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Transient ischemia of the retina results in massive degeneration of the retinotectal projection: long-term neuroprotection with brimonidine

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Abstract

In adult rats, we have induced retinal ischemia and investigated anterogradely labeled surviving retinal ganglion cell (RGC) afferents to the contralateral superior colliculus (SC). The animals received topically in their left eyes two 5-μl drops of saline or saline-containing 0.5% brimonidine (BMD), 1 h before 90 min of retinal ischemia induced by ligature of the left ophthalmic vessels. Two months after ischemia, the anterogradely transported neuronal tracer cholera toxin B subunit (CTB) was injected in the ischemic eyes and animals were processed 4 days later. As controls and for comparison, the retinotectal innervation of unlesioned age-matched control rats was also examined with CTB. In control and experimental animals, serial coronal sections of the mesencephalon and brainstem were immunoreacted for CTB and the area and thickness of the two most superficial layers of the SC containing densely CTB-labeled profiles were estimated with an image analysis system. Ninety minutes of ischemia resulted 2 months later in reduced density of CTB-labeled profiles in the contralateral SC of the vehicle-treated rats, representing less than one half the area occupied by CTB-labeled profiles in control rats. This resulted in shrinkage of these layers and in the presence of areas virtually devoid of CTB immunoreactivity, suggesting orthograde degeneration of retinal terminals and/or decrease of anterograde axonal transport. Topical pretreatment with BMD resulted 2 months later in CTB immunoreactivity that occupied the superficial layers of the contralateral SC in an area of approximately 86% of that observed in the unlesioned control group of animals, indicating that BMD protects against ischemia-induced degeneration of the retinotectal projection, and preserves anterograde axonal transport.

Keywords: Retinal ischemia; Neuroprotection; Orthograde tracers; Brimonidine; Retinotectal system; Alpha-2 selective agonists; Superior colliculus; Anterograde degeneration; Anterograde axonal transport; Retinal ganglion cell axons; Cholera toxin B subunit

Introduction

In mammals, visual information processed in the retina is conveyed to retinorecipient nuclei in the brain by retinal ganglion cell (RGC) axons. In the rat, the main retinorecipient target region in the brain is the superior colliculus (SC) or optic tectum, where virtually all RGCs project (Lund, 1965). This structure is involved in the generation of integrated responses to visual, auditory and somatosensory stimuli (reviewed in Sefton and Dreher, 1985). The SC is a bilateral structure of the brainstem that contains several layers; the superficial layers, also known as upper or visual layers, receive their inputs from retinal and primary visual cortex afferents and comprise the stratum zonale (SZ), stratum griseum superficiale (SGS) and stratum opticum (SO) (Huber and Crosby, 1943). In the SC, RGC axons are deployed in a very precise topographic ordered manner (Linden and Perry, 1983; Sauvé et al., 2001, 2002).

The retinotectal pathway is a suitable region of the central nervous system to investigate the effects of transient ischemia of the retina as well as to develop strategies toward its prevention (Vidal-Sanz et al., 2003). Using retrogradely transported neuronal tracers to identify the RGC population, we have recently shown that transient ischemia of the retina induces RGC loss, whose severity and duration is related to the initial ischemic interval (Lafuente et al., 2001, 2002a). Furthermore, a proportion of the RGC population that

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survive ischemia shows an impairment of the retrograde axonal transport (Lafuente et al., 2002c), implying that not all RGCs surviving retinal ischemia retain their normal physiological properties. Although the effects of transient ischemia on RGC survival have been studied extensively (Goldblum and Mittag, 2002; Junk et al., 2002; Lafuente et al., 2002a; Sellés-Navarro et al., 1996), very little is known about the impact of retinal ischemia on the primary optic pathway; the retinotectal projection. An understanding of the effects of ischemia on the retinofugal projection is important for future studies that may develop neuroprotective strategies, because these will need not only to increase RGC survival but also to preserve the integrity of their main output to the brain as well as their function.

The characterization of the pattern and course of RGC death that follows transient ischemia has allowed quantitative studies to investigate the neuroprotective effects of certain compounds to halt ischemia-induced RGC loss. Indeed, recent studies have documented that ischemia-induced RGC loss may be prevented with neuroprotective substances (Vidal-Sanz et al., 2000) including alpha-2 selective adrenergic agonists (α_2SA) (Lafuente et al., 2001, 2002b; Lai et al., 2002; Vidal-Sanz et al., 2001). A recent study has shown that treatment with brimonidine (BMD), an α_2SA, may also preserve retrograde axonal transport in RGCs after retinal ischemia (Lafuente et al., 2002c).

BMD is an α_2SA that is currently used to treat glaucoma patients because of its lowering effects on the intraocular pressure (Schuman et al., 1997). In addition to its intraocular pressure-lowering effects, BMD has been shown to have neuroprotective effects in a variety of injury-induced RGC death experimental models in adult rats, including ischemia–reperfusion (Lafuente et al., 2001, 2002a,b), chronic elevation of the intraocular pressure (WoldeMussie et al., 2001) and partial crush of the optic nerve (Yoles et al., 1999). Currently, BMD is under evaluation for its neuroprotective effects in several clinical trials of glaucoma-related diseases (Wheeler et al., 2003). α_2SA are thought to provide neuroprotection by activation of alpha-2 adrenoceptors (for review, see Wheeler et al., 2003). The mechanism by which this is accomplished is not well understood, but activation of alpha-2 adrenoceptors inhibits pro-apoptotic mitochondrial signaling (Tatton et al., 2001) and induces increased synthesis of survival factors such as basic fibroblast growth factor and BCL-2 in the retina (for review, see Wheeler et al., 2003).

In the present studies, we have studied the effects of transient retinal ischemia on the retinotectal projection and investigated the neuroprotective effects of BMD. We have used cholera toxin B subunit (CTB), a very sensitive anterogradely transported axonal tracer, to identify retinal afferents to the contralateral SC, 2 months after 90 min of transient ischemia induced by selective ligature of the ophthalmic vessels. We show that in saline-treated rats, retinal ischemia results 2 months later in a marked reduction of the retinotectal projection. Within the two most superficial layers of the contralateral SC, there was shrinkage as well as areas virtually devoid of retinal afferents. Approximately one half of the area normally filled with retinal terminals was lost in saline-treated animals, indicating an alteration of anterograde axonal transport in injured neurons or anterograde degeneration of retinal fibers whose parent neurons die following ischemia. Topical pretreatment with BMD resulted in preservation of the retinotectal innervation in an area that corresponds to approximately 86% of the area occupied by retinal afferents in control animals. This indicates long-term neuroprotective effects of BMD to prevent the loss of RGCs and the anterograde degeneration of their terminals, and implies that a major physiological property of these cells, the anterograde axoplasmic flow, is also preserved. (Parts of this work have been presented in abstract form; Vidal-Sanz et al., 2001; Villegas-Pérez et al., 2002.)

Materials and methods

Animals, anesthetics and drug administration

Experiments were performed on adult female albino (Sprague–Dawley) rats (180–200 g) obtained from the breeding colony of the University of Murcia (Murcia, Spain). The animals were fed ad libitum and maintained in cages in temperature-controlled rooms with a 12-h light–12-h dark cycle (light period from 8 AM to 8 PM; light intensity within the cages from 8 to 24 lx). All efforts were made to minimize the number of animals used and their suffering. Experiments were carried out in accordance with the European Union guidelines for the use of animals in scientific research and adhere to the ARVO statement for the use of animals in ophthalmic and vision research.

All the experimental manipulations, except induction of ischemia, were carried under general anesthesia with a mixture of ketamine (75 mg/kg, i.p., Ketolar; Parke-Davies, S.L., Barcelona, Spain) and xylazine (10 mg/kg, i.p., Rompún; Bayer, S.A., Barcelona, Spain). Because xylazine might activate α_2 adrenoceptors (Wen et al., 1996) and ketamine has neuroprotective effects against ischemia (Hoffman et al., 1992), for the induction of transient ischemia, rats were anaesthetized with 7% chloral hydrate (0.42 mg/g, i.p.). During recovery from anesthesia, the rats were placed in their cages, and a steroid–antibiotic ointment (Fludronef®; Iquinosa, Madrid, Spain) was applied on the cornea to prevent corneal desiccation. Animals were sacrificed with an overdose of the mixture of ketamine and xylazine.

Two groups of experimental animals were prepared for this study. The first group (*vehicle group*) was prepared to investigate the effects of retinal ischemia on the retinotectal innervation. These animals received on the left eye, 1 h before retinal ischemia, two 5-μl drops of 0.9% NaCl. The second group (*BMD group*) was prepared to investigate if the effects of retinal ischemia on retinotectal afferents

could be prevented with BMD, an α_2-selective adrenoceptor agonist also known as AGN190342 or UK-14,304 (Cambridge, 1981). BMD protects against transient ischemia-induced RGC death when administered 1 h before retinal ischemia or up to 4 h after retinal ischemia (Lafuente et al., 2001). Because pretreatment results in optimal prevention of ischemia-induced RGC death (Lafuente et al., 2001, 2002b,c), these animals received on the left eye, 1 h before retinal ischemia, two 5-μl drops of 0.9% NaCl containing 0.5% BMD. Both groups of animals were analyzed 2 months after ischemia; at this time, rats weighed around 300 g. We therefore examined the retinal afferents to the contralateral SC in an additional group of intact rats (280–310 g) (*unlesioned control group*). BMD was provided by Allergan (Irvine, CA) for these studies.

Surgical manipulations

Induction of transient ischemia

We have induced 90 min of ischemia in the left eye, an interval that results in consistent and predictable patterns of RGC loss (Lafuente et al., 2001, 2002a,b,c). Selective ligature of the ophthalmic vessels (SLOV) was induced following already described methods (Lafuente et al., 2001, 2002a; Otori et al., 1997; for review, see Vidal-Sanz et al., 2003). In brief, under deep anesthesia, the left optic nerve (ON) head was exposed in the orbit (Vidal-Sanz et al., 1987), the superior aspect of its dural sheath opened longitudinally, a 10/0 nylon suture introduced between the dural sheath and the ON and tied around the sheath, to interrupt blood flow through the ophthalmic vessels, which run in the inferior and nasal aspect within the sheath (Janes and Bounds, 1955; Morrison et al., 1999; Sugiyama et al., 1999). Care was taken not to damage the ON. Interruption of retinal blood flow during ischemia was assessed by direct ophthalmoscopy of the eye fundus through the operating microscope (Lafuente et al., 2001, 2002a). 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. Eye fundus inspection was facilitated because most eyes appeared mydriatic after the induction of transient ischemia. When necessary, a drop of 1% tropicamide (Colirio de Tropicamida, Alcon-Cusi Laboratorios, Barcelona, Spain) was applied topically to dilate the pupil.

Anterograde labeling of retinal afferents

To investigate the retinotectal projection, we have taken advantage of a method that demonstrates retinofugal projections with great sensitivity (Angelucci et al., 1996; Avilés-Trigueros et al., 2000; Wu et al., 1999). This consists of intraocular administration of the anterograde neuroanatomical tracer CTB, which is transported along the axonal projection, and the identification of retinal terminals in the brain by its immunolocalization. Four days before sacrifice, animals were anaesthetized and 5 μl of 1% solution of CTB (List Biological Laboratories, Campbell, CA, USA) was injected into the vitreous of the left eye.

Tissue processing and analysis

Two months after retinal ischemia, and 4 days after CTB application, rats were deeply anaesthetized and perfused transcardially through the ascending aorta first with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were dissected out from the skull, post-fixed overnight in the same fixative at 4°C, cryoprotected by immersion in a solution of 30% sucrose in PB for 48 h at 4°C and frozen in isopentane cooled in liquid nitrogen. Serial coronal sections (40-μm-thick) of the brain from the level of the anterior thalamus to the rostral pole of the cerebellum were obtained on a cryostat.

Immunohistochemistry

Orthogradely transported CTB was immunolocalized using previously described methods (Angelucci et al., 1996; Avilés-Trigueros et al., 2000). In brief, free floating sections were washed in PB, and endogenous peroxidase activity was blocked by immersing sections in 0.3% hydrogen peroxidase in PB for 20 min. Sections were rinsed again in PB, incubated for 30 min in 0.1 M glycine in PB and then overnight at 4°C in PB containing 0.5% Triton X-100, 4% normal rabbit serum (NRS) (Vector Laboratories, Burlingame, CA), and 2.5% bovine serum albumin (BSA) (Boehringer Mannheim, Mannheim, Germany). The following day, sections were incubated in a solution containing goat anti-CTB antibody (List Biological) diluted 1:4000 in a PB solution containing 2% NRS, 2.5% BSA and 2% Triton X-100 during 4 days at 4°C. Binding of primary antibody was visualized by incubating in biotinylated rabbit anti-goat IgG antibody (Vector Laboratories) diluted 1:200 in 2% NRS, 2.5% BSA and 2% Triton X-100 in PB for 1 h at room temperature, followed by an incubation in avidin–biotin peroxidase complex (Vectastain ABC Kit Elite; Vector Laboratories) diluted 1:100 in PB for 1 h; the peroxidase was detected using 0.025% 3,3′ -diaminobenzidine tetrahydrochloride (Sigma) in PBS as a chromogen. After 5 min, 0.004% H_2O_2 was added to the solution, and 3 min was allowed for development. Sections were rinsed thoroughly in PB at 4°C and then serially ordered and mounted on gelatinized slides, air-dried, dehydrated in a series of alcohols, defatted in xylene and coverslipped with DePeX.

Microscopic examination and analysis

In many mammals, including humans, the dorsal lateral geniculate nuclei (dLGN) are the major retinorecipient nuclei that relay visual information to the primary visual cortex (Lund et al., 1974). However, in rats, these nuclei

only receive afferents from approximately 35% of the RGC population (Martin, 1986), whereas the SC receive afferents from over 95% of the contralateral RGC population (Cusick and Lund, 1982; Dreher et al., 1985; Linden and Perry, 1983; Perry, 1981). Thus, for the present studies aimed at examining the fate of the retinal projection to the brain, as a whole, we decided to study retinal afferents to the contralateral SC. Retinal afferents and their distribution in the brain were inferred from immunohistochemical detection of CTB on the assumption that the presence of CTB indicates anterograde transport of this substance by RGC axons from the left eye to their axonal terminals in the SC.

The density of CTB-labeled profiles in the two most superficial layers of the contralateral SC was measured using computerized image analysis. Immunostained sections were homogeneously illuminated and examined under bright field microscopy (Axiophot, Carl Zeiss, Oberkochen, Germany). Digitized images were taken from 37 coronal consecutive sections in a mid-tectal location (the most rostral one being located at 1 mm from the habenular commissure), using image analysis software (ImagePro Plus version 4.5.1.27; Media Cybernetics, Silver Spring, MD). A fixed frame (2.5 mm × 1.87 mm, area 4.675 mm^2) (Fig. 1A), aligned on the left with the midline and on the top with the upper limit of the right SC was digitized (8 bits per channel of color RGB resolution) with the aid of a CCD camera (CoolSNAP™; RS Photometrics, Tucson, AZ) and its software (CoolSNAP™ v.1.2 program; RS Photometrics). Digital images were converted to 8 bits of grey resolution by using Adobe® Photoshop® version 7.0 (Adobe Systems, Mountain View, CA) for PC computers. A manual thresholding was carried out to binarize the images and determine the area of interest comprised between the upper limit of the SZ and the lower limit of the SGS (Fig. 1B). The percental area of each of the 37 sections was averaged to obtain a mean percent area for each animal. All measurements were taken without any filtering or contrast enhancement of the image, and in a masked fashion by the same investigator.

The thickness of both SZ and SGS was analyzed in the digitized images taken from 10 consecutive coronal sections of each animal at a middle level of the rostrocaudal and lateromedial axes of the right SC. Measurements were done on a predefined rectangular area (1.5 mm × 1.87 mm) positioned within the centre of the captured digitized image. Using the manual threshold, the upper limit of the SZ and lower limit of the SGS were defined using the auto-trace tool from the Image-Pro Plus™ (IPP) measurements menu. Average thickness for each section was determined using the curve thickness function of IPP, and the 10 measurements were averaged to obtain a mean thickness of both layers for each animal.

Measurements were imported into a spreadsheet (Microsoft® Excel® 97; Microsoft Corporation, Redmond, WA) for computation and graphing. Statistical analysis of the differences between groups of animals was performed using nonparametric ANOVA tests (Statistix V1.0; Analytical Software, Tallahassee, FL). Differences were considered significant when $P < 0.05$.

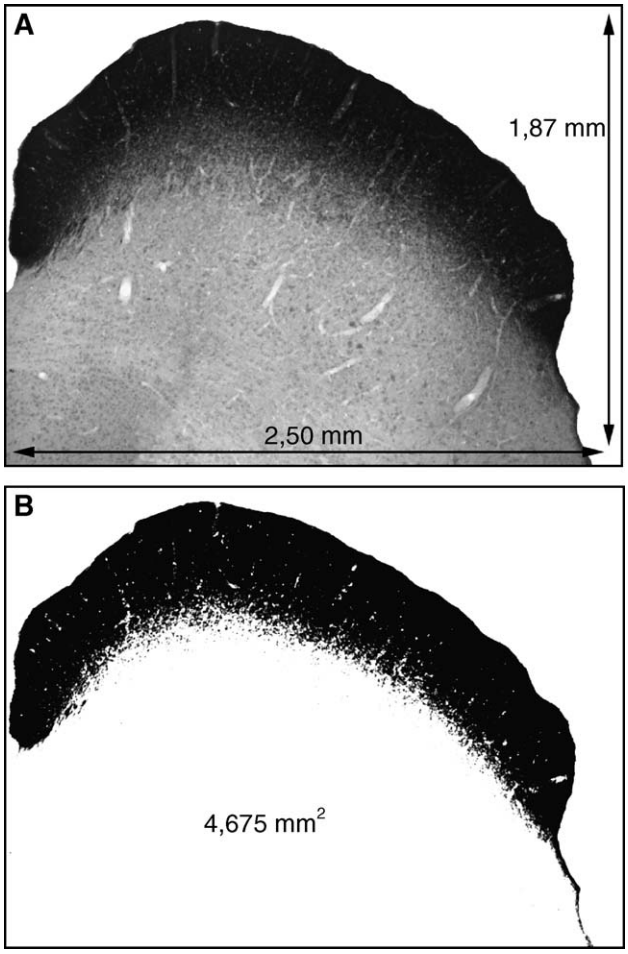

Fig. 1. Light micrograph of a 40-μm-thick cryostat coronal section through the midbrain showing labeled retinal afferents in the superficial layers of the right superior colliculus 4 days after CTB injection into the contralateral eye. (A) The picture shows a standard area of 4.675 mm^2 that was captured for image analysis. (B) A manual thresholding was carried out to binarize the image and define the limits between the SGS and SO to measure by densitometry the percentage of the area densely occupied by CTB–retinal afferents in the two most superficial layers of the SC. Dorsal is to the top and medial to the left.

Results

Using qualitative and quantitative neuroanatomical techniques, the current studies were conducted to investigate the effects of 90 min of retinal ischemia on retinal afferents that project to the visual layers of the main rat retinorecipient target in the brain: the SC. In addition, we have investigated the neuroprotective effects of BMD against ischemia-induced degeneration of the retinotectal projection. The main findings of this study can be summarized as follows: (i) In control animals, CTB injection into the left eye results 4 days later in very dense CTB immunoreactivity within the

superficial layers of the right SC. (ii) In saline-treated animals, 2 months after 90 min of retinal ischemia, CTB immunoreactivity occupying the two most superficial layers of the contralateral SC was reduced to less than half of the values observed in the unlesioned control group of animals. This marked diminution in CTB immunoreactivity resulted in shrinkage of these layers and in the presence of areas that were virtually devoid of CTB immunoreactivity. (iii) Topical pretreatment with a single dose of BMD resulted 2 months later in preservation of the retinotectal innervation in an area that corresponds to approximately 86% of the area occupied by retinal afferents in control animals.

Unlesioned control group of animals

Injection of CTB into the vitreous of intact control animals resulted 4 days later in the presence of CTB immunoreactivity within the retinorecipient regions of the mesencephalon and brainstem, as has been previously reported in detail using similar techniques for other rodents (Ling et al., 1998; Vercelli et al., 2000). CTB immunoreactivity was very intense within the contralateral retinorecipient nuclei and scarce in the ipsilateral nuclei. In the present studies, we have analyzed the contralateral SC, which in the albino rat receives axons from over 95% of the contralateral RGC population (Lund, 1965), and the small proportion of retinal afferents that project ipsilaterally (Linden and Perry, 1983; Vercelli et al., 2000) was not analyzed in detail. In the unlesioned control animals, CTB-labelled neurons in the oculomotor nuclei and their axons were found consistently labeled. Such labeling, which was also present in the experimental groups of animals, is interpreted as the retrograde labeling of these neurons by the tracer that leaked out of the eye into the extraocular muscles at the time of CTB intraocular injection (Avilés-Trigueros et al., 2000; Vercelli et al., 2000).

There was little variability in the extent or intensity of CTB labeling throughout the SC. The visual layers of the contralateral SC appeared intensely labeled with CTB, with highest densities of CTB immunoreactivity in the SZ and SGS, where RGC axons arborize, differentiate and establish synaptic contacts with target neurons. The limits between the SGS and the SO were outlined by a difference in the intensity of CTB immunoreactivity. In the SO, the immunoreactivity was mostly confined to its upper half, from which the density decreased steadily in a dorsoventral direction so that CTB immunoreactivity delineated only a few scattered retinal axons in the lower SO. There was very little label if any within deeper non-visual layers of the SC. As is characteristic of dense projections labeled with CTB (Angelucci et al., 1996), the intense labeling within SZ and SGS of the contralateral SC resulted in a continuous band that precluded the identification of single terminals or axonal arborizations (Figs. 1A, 2A). However, within these same layers on the ipsilateral SC, CTB immunoreactivity often delineated processes that could be resolved into single retinal arborizations (data not shown) (Ling et al., 1998; Lund, 1969; Lund et al., 1980; Vercelli et al., 2000). Because CTB was injected into the left eye 4 days before animal processing, we interpret CTB immunoreactivity as RGC terminals that have become labeled by anterograde transport of the tracer from the retina toward their target region in the brain.

In the unlesioned control group of rats, CTB-labeled terminals in each animal appeared in 68–76 consecutive 40-μm-thick coronal sections of the SC, which corresponds to the rostrocaudal extent of the SC comparable to previous studies (LeVere, 1978). The mean area of the digitized frame occupied by CTB-labeled retinal afferents in the SZ and SGS of the contralateral SC (Fig. 2A) (expressed as percentage of the 4.675 mm^2 analyzed for each section) was 22.9%, 24.6%, 23% and 18%, respectively, for each of the control animals, with a mean value of 22.1 ± 2.9% (mean ± SD; $n = 4$) (Fig. 3). This represents approximately one fourth of the digitized frame (Fig. 1). A typical pattern and distribution of CTB-labeled retinal afferents in one of these unlesioned control animals is illustrated in Fig. 2A.

The thickness of the two most superficial layers of the contralateral SC was 430, 427, 424 and 299 μm, respectively, for each of the control animals, with a mean value of 395 ± 64 μm (mean ± SD; $n = 4$). This mean thickness is comparable to that previously reported by other authors (García del Caño et al., 2002; Warton and Jones, 1985).

Vehicle-treated group of animals

In the vehicle-treated group of animals, there was some variability in the extent and intensity of CTB labeling throughout the right SC of individual animals but, overall, the results of this group of animals were rather consistent. When compared to intact age-matched controls, there was a marked reduction in the amount of CTB-labeled retinal afferents in the two most superficial layers of the contralateral SC of the vehicle-treated animals. The percentage of the digitized frame occupied by CTB-labeled retinal afferents in the SZ and SGS was 6.5%, 5.2%, 14.9%, 5.5%, and 1.4%, respectively, for each of the vehicle-treated animals, with a mean group value of 6.7 ± 4.9% (mean ± SD; $n = 5$) (Fig. 3). These values, which corresponded to approximately 1/10 of the area analyzed (Fig. 1), were significantly smaller than those obtained in the unlesioned control group of age-matched animals (Mann–Whitney test, $P = 0.007$). A typical pattern and distribution of CTB-labeled retinotectal afferents for a representative vehicle-treated rat is illustrated in Fig. 2B.

In this group of animals, within the SZ and SGS of the right SC, there were small patches in which CTB labeling was much reduced, allowing observation of individual axons and their terminal arborizations (Figs. 4A, B). In addition, there were areas with very little to virtually none CTB immunoreactivity (Figs. 4A, B). These areas varied in size and shape within different consecutive coronal sections

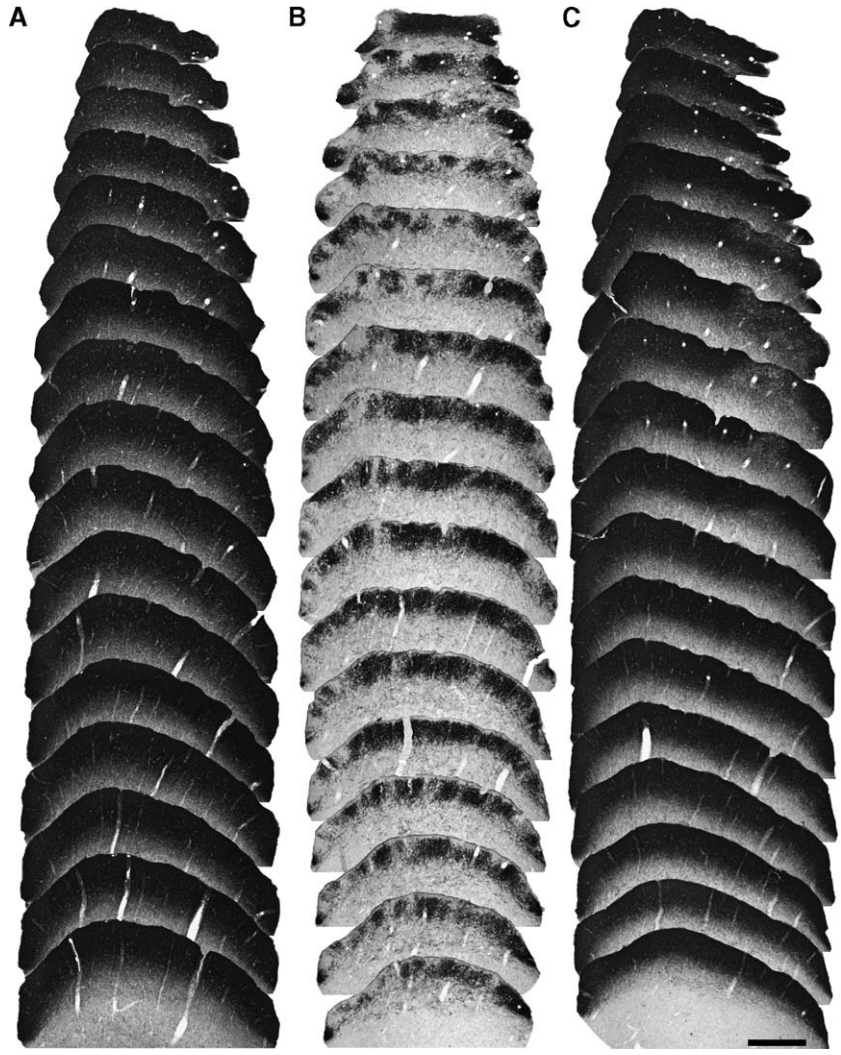

Fig. 2. Light micrographs illustrating every fourth 40-μm-thick cryostat serial coronal section through the mid-SC in its rostro- (top) caudal (bottom) extent, from one representative control unlesioned rat (A) or rats that were treated 1 h before 90 min of retinal ischemia in the left eye with two 5-μl drops of saline (B), or saline-containing 0.5% BMD (C). To identify retinotectal afferents, the left eye was injected with the tracer CTB 4 days before animal processing and 2 months after retinal ischemia. The side contralateral to the eye injection shows density and distribution of CTB-labeled retinal afferents in the superficial layers of the SC. Brown dark areas correspond to RGC terminals anterogradely labeled with CTB. In the right SC of the unlesioned rat, very densely labeled retinal axons fill the superficial layers (A). In contrast, note the reduction of CTB-labeled profiles in the superficial layers of the right SC in the vehicle-treated rat (B), which results in reduced thickness and in areas virtually lacking retinal axons. The densities of CTB-labeled profiles in the BMD-treated group of rats (C) were significantly greater than those of the vehicle-treated group of rats (B). The mean area occupied by CTB-labeled retinal afferents to the contralateral SC of the individual rats shown in A, B, or C (expressed as percentage of the area analyzed) was 22.8%, 5.29%, or 20%, respectively. Dorsal is to the top and middle is toward the left. Scale bar = 500 μm.

of the same animal and within individual animals. In some cases, the size of these areas was restricted to small patches within the upper or the lower half of the SGS, but often presented the form of a column extending in the dorso-ventral axis from the lower limit of the SGS to the upper limit of the SZ, in a manner that resembles the deployment of axon terminals in the SC of the rodent (Ling et al., 1998), suggesting degeneration of retinal axons and their terminal arborizations. The lateral extension of these areas varied from a small narrow column to almost one half of the medio-lateral extension of the SC (Fig. 4), whereas in the rostro-caudal extension of the SC, the patches varied from being observed in a few consecutive serial coronal sections to almost 10–12 consecutive coronal sections (Fig. 2B).

In this group of animals, the thickness of the SZ and SGS taken together was 142, 118, 254, 152 and 18 μm, respectively, for each individual animal, with a mean thickness for this group of 136 ± 84 μm (mean ± SD). When compared to the unlesioned control group, these values are significantly smaller (Mann–Whitney test, $P = 0.007$) and represent a reduction of over one half of its normal value (Fig. 2B). These animals also showed a qualitative reduction in the number of CTB-labeled retinal afferents in the SO (Figs. 2B, 4).

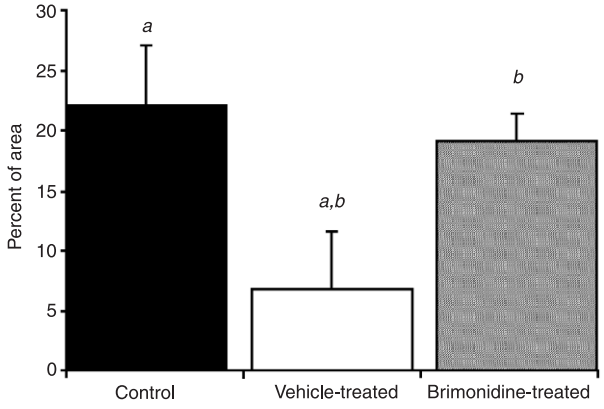

Fig. 3. Bar histograms showing mean (± SD) percent of the area that was occupied by heavily CTB–retinal afferents to the contralateral superior colliculus (SC) in 37 consecutive coronal sections (see Fig. 1). Experimental rats were treated 1 h before 90 min of retinal ischemia with two 5-μl drops of saline (vehicle-treated), or saline-containing 0.5% BMD (BMD-treated) and were processed 2 months after retinal ischemia to quantify retinotectal afferents orthogradely labeled with CTB. Retinal afferents to the contralateral SC in four intact age-matched rats (unlesioned control) were also examined. Ninety minutes of retinal ischemia in the left eye resulted 2 months later in marked reductions of the density of CTB-labeled profiles in the contralateral SC of the vehicle-treated group of rats, representing less than one half the area occupied by CTB-labeled profiles in the unlesioned control group of rats. Pretreatment with BMD 1 h before retinal ischemia resulted in significantly greater densities of CTB-labeled profiles when compared to the vehicle-treated groups. Unlesioned control group, $n = 4$; vehicle-treated group, $n = 5$; BMD-treated group, $n = 6$. [a]Significantly different (Mann–Whitney test, $P = 0.007$). [b]Significantly different (Mann–Whitney test, $P = 0.002$).

BMD-treated group of animals

The vehicle-treated and unlesioned control group of animals established the baseline to examine the extent to which ischemia-induced degeneration of the retinotectal projection could be diminished with BMD pretreatment. In the BMD-treated group of animals, the distribution of CTB-labeled retinal afferents within the contralateral SC was qualitatively rather comparable to that found in the unlesioned control group of animals and most sections showed intense, normal looking, CTB immunoreactivity within the upper layers of the right SC. There were, however, some coronal sections showing small areas with reduced intensity, but not devoid of CTB immunoreactivity (Fig. 2C).

The percentage of the digitized images occupied by CTB immunoreactivity in the SZ and SGS was 20.2%, 17.6%, 21.7%, 20%, 15.5% and 19.9%, respectively, for each of the BMD-treated animals, with a mean group value of 19.1 ± 2.2% (mean ± SD; $n = 6$) (Figs. 2C, 3). These values represented approximately 86% of the values obtained in the unlesioned control group and were slightly but not significantly smaller (Mann–Whitney test, $P = 0.057$). When compared to the values obtained for the vehicle-treated group of animals, the innervation of the SC in the BDM-treated group of animals was significantly greater (Mann–Whitney test, $P = 0.002$), representing almost a 3-fold increase. A typical pattern and distribution of CTB immu-

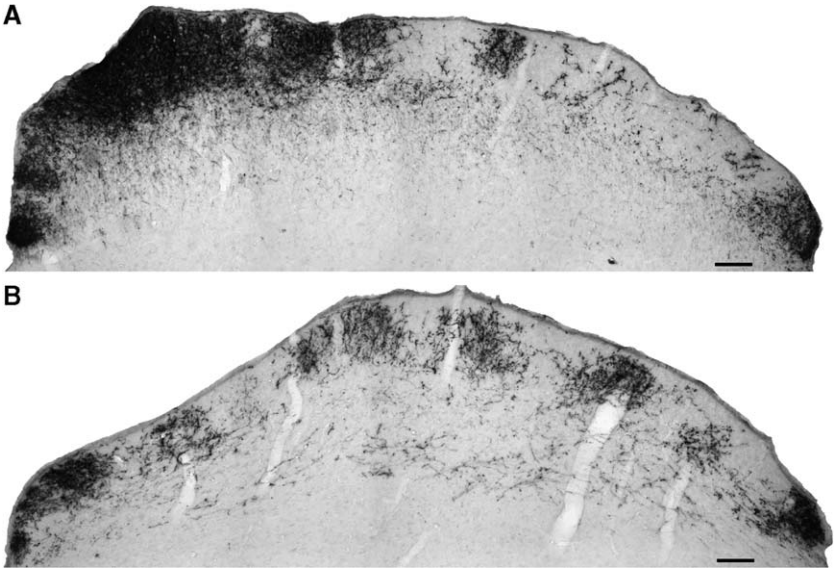

Fig. 4. Light micrographs illustrating at higher power the density and distribution of CTB-labeled retinal axons and terminals on two representative coronal sections through the right mid-SC of two different rats that were treated, 1 h before 90 min of retinal ischemia in the left eye, with two 5-μl drops of saline and processed 2 months later. Retinotectal afferents were visualized with CTB. Brown dark areas correspond to RGC axons and their terminals anterogradely labeled with CTB. In contrast to unlesioned control animals (see Fig. 1) in which the density of the contralateral retinotectal projection is very dense for the two most superficial layers of the SC, in the vehicle-treated animals, there was a marked reduction of CTB-labeled profiles. In addition, there were areas virtually lacking CTB-immunoreactive fibers; these areas varied in size and extension but often had the form of a column (A, B). Dorsal is to the top and middle is toward the left. Scale bar, A, B = 100 μm.

noreactivity for a representative BMD-treated rat is illustrated in Fig. 2C.

The thickness of SZ and SGS in the right SC of these animals was 356, 287, 354, 350, 242 and 282 μm, respectively, for each of the BMD-treated animals, with a mean value of 311 ± 48 μm (mean ± SD). This mean thickness represents 78% of the thickness obtained in the unlesioned control group of animals and is significantly smaller than that found in controls (Mann–Whitney test, $P = 0.03$), but significantly greater than that found in the vehicle-treated animals (Mann–Whitney test, $P = 0.004$).

Discussion

Using CTB as an anterograde neuronal tracer to identify retinal afferents, we have examined the retinofugal projection to the contralateral SC in control rats, and observed the degenerative changes induced in this projection by 90 min of retinal ischemia. Our results show that this injury leads 2 months later to a profound degeneration of the retinotectal projection resulting in a decrease of density and area occupied by contralateral retinotectal afferents. The present study also documents that ischemia-induced degeneration of retinal afferents may be prevented with topical administration of BMD.

Retinal ischemia leads to anterograde degeneration of retinotectal afferents

The automated densitometric analysis of the visual layers in the contralateral SC of vehicle-treated rats documents that transient ischemia of the retina results 2 months later in large reductions of CTB immunoreactivity. Because in all the animals analyzed we have consistently found ocular motoneurons that were CTB labeled with a similar degree of intensity, small variations in the efficiency of the immunohistochemical reaction to demonstrate the presence of CTB cannot explain the reduction of CTB immunoreactivity found in the vehicle-treated animals. This assumption is further supported by the consistency of the CTB immunoreactivity found in coronal sections from both unlesioned control and BMD-treated groups of animals, and by the intensity of CTB staining in those regions of the SC of the vehicle-treated group where the immunoreaction was present. Variations in intensity of the immunohistochemical reaction were minimized by processing sections from four different animals together. In addition, differences in background intensities were controlled with the threshold of the image analysis program which allowed differentiation of specific CTB labeling from background labeling. Thus, a more likely explanation for the decrease in CTB immunoreactivity following retinal ischemia is either a functional impairment of the anterograde axonal transport of CTB from the retina toward its terminals in the SC or the anterograde degeneration of retinal afferents following death of their parent neurons.

The reduction of CTB-labeled retinal terminals observed in the SC of the vehicle-treated animals could be explained by an impairment of the RGCs anterograde axonal transport. Marked, selective and persistent alterations in the slow component of the anterograde axonal transport (McKerracher et al., 1990a,b, 1993) and in the synthesis of cytoskeletal proteins (McKerracher et al., 1993) have been reported following injury to the optic nerve in adult rats. In addition, transient ischemia of the retina induces alterations of the retrograde axoplasmic transport in rats (Lafuente et al., 2002c) and monkeys (Levy and Adams, 1975; Radius, 1980; Radius and Anderson, 1980). In rabbits, transient ischemia of the spinal cord has been shown to slow down both the slow (Chavco et al., 1987) and the fast axonal transport (Malatova et al., 1989) and to alter microtubule stability (Shackelford and Nelson, 1996). Thus, we cannot rule out the possibility that in the present studies, retinal ischemia may have altered anterograde axoplasmic transport of CTB from the RGCs in the retina toward their axon terminals in the SC, resulting in diminished levels of immunoreactivity.

In the vehicle-treated group of animals, there was also an important reduction in thickness of the two most superficial SC layers. Although this finding could be explained by an impairment of anterograde transport, it is more likely due to the death of RGCs and the associated anterograde degeneration of their axons and terminals. Indeed, shrinkage of the visual layers of the SC has been reported following eye removal in the adult rat, which also leads to anterograde degeneration of RGC axonal arborizations and terminals in the visual layers of the contralateral SC (García del Caño et al., 2002; Lund, 1969; Lund and Lund, 1971a,b; Tsang, 1937). Moreover, previous studies from this laboratory have documented that in adult rats, 90 min of retinal ischemia induce 2 weeks later the loss of approximately one half of the RGC population (Lafuente et al., 2001). Thus, it is likely that ischemia-induced RGC loss is responsible for the shrinkage of the SZ and SGS in the SC of the vehicle-treated rats. Because optic nerve fiber loss has been shown to be accompanied by degeneration of relay neurons and their circuits in the lateral geniculate nucleus (Weber et al., 2000; Yücel et al., 2000) and SC (Lund, 1969; Lund and Lund, 1971a,b; Tsang, 1937), it is also possible that shrinkage of the visual layers in the vehicle-treated rats may be due not only to RGC axonal degeneration but also to transneuronal degeneration within the SC itself.

In the vehicle-treated animals, there were areas within the superficial layers of the SC virtually devoid of CTB-labeled retinal terminals. Retinal axons project to the SC in a precise topographic ordered manner (Linden and Perry, 1983; Sauvé et al., 2001, 2002). Because of this retinotopy, the observation of areas virtually devoid of CTB-labeled retinal terminals would imply the death of neighbouring RGCs within corresponding areas of the retina. In a recent study, in which

RGCs were retrogradely labeled 1 week after retinal ischemia to identify surviving RGCs capable of retrograde axonal transport (Lafuente et al., 2002c), it was found that RGC loss was not evenly distributed throughout the retina, but there were patches of the retina in which RGCs were sparsely distributed (see Figs. 2A, B and 3A, B in Lafuente et al., 2002c). The location and extension of these patches varied within different quadrants of the retina and between animals (Lafuente et al., 2002c). Thus, it is very likely that the areas lacking CTB-labeled retinal terminals in the SC of the vehicle-treated group of animals in the present study represent the lack of anatomical projection from such retinal patches lacking RGCs in our previous report (Lafuente et al., 2002c).

The present studies do not allow us to estimate the amount of RGC loss that is responsible for the reduction in the area occupied by retinal afferents observed in the SC of the vehicle-treated rats, nor whether these degenerative changes represent an ongoing process. Anterograde tracing with CTB has been demonstrated to be very sensitive and capable of identifying even the finest projections both in the intact (Angelucci et al., 1996; Ling et al., 1998) and regenerated (Avilés-Trigueros et al., 2000; Carter and Jhaveri, 1997) rodent visual pathway. However, for large projections, such as the retinotectal projection, CTB results in a dense band that precludes identification of single retinal terminal arborizations. Thus, a normal-looking density of CTB immunoreactivity only implies that a substantial proportion of retinal afferents innervate the SC, and at present, we do not know how many RGCs need to degenerate before we can observe a clear deficit in the retinotectal projection. On the other hand, it is known that retinal deafferentation of the SC might induce sprouting and neoformation of synaptic boutons by remaining retinal fibers in the visual layers of the adult rat SC (García del Caño et al., 2002; Lund and Lund, 1971a,b). Thus, we cannot rule out the possibility that 2 months after retinal ischemia, the surviving retinal afferents may have also undergone some degree of terminal reorganization and expansion into neighbouring areas of the SC that have become visually deafferented as a consequence of retinal ischemia. An estimation of the time course and an accurate correlation between the amount of RGC loss and the associated reduction in retinotectal innervation would require additional studies analyzed at different survival intervals and using double labeling techniques to identify in the same animal the population of surviving RGCs in the retina and its retinofugal projection in the contralateral SC. Based on previous studies from this laboratory showing that RGC loss after retinal ischemia is an ongoing process that induces by 2 months the death of approximately 65% (Lafuente et al., 2002a), it is reasonable to predict that the degenerative changes observed in the retinotectal projection are a dynamic process and that at 2 months, the actual RGC loss in these experiments approximates 65% of the original RGC population.

BMD protects from ischemia-induced degeneration of retinotectal afferents

In addition to providing new information on the sequelae of retinal ischemia on the bulk of the retinal projection, our data provide the basis to examine the effects of pretreatment with BMD to prevent or diminish degeneration of retinotectal afferents. In the BMD-treated group of animals, CTB-labelled retinal afferents occupied an area of the SC that represented approximately 86% of that found in control animals. Previous studies from this laboratory have shown that pretreatment with BMD may prevent RGC loss (Lafuente et al., 2001) in a dose-dependent manner (Lafuente et al., 2002b) after retinal ischemia. A recent study, in which RGCs were retrogradely labeled from the SC 1 week after retinal ischemia and vehicle- or BMD-treated animals were analyzed 1 week later, showed that a proportion of RGCs surviving retinal ischemia in the vehicle-treated group had their retrograde axonal transport impaired, while pretreatment with BMD prevented such alteration (Lafuente et al., 2002c). Although this short-term study suggested that BMD could protect retinotectal axons from degeneration after retinal ischemia, our present results show unequivocally that the degeneration of the retinotectal projection induced by transient ischemia of the retina may be largely limited with BMD pretreatment. Furthermore, the presence of CTB-labeled retinal terminals in the SC, 4 days after intraocular injection of CTB into the contralateral eye, implies that surviving RGCs maintain their physiological property of anterograde axonal transport, even 2 months after the ischemic insult.

The techniques employed in the present experiments document that BMD prevents ischemia-induced degeneration of retinotectal afferents, but do not allow to quantify the number of RGC axons that were rescued with BMD. Further doubly labeled experiments would be required to correlate the retinotectal innervation preserved with BMD with the population of RGCs contributing to this projection. Our studies do not address the issue of whether BMD rescued retinotectal axons from ischemia-induced degeneration preserve or recover their normal visual input to the SC. However, the apparent normal retinotectal innervation of the SC in the BMD-treated group of animals may provide the anatomical substrate to further examine point-to-point sensitivity values across the tectum with physiological techniques that resemble human perimetry measurements (Sauvé et al., 1998, 2001) in these BMD-treated animals. Visual behaviors are largely mediated by the dLGN, the major retinorecipient nuclei relaying visual information to the primary cortex (Lund et al., 1974). Because in rats the dLGN is innervated by collaterals of the retinotectal axons (Martin, 1986), it is conceivable that innervation of the dLGN is also preserved in the animals treated with BMD. Indeed, although not analyzed in detail, the coronal sections of the mesencephalon showed an apparent normal retinal innervation of the dLGN in the BMD-treated group of

animals, and this would also provide the anatomical substrate for future studies designed to investigate visually related functions in these BMD-treated animals (Coffey et al., 2002; Lund et al., 2001).

Understanding the pattern of degeneration that occurs in the major retinofugal pathway following retinal ischemia will benefit ongoing studies conducted to develop neuroprotectant-based treatment strategies, and may provide insights into other progressive diseases that may also cause ischemia, such as glaucomatous optic neuropathy. The present studies show that following transient ischemia of the retina, there is considerable degeneration of retinal terminals in the main retinorecipient target region in the brain. Our studies do not allow us to differentiate between the relative contributions of an impaired anterograde axonal transport or retrograde RGC death in the diminution of CTB-labeled retinal afferents in the SC. However, our previous observations on the RGC loss in similarly prepared animals and the degree of degeneration in the retinal projection observed in the present studies suggests a link between loss of RGCs and degeneration of their axon terminals. Using this very same paradigm, we have also shown the neuroprotective effects of BMD in preventing degeneration of the retinotectal innervation.

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