONCLUSIONS. LOV induces degeneration of the inner retinal layers and the retinotectal projection 3 months after the insult. BMD administration significantly protected against LOV-induced retinal damage and degeneration of retinal projection.

(I Invest Ophthalmol Vis Sci. 2005;46:3825–3835) DOI: 10.1167/iovs.05-0392

From the 1Departamento de Oftalmología, Facultad de Medicina, Universidad de Murcia, Campus de Espinardo, Murcia, Spain; and the 2Departamento de Fisiología, Facultad de Medicina, Universidad de Alcalá de Henares, Madrid, Spain.

Supported by Regional Government of Murcia Grant PI 92/00540/FS/01, Spanish Ministry of Science and Technology Grants BFIF2002-03742 and SAF2001-01038, Ministry of Health Grant C03/13 and FIS PI02047, European Union Grant QLK6-CT-2001-00385, and an unrestricted grant from Allergan Inc.

Submitted for publication March 29, 2005; revised April 26, 2005; accepted July 5, 2005.

Disclosure: S. Mayor-Torroglosa, None; P. De la Villa, None; M.E. Rodríguez, None; M.P. Lafuente López-Herrera, None; M. Avilés-Trigueros, None; A. García-Avilés, None; J. Miralles de Imperial, None; M.P. Villegas-Pérez, None; M. Vidal-Sanz, Allergan Inc. (F)

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Manuel Vidal-Sanz, Departamento de Oftalmología, Facultad de Medicina, Universidad de Murcia, Campus de Espinardo, E-30100 Espinardo, Murcia, Spain; ofmvm01@um.es.
Several laboratories have shown the capacity of different substances to prevent degeneration induced by retinal and optic nerve injuries. Among these neuroprotectants, attention has been focused on \( \alpha_2 \)-selective adrenergic agonists. These agonists, which are currently used to treat glaucoma because of their intraocular-pressure-lowering effects, have been shown to have neuroprotective effects in rodents against various types of retinal injuries, including partial crush of the optic nerve and laser-induced chronic elevation of the intraocular pressure and retinal ischemia. Work from our laboratory has shown that pretreatment with brimonidine (BMD) as \( \alpha_2 \)-selective adrenergic agonist is effective in protecting the RGC population from LOV-induced cell death, in a dose-dependent manner. However, most of these studies were conducted at early postischemic intervals (i.e., 1–3 weeks) leaving the long-term efficacy of the compound far less frequently studied. Using functional (ERG), structural (histologic analysis of cross sections of the retina) and neuroanatomical tracing techniques (combined with immunohistochemistry), we investigated the effects of transient ischemia of the retina 3 months after the insult. Moreover, we investigated whether BMD provides long-lasting neuroprotection against ischemia-induced degeneration of the primary visual pathway.

**Material and Methods**

**Animal Groups, Anesthetics and Drug Administration**

Adult female albino Sprague-Dawley rats (180–200 g) from the breeding colony of the University of Murcia (Murcia, Spain) were fed ad libitum and maintained in cages in temperature-controlled rooms with a 12-hour light–dark cycle (light period from 8 AM to 8 PM; light intensity within cages was 8–24 lux). All procedures were performed in accordance with the European Union guidelines for the use of animals in scientific research and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental manipulations were performed in rats under anesthesia with a mixture of ketamine (75 mg/kg, intraperitoneal; Ketolar; Parke-Davis, SL, Barcelona, Spain) and xylazine (10 mg/kg intraperitoneal; Rompun; Bayer, SA, Barcelona, Spain). For the induction of transient ischemia and for ERG recordings, rats were anesthetized with 7% chloral hydrate (0.42 mg/g, intraperitoneally). During recovery from anesthesia, rats were placed in their cages and a topical steroid antibiotic ointment (Fludronef; Iquinosa, Madrid, Spain) was applied to prevent corneal desiccation. Animals were killed with an overdose of intracardiac pentobarbital (Dolethal, Vetoquinol; Especialidades Veterinarias SA, Alcobendas, Madrid, Spain).

One hour before retinal ischemia, two 5-µL drops of 0.9% NaCl alone (vehicle-treated group) or containing 0.5% brimonidine (BMD; brimonidine-treated group) were administered to the left eyes of the rats. Inner and outer retinal function was examined by electroretinogram (ERG) recordings at 1 week and 2 or 3 months after ischemia. After ERG recordings, animals were used to examine the histoarchitecture of the retina, or to determine the total volume of the superficial layers of the contralateral SC occupied by retinal afferents. Animals survived 3 months after ischemia. At this time, the rats weighed ~300 g. In control experiments and for comparison, we examined retinal afferents in an additional group of intact rats (290–320 g; control group; \( n = 11 \)). Brimonidine was dissolved in 0.9% NaCl (0.5 mg BMD in 100 µL of 0.9% NaCl) and was provided by Allergan Inc. (Irvine, CA) for these studies.

**Induction of Transient Ischemia**

Transient ischemia of the left retina was induced by selective ligature of the ophthalmic vessels (LOV) for 90 minutes, according to published methods. In brief, the left optic nerve head was exposed in the orbit and the dural sheath was opened longitudinally. A 10-0 nylon suture was introduced between the optic nerve and the sheath and tied around the latter to interrupt blood flow through the ophthalmic vessels, which run in the inferior and nasal aspect within the sheath. Care was taken not to damage the optic nerve. Interruption of retinal blood flow during ischemia was assessed by direct ophthalmoscopy of the eye fundus through the operating microscope. At the end of the ischemic period, the ligature was released, and retinal reperfusion was assessed through the operating microscope. The animals that did not show complete recovery of the retinal blood flow within the first few minutes after releasing the ligature were discarded. This ischemic interval was chosen on the basis of earlier studies reporting consistent RGC loss after this period of transient ischemia in rat retina.

**ERG Recordings**

ERGs were recorded from a first group of age-matched untouched control rats \( (n = 8) \) and from experimental groups of vehicle- \((n = 12)\) or BMD-treated \((n = 13)\) animals at 3 months after LOV. Because the vehicle-treated group of animals showed significant decreases in the amplitudes of the b-wave, additional groups of animals were prepared, to study the time course of these changes. This second group of vehicle- \((n = 7)\) or, BMD- \((n = 8)\) treated animals was examined at 1 week, 2 months, and 3 months after SLOV. Before recordings were made, animals were dark adapted overnight. Rats were anesthetized and their pupils dilated with a topical drop of 1% tropicamide (Colirucis, Tropicamida; Alcon Cusi, SA, El Masnou, Barcelona, Spain). To optimize electrical recording 2% methocel (Ciba Vision AG, Hettlingen, Switzerland) was instilled in each eye immediately before placing the corneal electrode. Animals were placed on a Faraday cage, and experiments were conducted in absolute darkness. The nonregistered eye was covered with an opaque contact lens. Bipolar recording was performed between an AgAgCl electrode fixed on a corneal lens and a reference electrode located on the head skin; ground electrodes were located in the tail and nose. Scotopic flash ERGs were recorded from each eye in response to light stimuli that consisted of light emission diodes (LED-white light) centered on the visual axis and located 5 mm away from the cornea. Light stimuli were presented for 5 ms at five different increasing intensities (ranging 10–3–10–7 cd/m²). Four to eight consecutive recordings were averaged for each light presentation. Interval between light flashes was 10 seconds. The ERG signals were amplified, band-pass filtered between 0.3 and 1000 Hz and digitized at 10 kHz with a data-acquisition board (Power Laboratory-2ST, ADInstruments Pty. Ltd., Oxfordshire, UK). Recordings were saved on a computer and analyzed off-line by an investigator blinded to the experimental condition of the animal.

**Analysis of the Retinal Layer Thickness**

After ERG recordings, one half of the animals that were only examined at 3 months after LOV (vehicle- \( n = 6 \)) or BMD- \( n = 7 \) (treated animals) were processed for retinal histarchitecture in paraffin-embedded cross sections. In brief, rats were perfused through the left cardiac ventricle with saline, followed by 0.1 M PBS containing 4% paraformaldehyde and 0.25% glutaraldehyde. Both eyes were enucleated, and the superior aspect of the eyes was marked with chin ink as a landmark. Three-µm-thick sagittal sections containing the whole retina, including the optic nerve head, were cut on the microtome (model HM340E; Microm International GmbH, Walldorf, Germany), dried at 37°C, and stained with hematoxylin and eosin (Sigma-Aldrich Chemical Co., St. Louis, MO). Retinal layer thickness were analyzed in eight alternate sections from each eye using bright-field microscopy (Axioskop; Carl Zeiss Meditec, Inc., Oberkochen, Germany) and image analysis software (ImagePro Plus, ver. 4.5.1.27; Media Cybernetics, Silver Spring, MD). For each section, six rectangular frames (420 µm in length) at approximately 600, 1800, and 3000 µm from the optic nerve head, on both sides (superior and inferior), were captured with a digital camera (Axio-
FIGURE 1. Intensity-response ERGs in control and experimental rats 3 months after ischemia. ERG recordings were obtained from the left (LE) and right (RE) eyes of a control group (A) and two groups of experimental Sprague-Dawley (B, C) rats 3 months after a 90-minute LOV. One hour before LOV, experimental rats were pretreated topically in the left eyes, with two 5-μL drops of saline alone (B) or saline containing 5% brimonidine (C). Representative average (mean ± SD) intensity–response curves to light stimuli of increasing intensity (from $10^{-4}$ to 10 cd/m$^2$) for the ERG a- and b-wave from the control (A, n = 8), vehicle (B, n = 12), and brimonidine-(C, n = 13) treated groups of animals. In the vehicle-treated group of rats, the mean b-wave amplitudes in the left eyes were significantly smaller when compared with that in the contralateral eyes, at all light intensities (Mann-Whitney test, $P < 0.01$). There were no significant differences in the control (A) or in the BMD-treated (C) groups of rats.

**Analysis of the Retinotectal Projection**

After ERG recordings, the animals that had been used for time-course analysis of ERG changes (vehicle- $n = 7$) or BMD- $n = 8$ treated animals) as well as one half of the animals in the other group (vehicle- $n = 6$) or BMD-treated $n = 6$ animals) were processed to examine the retinotectal innervation. In addition, and for comparison, the volume of the retinotectal projection was determined in a group of untouched aged-matched rats ($n = 11$).

**Anterograde Labeling of Retinal Afferents**. Retinal afferents were investigated by using a very sensitive method to demonstrate retinotectal projections. Control and experimental animals were anesthetized and 5 μL of 1% solution of cholera toxin B subunit (CTB; List Biological Laboratories, Campbell, CA) were injected into the vitreous of the left eye for anterograde labeling of the retinal terminals. Four days later, serial coronal sections (40-μm-thick) extending through the diencephalon and brain stem were obtained on a freezing microtome, according to previously described methods. The rostro-caudal extension of the SC comprised 71 to 79 (71 ± 3; mean ± SD), 67 to 82 (72 ± 5), or 71 to 78 (73 ± 6) sections, for the vehicle- or BMD-treated or control group, respectively, corresponding to a rostro-caudal extension of the SC comparable to previous studies.

**Immunohistochemistry**. Orthogradely transported CTB was immunolocalized in free-floating sections by using previously described methods. Immunostained sections were serially ordered, mounted on gelatinized slides and coverslipped with DePeX (Electron Microscopy Sciences, Fort Washington, PA). Immunostained sections were examined by using a very sensitive method to demonstrate retinotectal projections.

**Microscopic Examination, Densitometry Analysis, and Estimation of the Volume of the SC**. An estimate of the volume of the retinotectal projection was obtained for retinal afferents to the contralateral SC, which in the rat receives from more than 95% of the contralateral RGC population. The total volume occupied by intensely CTB-labeled profiles in the stratum zonale (SZ) and the stratum griseum superficiale (SGS), the two most superficial visual layers of the contralateral SC, was estimated for each animal by using computerized image and mathematical analysis. Immunostained sections were examined under bright-field microscopy (Axiophot; Carl Zeiss Vision GmbH). A fixed frame (2.5 mm × 1.87 mm; area 4.675 mm$^2$) including the visual layers of the right SC was obtained with a digital camera and its software. Digitized images taken from every serial consecutive section of the SC of each animal analyzed for CTB labeling were converted to 8 bits of gray resolution (Photoshop, ver. 7.0; Adobe Systems, Mountain View, CA). Using image analysis (ImagePro; Media Cam HR; Carl Zeiss Vision GmbH, München-Hallbergmoos, Germany) and its software (AxioVision Software Release 3.1, ver. 3-2002; Carl Zeiss Vision Imaging Systems). Measurements included thickness of the outer nuclear (ON) layer, and of the inner nuclear and inner plexiform (IN and IP) layers combined. For each digitized image, a line was traced over the outer and inner borders of the designated layers, and the image analysis program averaged the thickness over the 420-μm length of the frame. All measurements from each of the eight different sections were pooled together to provide a mean value for each eye. Mean values from ischemic eyes in each group were pooled together and compared with those of the nonischemic fellow eyes. No attempt was made to analyze for differences in regional areas (central versus middle or periphery) of the retina.

**Microscopic Examination, Densitometry Analysis, and Estimation of the Volume of the SC**. An estimate of the volume of the retinotectal projection was obtained for retinal afferents to the contralateral SC, which in the rat receives from more than 95% of the contralateral RGC population. The total volume occupied by intensely CTB-labeled profiles in the stratum zonale (SZ) and the stratum griseum superficiale (SGS), the two most superficial visual layers of the contralateral SC, was estimated for each animal by using computerized image and mathematical analysis. Immunostained sections were examined under bright-field microscopy (Axiophot; Carl Zeiss Vision GmbH). A fixed frame (2.5 mm × 1.87 mm; area 4.675 mm$^2$) including the visual layers of the right SC was obtained with a digital camera and its software. Digitized images taken from every serial consecutive section of the SC of each animal analyzed for CTB labeling were converted to 8 bits of gray resolution (Photoshop, ver. 7.0; Adobe Systems, Mountain View, CA). Using image analysis (ImagePro; Media Cam HR; Carl Zeiss Vision GmbH, München-Hallbergmoos, Germany) and its software (AxioVision Software Release 3.1, ver. 3-2002; Carl Zeiss Vision Imaging Systems). Measurements included thickness of the outer nuclear (ON) layer, and of the inner nuclear and inner plexiform (IN and IP) layers combined. For each digitized image, a line was traced over the outer and inner borders of the designated layers, and the image analysis program averaged the thickness over the 420-μm length of the frame. All measurements from each of the eight different sections were pooled together to provide a mean value for each eye. Mean values from ischemic eyes in each group were pooled together and compared with those of the nonischemic fellow eyes. No attempt was made to analyze for differences in regional areas (central versus middle or periphery) of the retina.

**Analysis of the Retinotectal Projection**

After ERG recordings, the animals that had been used for time-course analysis of ERG changes (vehicle- $n = 7$) or BMD- $n = 8$ treated animals) as well as one half of the animals in the other group (vehicle- $n = 6$) or BMD-treated $n = 6$ animals) were processed to examine the retinotectal innervation. In addition, and for comparison, the volume of the retinotectal projection was determined in a group of untouched aged-matched rats ($n = 11$).

**Anterograde Labeling of Retinal Afferents**. Retinal afferents were investigated by using a very sensitive method to demonstrate retinotectal projections. Control and experimental animals were anesthetized and 5 μL of 1% solution of cholera toxin B subunit (CTB; List Biological Laboratories, Campbell, CA) were injected into the vitreous of the left eye for anterograde labeling of the retinal terminals. Four days later, serial coronal sections (40-μm-thick) extending through the diencephalon and brain stem were obtained on a freezing microtome, according to previously described methods. The rostro-caudal extension of the SC comprised 71 to 79 (71 ± 3; mean ± SD), 67 to 82 (72 ± 5), or 71 to 78 (73 ± 6) sections, for the vehicle- or BMD-treated or control group, respectively, corresponding to a rostro-caudal extension of the SC comparable to previous studies.

**Immunohistochemistry**. Orthogradely transported CTB was immunolocalized in free-floating sections by using previously described methods. Immunostained sections were serially ordered, mounted on gelatinized slides and coverslipped with DePeX (Electron Microscopy Sciences, Fort Washington, PA). Using image analysis (ImagePro; Media Cam HR; Carl Zeiss Vision GmbH, München-Hallbergmoos, Germany) and its software (AxioVision Software Release 3.1, ver. 3-2002; Carl Zeiss Vision Imaging Systems). Measurements included thickness of the outer nuclear (ON) layer, and of the inner nuclear and inner plexiform (IN and IP) layers combined. For each digitized image, a line was traced over the outer and inner borders of the designated layers, and the image analysis program averaged the thickness over the 420-μm length of the frame. All measurements from each of the eight different sections were pooled together to provide a mean value for each eye. Mean values from ischemic eyes in each group were pooled together and compared with those of the nonischemic fellow eyes. No attempt was made to analyze for differences in regional areas (central versus middle or periphery) of the retina.

**Analysis of the Retinotectal Projection**

After ERG recordings, the animals that had been used for time-course analysis of ERG changes (vehicle- $n = 7$) or BMD- $n = 8$ treated animals) as well as one half of the animals in the other group (vehicle- $n = 6$) or BMD-treated $n = 6$ animals) were processed to examine the retinotectal innervation. In addition, and for comparison, the volume of the retinotectal projection was determined in a group of untouched aged-matched rats ($n = 11$).

**Anterograde Labeling of Retinal Afferents**. Retinal afferents were investigated by using a very sensitive method to demonstrate retinotectal projections. Control and experimental animals were anesthetized and 5 μL of 1% solution of cholera toxin B subunit (CTB; List Biological Laboratories, Campbell, CA) were injected into the vitreous of the left eye for anterograde labeling of the retinal terminals. Four days later, serial coronal sections (40-μm-thick) extending through the diencephalon and brain stem were obtained on a freezing microtome, according to previously described methods. The rostro-caudal extension of the SC comprised 71 to 79 (71 ± 3; mean ± SD), 67 to 82 (72 ± 5), or 71 to 78 (73 ± 6) sections, for the vehicle- or BMD-treated or control group, respectively, corresponding to a rostro-caudal extension of the SC comparable to previous studies.

**Immunohistochemistry**. Orthogradely transported CTB was immunolocalized in free-floating sections by using previously described methods. Immunostained sections were serially ordered, mounted on gelatinized slides and coverslipped with DePeX (Electro...
Cybernetics) an identical thresholding was performed to binarize all the images and determine the area between the upper limit of the SZ and the lower limit of the SGS that was intensely labeled with CTB. Binarized images did not include area occupied by blood vessels. The area occupied by CTB-labeled retinal afferents in the SZ and SGS of the contralateral SC in each serial coronal 40-μm-thick section was fed into a worksheet (Sigma Plot 2002 for Windows; ver. 8.0 SPSS Inc., Chicago, IL) and a polynomial regression line (order 5; with an $r^2 > 0.91$ in all cases studied) was obtained for each individual SC and the integral of the function yielded the volume of the SC occupied by intense CTB labeling in each animal. This mathematical analysis allowed calculation of the volume of the SC, even in the few instances in which artifacts associated with histologic mounting (e.g., wrinkles, tears, folds, and tissue debris) made few serial sections unusable for measurement. Measurements were imported into a spreadsheet (Excel 97; Microsoft Corporation, Redmond, WA) for computation and graphing.

Statistical Analysis of Data

All averaged data are presented as means with standard deviations (SD). All morphometric and functional measurements and analysis were performed in a masked fashion. Statistical significance was assessed using nonparametric ANOVA (Kruskal-Wallis followed by Mann-Whitney) tests (Statistix V1.0; Analytical Software, Tallahassee, FL). Differences were considered significant when $P < 0.05$.

RESULTS

ERG Recordings

ERGs 3 Months after LOV. Figure 1A shows the results obtained for the group of untouched age-matched albino SD rats. In the group of animals analyzed at 3 months after LOV, averaged data from the vehicle-treated group of rats showed similar values for the a-wave in experimental (ischemic) and control eyes (Fig. 1B). The b-wave amplitudes from vehicle-treated eyes, however, were significantly smaller at all light intensities when compared with the contralateral eyes (Fig. 1B). The BMD-treated group showed comparable a- and b-wave amplitudes in both eyes (Fig. 1C).

Consecutive ERGs at 1, 8, and 12 Weeks after LOV. To determine whether the changes in b-wave amplitudes appeared soon after LOV or were an evolving event, we examined additional groups of vehicle- and BMD-treated animals at increasing survival intervals. Figure 2 shows the average amplitudes of a- and b-waves from the vehicle-(Figs. 2A–C) and BMD-(Figs. 2D–F) treated groups recorded at 1, 8, and 12 weeks after ischemia (Figs. 2A, 2D, and 2E, respectively) and 12 weeks after a 90-minute LOV. A significant decrease in the mean b-wave amplitude was observed at 8 and 12 weeks after ischemia ($^{*}P < 0.01$) and in the mean a-wave amplitude at 12 weeks after ischemia ($P < 0.05$). (D–F) Data from left (experimental) and right (control) eyes in rats pretreated topically in their left eyes 1 hour before transient ischemia with two 5-μL drops of saline (vehicle-treated rats; $n = 7$). ERG recordings were performed 1 (A), 8 (B), and 12 (C) weeks after the 90-minute LOV. A significant decrease in the mean b-wave amplitude was observed at 8 and 12 weeks after ischemia ($^{*}P < 0.01$) and in the mean a-wave amplitude at 12 weeks after ischemia ($P < 0.05$). (D–F) Data from left (experimental) and right (control) eyes in rats pretreated topically in the left eyes 1 hour before transient ischemia with two 5-μL drops of 0.5% brimonidine (brimonidine-treated rats; $n = 8$). ERG recordings were performed 1 (D), 8 (E), and 12 (F) weeks after a 90-minute LOV. There were no significant differences in the mean amplitudes of the a- or b-waves in this group of rats at any time interval.
were no differences in the a- and b-wave amplitudes recorded from ischemic and contralateral eyes (Fig. 2F).

Morphometric Analysis

Representative photomicrographs from paraffin-embedded sections 3 months after LOV are shown in Figure 3. Compared with the untouched eyes, there was a marked reduction in the thickness of the IN and IP layers in the ischemic eyes of the vehicle-treated rats. This was not observed in the BMD-treated rats. Morphometric analysis of the control nonischemic eyes of the vehicle- or BMD-treated groups showed comparable mean thickness of the ON layer (Mann-Whitney test, \( P = 0.158 \)) or of the IN and IP layers (Mann-Whitney test, \( P = 0.149 \); Fig. 4). In the vehicle-treated eyes, there was a significant decrease (approximately one third) in mean IN and IP layer thicknesses, when compared with the fellow nonischemic eyes (42.8 ± 6 \( \mu \)m vs. 60.8 ± 3.6 \( \mu \)m; \( n = 6 \); Mann-Whitney test, \( P = 0.005 \); Fig. 4A), whereas in the BMD-treated group of animals the mean thickness of IN and IP layers were comparable in the ischemic and fellow eyes (62.8 ± 6.8 \( \mu \)m vs. 64.3 ± 8.2 \( \mu \)m; \( n = 7 \); Mann-Whitney test, \( P = 0.68 \); Fig. 4B). The mean ON layer thickness of the ischemic and fellow eyes in the vehicle- or BMD-treated groups were comparable (Kruskal Wallis test, \( P = 0.15 \); Fig. 4).

Retinotectal Projection

Control Group. To determine the normal volume of the retinotectal innervation we measured CTB-immunostained areas in the two most superficial visual layers of the contralateral SC in 11 unlesioned rats. Injection of CTB into the left eye resulted 4 days later in CTB immunoreactivity that was characteristically confined to the pretectal region, the dorsal lateral geniculate nuclei, and the SC, all of which are the normal retinorecipient regions in the mesencephalon and midbrain.20,33–35,39,40 There was little variability in the extent or intensity of CTB labeling throughout the SC. One characteristic of CTB labeling33 is the intense staining within the two most superficial layers of the contralateral SC, the SZ and SGS, where RGC axons arborize and establish synaptic contacts with target neurons. This resulted in a continuous dark band that precluded identification of individual terminals or axonal arborizations (Fig. 5A). A typical distribution of CTB-labeled retinal afferents in a representative control animal is illustrated in Figure 5A. In the ipsilateral SC and within these same layers, where retinal afferents are known to be more sparse,1,20,39–41 CTB-immunoreactivity often delineated processes that could be resolved into single retinal arborizations (data not shown). In the present studies we analyzed the contralateral SC, which in the albino rat receives axons from more than 95% of the contralateral RGC population.1,2 Overall, taking into account all cryostat-sectioned midbrains (control and experimental), the rostrocaudal extension of the SC comprised 72 ± 4 (mean ± SD; \( n = 38 \)) consecutive sections, which correlates well with previous studies.20,35 The methodology used allows a quantitative estimation of the entire volume occupied by CTB-labeled axon terminals in the superficial layers of the contralateral SC. In the control group, this averaged 2.28 ± 0.18 mm\(^3\) (\( n = 11 \); Fig. 6A).
Vehicle-Treated Group. In this group, there was some variability in the extent of CTB-labeled terminals throughout the contralateral SC of individual animals; the mean volumes ranged from 0.399 to 1.750 mm³. Overall, however, the results in this group were rather consistent with a mean volume of CTB-labeled terminals of 1.261 ± 0.33 mm³ (n = 13; Fig. 6B). These volumes were significantly smaller than those in the control group of animals (Mann-Whitney test, P = 0.0000) and represented a reduction of approximately 54% in the volume of CTB-labeled terminals for this group of animals was 1.917 ± 0.22 mm³ (n = 14; Fig. 6C). When compared, these values were significantly greater than those obtained for the vehicle-treated group of animals (Mann-Whitney test, P = 0.00001) than those of the control group (Fig. 7).

Brimonidine-Treated Group. The distribution of CTB-labeled retinal afferents in the contralateral SC of the BMD-treated groups of animals was comparable to that found in the control animals. Most of the coronal sections showed typical dense CTB labeling of the superficial layers throughout the extent of the SC. A few coronal sections, however, showed small areas in which CTB-labeling was reduced in intensity, with occasional small areas devoid of it (Fig. 6C). The mean volume of CTB-labeled terminals for this group of animals was 1.917 ± 0.22 mm³ (n = 14; Fig. 6C). When compared, these values were significantly greater than those obtained for the vehicle-treated group of animals (Mann-Whitney test, P = 0.0000) and smaller (Mann-Whitney test, P = 0.0001) than those of the control group (Fig. 7).

DISCUSSION

Previous studies using retrogradely transported tracers to identify the population of RGCs that survive transient ischemia of the retina indicated that RGC loss is an ongoing process that may last up to 3 months after the initial insult. Thus, histologic measurements or functional assessments performed shortly after ischemia may underestimate the evolving anatomic and functional consequences of injury and thus the ultimate extent of injury and/or achieved protection. Using a combination of functional, morphometric, and densitometric analyses, we designed the present studies to investigate long-term effects of selective LOV on the outer and inner layers of the retina and on the main retinal output to the brain. In addition, we have further investigated the protective effects of BMD against long-term LOV-induced degeneration of the retina and its major projection to the brain. The main findings of this study can be summarized as follows. (1) ERG recordings 1 week after LOV revealed no changes in the amplitudes of the a- or b-waves in the saline- or BMD-treated groups of rats when compared with the respective nonischemic fellow eyes. At 2 months, however, there were marked reductions in the mean amplitudes of the b-waves in the saline-treated animals that represented approximately one half of the amplitudes recorded in the contralateral eyes. These reductions where also present at 3 months. In the BMD-treated group, however, the a- and b-waves of ischemic eyes were comparable to those recorded from the nonischemic fellow eyes, at both 2 and 3 months after ischemia. (2) Morphometric analysis of retinal layers at 3 months after LOV showed a mean reduction in thickness of the IN and IP layers of the left eyes in the vehicle-treated animals to approximately two thirds of the thickness in the contralateral eyes. These reductions were also present at 3 months. In the BMD-treated group, there were no reductions of retinal layer thickness. (3) In saline-treated animals, 3 months after 90 minutes of retinal ischemia, CTB-immunoreactivity in the visual layers of the contralateral SC had diminished to approximately one half of the values observed in the control group of animals. This marked diminution in CTB immunoreactivity resulted in areas that were virtually devoid of CTB immunoreactivity, reflecting ischemia-induced death of the parent neurons and the degeneration of their axons, arborizations, and synaptic terminals. Topical pretreatment with BMD resulted in preservation of the retinotectal innervation at 3 months in a volume that corresponded to approximately 84% of that occupied by retinal afferents in the control animals, indicating that BMD administration resulted in significant lasting protection against ischemia-induced degeneration of the retinotectal projection.
Correlative Functional and Morphologic Analyses of Retinal Layers

Functional assessment of outer and inner retina involved ERG recordings and examination of the a- and b-waves. A first group of vehicle-treated animals showed, at 3 months, significant decreases in the amplitude of the b-waves of the ERG. Another group of animals was then prepared and examined at progressive survival intervals after ischemia. This latter group had a substantial decrease in the b-wave amplitude in the saline-treated groups of rats between 1 and 8 weeks after injury. This indicates evolving functional changes and is consistent with previous studies showing that LOV induces progressive retinal damage. Although a significant decrease in the amplitude of the mean a-wave of the ERGs was recorded from the group of animals analyzed serially at 12 weeks after LOV, such a decrease was not found in the other group of animals that were recorded only at 3 months. Consistent with the later finding, the analysis of the retinas from one half of these animals did not show significant diminution in thickness of the ONL. Thus, our results are not conclusive, and further studies, perhaps at even longer survival intervals, are needed to determine whether the loss of photoreceptors is also involved in retinal injury induced by LOV, as has been shown in other models involving chronic bilateral carotid artery occlusion. Many studies have reported significant changes in the b-wave amplitude of the ERG recorded at 1 week after transient ischemia of the retina, but this was not found in our ERG recordings in the saline-treated group of rats at 1 week (Fig. 2A). This discrepancy could be due to the different techniques used to produce transient ischemia of the retina. We used selective ligation of the
ophthalmic vessels\textsuperscript{10,15} and all other previous investigators have used transient elevation of intraocular pressure above systolic levels to induce retinal ischemia, which results in more severe retinal damage.\textsuperscript{9–11,46} This methodologic difference could explain early severe alterations of the ERG.

**LOV and Degeneration of the Retinotectal Projection**

As previously shown by others\textsuperscript{33,46} and by this laboratory,\textsuperscript{20,34} intraocular injections of CTB in the control group of rats results in dense labeling of the visual layers in the contralateral SC, throughout its rostrocaudal and mediolateral extent (Fig. 6A). This was in contrast with the findings in the saline-treated groups of rats, in which large areas devoid of CTB labeling were found in the contralateral visual layers of the SC (Fig. 6B), resulting in reductions of the retinotectal projection to approximately one half of the values found in control animals (Fig. 7). LOV results not only in a diffuse\textsuperscript{10,14} but also in a focal pattern\textsuperscript{15} of RGC loss. Because in the rat, the SC represents the entire visual field of the contralateral eye and retinal axons deploy in the SC in a precise, topographically ordered manner\textsuperscript{2–4} one could expect to find a mirrored situation in the
visual layers of the tectum. Indeed, both diffuse diminution in density of CTB-labeled profiles and focal patchy, column-like areas virtually devoid of CTB-labeled terminals (Fig. 6B) were found. Although an impairment of the orthograde axonal transport of CTB from the retina toward the SC could explain the lack of labeling, it is more likely that the absence of CTB-label in the visual layers is a consequence of LOV-induced degeneration of retinal axons and death of the parent neurons, as has been shown in visual deafferentation studies. Previous studies in this laboratory with retrogradely transported neurotrophic factors used to identify RGCs, have shown that LOV results in the loss of approximately 70% of the original RGC population at 3 months. The present findings are in agreement with our previous study in which we used a sampling approach to estimate retinotectal afferents and showed that LOV induced the loss of approximately half of the retinotectal afferents 2 months after insult. Moreover, the present study further documents that LOV resulted 3 months after injury, at a time when RGC loss has reached its plateau, in massive loss of retinal afferents to the visual layers of the SC, the main retinorecipient region in the brain in rodents, involved in the integration of visual and other sensorial inputs such as orienting responses and head tracking to moving stripes.

**Protective Effect of BMD against LOV-Induced Retinal Damage**

In this study we also examined the effectiveness of BMD in preventing retinal damage, by analyzing ERGs as a functional index of the inner and outer nuclear layers, by measuring the thickness of inner retinal layers as a morphometric parameter of structural retinal damage, and by estimating the volume of the retinotectal projection to the contralateral SC as an indication of the integrity of the major retinal output to the brain in animals that were pretreated with saline or BMD. In the BMD-treated groups of rats, the amplitudes of the b-waves from both eyes were comparable at all time intervals. Accordingly, the retinas analyzed morphometrically showed no significant reductions in the thickness of their IN and IP layers. Thus, as for the vehicle-treated group of rats, there was a good correlation between the physiological and anatomic results (see Figs. 2, 4). Previous studies have shown the effectiveness of BMD in protecting against LOV-induced RGC loss in a dose-dependent manner. The present study reveals the lasting effects of BMD to prevent LOV-induced inner retinal damage, as assessed both functionally and morphometrically. The remaining animals from the BMD-treated groups were processed to estimate the volume of their retinotectal projection. Whereas at 3 months after injury, LOV resulted in the vehicle-treated animals in reduction of retinal afferents that approximates half of the normal projection, in the BMD-treated group of animals, the volume of the retinotectal projection approximated 84% that found in control animals (Fig. 7), thus showing that BMD provides persistent neuroprotection against LOV-induced degeneration of retinotectal afferents. The apparent discrepancy between the morphometric analysis and the volumes of retinotectal innervation in vehicle- versus BMD-treated groups of rats (two thirds versus no reduction in thickness, but a 50% vs. 16% reduction in volumes) probably reflects a worse preservation of the IN and IP layers and RGCs in the vehicle-treated rats versus the protection observed in the BMD-treated group.

The mechanisms underlying the long-lasting neuroprotective effects of BMD observed in the present studies are not fully understood, but it is plausible that BMD may halt the early RGC loss that follows retinal ischemia and this may result in prevention of further damage to the retina. Moreover, the sustained effects observed in this study may be related not only to the initial rescue of RGCs but to the preservation of other non-RGC neurons located in the INL. Previous studies have documented the neuroprotective effects of BMD against a variety of retinal injuries, and several mechanisms have been postulated to explain the neuroprotective effects of 2-adrenergic agonists, including activation of several intrinsic pathways toward cell survival after injury. It is known that 2-selective adrenergic agonists upregulate retinal levels of endogenous basic fibroblast growth factor (bFGF) and brain-derived neurotrophic factor (BDNF) and diminish intracellular levels of glutamate and aspartate after retinal ischemia. Moreover, topical administration of BMD, which results in upregulation of antiapoptotic factors such as bcl-2 and bcl-xl, may halt apoptotic degradation by maintaining the mitochondrial permeability and avoiding release of degradation-signaling factors, suggesting that BMD may act early on the cascade of events leading to apoptosis. Finally, the neuroprotective effects of BMD may also be explained by regulation of survival pathways in the retina through the activation of Müller cells which in response to injury may upregulate the expression of the neuroprotective protein Bcl-2. It is important that a neuroprotective compound not only prevent injury-induced neuronal degeneration but also maintain function. Optic nerve fiber loss has been shown to be accompanied by degeneration of relay neurons and their circuits in the main retinorecipient target regions of the brain: the SC and the lateral geniculate nucleus. Preservation of retinal afferents to the main target territories in the brain would ensure not only a normal visual input to retinorecipient layers of the SC but also the prevention of further secondary degeneration within retinorecipient target regions of the brain. Although BMD significantly diminished the loss of retinal afferents, it remains to be shown that it was also capable of preventing any further transneuronal degeneration of central visual targets.

**Conclusion**

In summary, our study shows that 3 months after a 90-minute LOV, there is profound degeneration of IN and IP retinal layers...
and of its major retinotectal projection. In addition, our results provide additional evidence to support the lasting neuroprotective effects of BMD against transient ischemia-induced retinal injury. Understanding degeneration after ischemia may provide insights into other progressive neurodegenerative diseases that may be associated with ischemia, such as glaucomatous optic neuropathy and are essential for the design of therapeutic strategies to prevent loss of sight.

Acknowledgments

The authors thank Angel Ferrández-Izquierdo for help with the mathematical analysis of the data and José M Bernal, María E. Aguiler, Rima Barhoum, and Isabel Cánovas for technical support.

References


