Acute Endothelin-1 Application Induces Reversible Fast Axonal Transport Blockade in Adult Rat Optic Nerve

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Purpose. To investigate the effects of endothelin (ET)-1 on fast axonal transport in the optic nerve.

Methods. Sterile sponge soaked in 1 nM ET-1 was applied to the optic nerve surface of adult Brown Norway rats for 1 hour, after which 20% horseradish peroxidase (HRP) was placed over the superior colliculi (SC). Rats were killed 2, 4, 6, 24, and 48 hours later; the retinas and optic nerves were removed and fixed; and cut sections were processed histochemically, to visualize the time course of HRP transport by light microscopy. Naive and saline controls were processed identically. Retinal ganglion cell (RGC) survival after acute ET-1 application was investigated in another group of animals. After retrograde labeling of RGCs with the fluorescent neurotracer Fluorochrome (FG; Fluorogold; Fluorochrome Inc., Denver, CO), 1 nM ET-1 was applied to the optic nerve. Rats were then killed 5, 10, and 21 days later. The retinas were whole mounted and FG-positive RGCs were imaged and quantified with fluorescence microscopy.

Results. In naive controls, HRP labeling was observed over the entire nerve at 2 hours but had cleared by 48 hours. HRP labeling of RGCs started at 6 hours, and by 48 hours, uniform labeling was seen throughout the retina. In ET-1-treated optic nerves, transport of HRP was arrested at the distal portion of the nerve at 2 hours. Recovery of transport was evident from 4 hours. At 6 and 24 hours, all nerves showed full recovery with HRP-positive RGCs in the retina, but the ratio of the RGC counts in treated versus fellow untreated eyes (0.66 ± 0.13 and 0.67 ± 0.17, respectively) was less than that of the naive control (1.02 ± 0.28 and 1.05 ± 0.13, respectively) animals. At 48 hours, recovery was complete, and there was no significant difference in the ratio of RGC counts between ET-1 and naive control groups. No RGC loss was observed after ET-1 application.

Conclusions. Local acute application of ET-1 produces a reversible blockade of rapid axonal transport in optic nerve. (*Invest Ophthalmol Vis Sci.* 2008;49:961–967) DOI:10.1167/iovs.07-1243

Endothelin (ET)-1 is a peptide produced mainly by the vascular endothelium and released primarily abluminally.[1,2] As a potent vasoconstrictor regulating blood flow to tissues and organs, it has key physiological functions in the cardiovascular, renal, intestinal, and central nervous systems.[3–5] It has also been implicated in many diseases associated with blood flow disorders, including ischemic artery and heart disease,[6,7] diabetes,[8] Raynaud's disease,[9] cerebral vasospasm,[10,11] brain ischemia, and stroke.[12,13] In the eye, ET-1 is an important vasoactive peptide and acts as a mediator of many ocular physiologic and pathologic conditions.[2,14] It is synthesized and released mainly from iris–ciliary processes, vascular endothelial cells, and neurons, although ET-1 receptors are found throughout the anterior eye and on a variety of retinal cells.[15–17] Recent studies have suggested involvement of ET-1 in the pathogenesis of many eye disorders, including diabetic retinopathy,[18,19] light-induced retinal degeneration,[20] and exfoliation syndrome.[21] In the past decade many investigators have also suggested an important role for ET-1 in the pathogenesis of glaucoma. Elevated ET-1 levels have been found in the aqueous humor of patients with primary open-angle glaucoma and in a rat model of glaucoma,[22–24] whereas a higher concentration of plasma ET-1 has been shown in patients with glaucoma at either baseline or under stress, such as cold.[25,26] In monkey and rabbit chronic ET-1 application to the optic nerve causes a reduction in optic nerve blood flow and demonstrable optic nerve damage without elevation of intraocular pressure.[27–29] Our laboratory showed similar results in a rat model of optic nerve damage.[30] Recently, Lau et al.[31] reported that acute injections of ET-1 into rat eyes produced retinal ganglion cell (RGC) death. Several studies have shown blockade of axoplasmic transport in human glaucoma and experimental glaucoma in monkeys and rodents associated with RGC loss.[32–37] However, the mechanisms that lead to RGC loss after ET-1 delivery and features shared with pressure-induced damage are not known. As a first step, the purpose of this study was to investigate the effects of acute ET-1 application in rat optic nerve on fast axonal transport and possible effects on RGC survival.

Materials and Methods

Animals

Adult male Brown Norway rats (250–300 g) were used for all experiments. They were housed in a 12-hour light–dark cycle environment and given food and water ad libitum. All procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and ethics board approval was obtained from the Dalhousie University Committee on Laboratory Animals. Animal were anesthetized for surgery with a ketamine-xylazine-acepromazine cocktail and killed with an overdose of pentobarbital sodium (340 mg/mL). Both drugs were administered intraperitoneally.

A total of 68 animals were used for two different experiments: axonal transport and RGC survival. Details of the number of animals used in each experiment are shown in Table 1.

ET-1 Application

One week after acclimation, the rats were anesthetized, and the right optic nerve was carefully exposed by blunt dissection through an incision over the orbital ridge. A 1-mm^2 pellet of sterile water-insoluble

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TABLE 1. Number of Animals Used in Each Group of Experiments at Each Time Point

Groups	Time Point				
	2 h	4 h	6 h	24 h	48 h
Axonal transport after HRP application					
ET-1	4	4	4	4	4
Saline control	3	3	0	0	0
Naive control	3	3	4	4	4
		5 d		10 d	21 d
RGC survival after ET-1 application					
ET-1		4		4	4
Saline control		4		4	4

Data are the number of animals used at each time point.

sponge (Surgifoam; Ethicon, Inc., Somerville, NJ) soaked in 1 nM ET-1 was applied to the surface of the exposed nerve at ~1.5 mm behind the globe. Care was taken to minimize contact with the nerve. After 1 hour, the pellet was removed, and the exposed nerve was rinsed with saline. ET-1 was applied only to the right optic nerve.

Analysis of Axonal Transport

Animals were divided into three groups for axonal transport analysis: ET-1, saline control, and naive control groups. The number of animals used in each group is shown in Table 1. In the ET-1 group, the effects of ET-1 on axonal transport in the optic nerve was monitored by observing the retrograde transport of horseradish peroxidase (HRP Type VI; Sigma-Aldrich, St. Louis, MO). Immediately after ET-1 had been applied to the right optic nerve and the latter rinsed, sterile sponge soaked in 20% HRP was placed over both superior colliculi (SC) after the overlying cortex was aspirated. Rats were then killed after 2, 4, 6, 24, and 48 hours. For the saline control group, saline was used instead of ET-1 and animals were killed at 2 and 4 hours after HRP application. In the naive control group, RGCs and their axons were retrogradely labeled with HRP without application of ET-1 or saline to the optic nerve. Animals were killed at 2, 4, 6, 24, and 48 hours after HRP application.

Changes in the density of HRP labeling of optic nerves was assessed by measuring the gray level at each pixel along a line (1 pixel wide) drawn through the central 10 mm of digital images (16 bit, 9.4 pixels/mm) of the nerve using ImageJ (National Institutes of Health, Bethesda, MD). The density was corrected for changes in illumination by dividing the gray value at each point along the nerve by the gray value of a parallel line segment where no nerve was present to yield a ratio value. Changes in the density of labelling along the length of the nerve were calculated from the change of ratio from proximal to distal portions of the nerve.

Tissue Preparation and Histology

All animals were perfused with saline followed by 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After perfusion, the retina and entire length of both optic nerves were removed and postfixed with a solution of 1.25% glutaraldehyde and 1% paraformaldehyde in 0.1 M PB, for 30 minutes. The optic nerves were cryoprotected in 20% sucrose and embedded in OCT (Sakura Finetek USA., Inc. Torrance, CA), and 25-μm-thick sections were cut in the longitudinal plane. The retina was flattened with four radial cuts. Both the retina and optic nerve sections were reacted for HRP according to the tetramethylbenzidine procedure.[38] After HRP staining, the retina and optic nerve sections were placed on glass slides, dehydrated, coverslipped with mounting medium (Entellan; BDH, E. Merck, Darmstadt, Germany) and

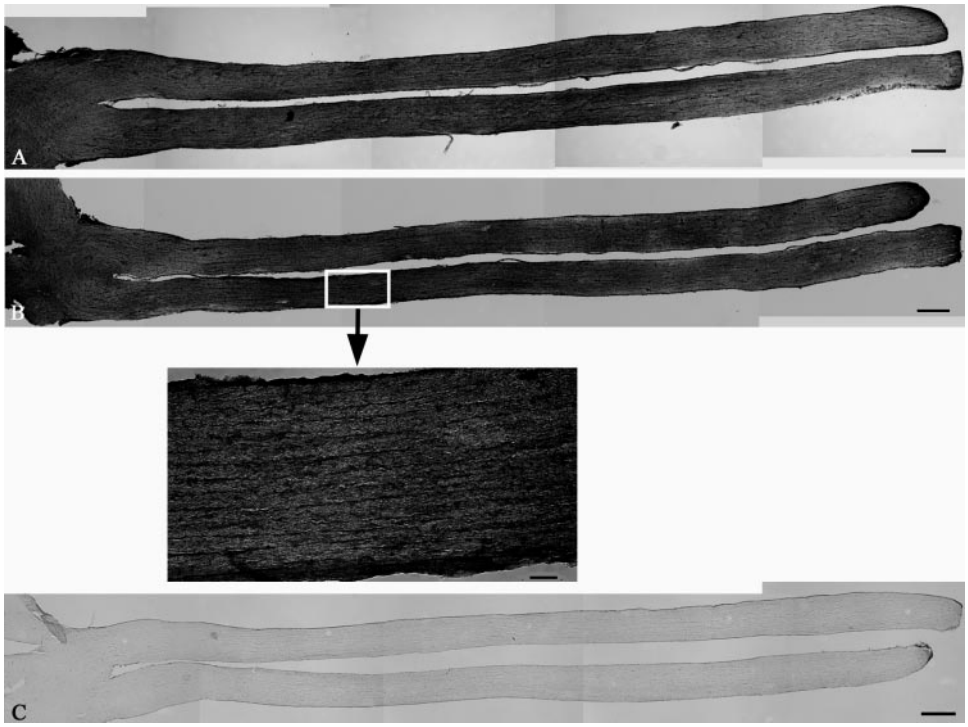

FIGURE 1. Longitudinal sections of optic nerve in naive animals killed at various time points after HRP application to the SC. At 2 hours (**A**), the axons over the entire length of the nerve were labeled evenly by HRP. The density of labeling increased slightly at 6 hours (**B**). *Inset*: high magnification of boxed area in (**B**) showing HRP-labeled axons in the optic nerve. At 48 hours (**C**), HRP was no longer evident within the optic nerve. Scale bars: (**A–C**), 500 μm; *inset*: 100 μm.

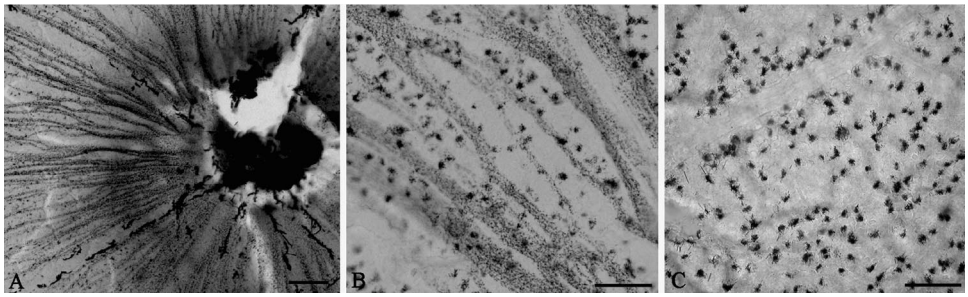

FIGURE 2. Low-magnification photomicrograph showing HRP-positive axons present throughout the whole retina of naive control animal 6 hours after HRP application to the SC (**A**). Higher magnification photomicrographs showing HRP labeled RGCs and axons in wholemount retinas of naive control animals 6 (**B**) and 24 (**C**) hours after HRP application to the SC. Scale bars, 100 μm.

examined with a motorized microscope (Axioplan 2; Carl Zeiss, Jena, Germany).

RGC Survival

RGC survival was investigated in another group of animals. Rats were divided into two equal clusters: an ET-1 group and a saline control group. RGCs were first labeled with the fluorescent neurotracer Fluorochrome (FG; Fluorogold, Fluorochrome, Inc., Denver, CO), as described previously.[30] After 7 days, ET-1 (1 nM) was applied to the optic nerve. The rats were killed 5, 10, and 21 days later ($n = 4$, for each group, shown in Table 1). The globe was fixed in 4% paraformaldehyde in 0.1 M PB for 3 hours. The retina was carefully dissected, flattened with four radial cuts, placed on a glass slide, and coverslipped with antifade medium (Citifluoro; Mirivac, Halifax, Nova Scotia, Canada). FG-labeled RGCs were visualized using the UV-2A filter of the motorized microscope (Axioplan 2; Carl Zeiss). Saline control rats underwent the same procedures, but with saline application instead of 1 nM ET-1.

Quantification of RGC Labeling and Survival

For all the retinas, digital photomicrographs (350 × 275 μm) were taken centered at 1, 2, and 3 mm from the optic disc center in each quadrant after carefully focusing on the RGC layer. HRP- and FG-positive RGCs were manually counted as described previously.[30] For each eye, RGC counts were averaged across each quadrant for the three retinal eccentricities, and RGC labeling and survival were expressed as a ratio of the counts in the experimental to fellow untreated eyes.

RESULTS

Axonal Transport

In naive control rats, HRP labeling was observed over the entire length of the optic nerve as early as 2 hours after application to the SC and remained labeled evenly up to 6 hours (Figs. 1A, 1B). At 24 hours, the density of labeling decreased over the entire length of the nerve, and only a few HRP-positive axons were found. HRP activity in the optic nerve was not apparent at 48 hours after the application to the SC (Fig. 1C). HRP-positive RGCs and their axons were first observed in all four retinal quadrants at 6 hours (Figs. 2A, 2B). Although at 24 hours there were virtually no HRP-labeled axons in the retina, RGCs labeled positively and evenly across the retina for HRP, indicating that the tracer had moved from the axons into the cell bodies (Fig. 2C). These findings were also evident at 48 hours.

In ET-1-treated optic nerves, all four rats showed clear interruption of HRP transport at 2 hours. The blockade started at the point of ET-1 application and extended back one fourth to one third of the optic nerve length toward the chiasm (Figs. 3A, 4A). At the same time point, control nerves treated with saline showed no blockade of HRP transport (Figs. 3B, 4D). Transport recovery started at 4 hours (Fig. 4B), but the density of labeling was slightly weaker when compared with the naive control. At 6 hours, the axonal transport completely recovered (Figs. 3C, 4C), and HRP labeling in the optic nerve was no different from that of the naive control (Fig. 1B). These findings were verified with the densitometric measurements (Fig. 4E).

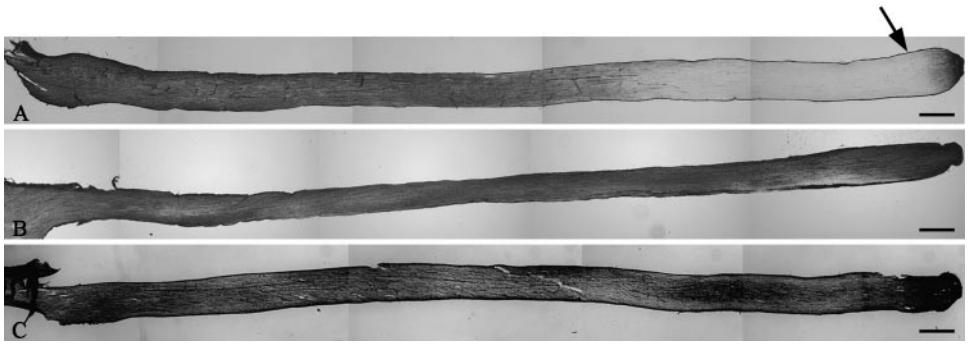

FIGURE 3. Photomicrographs illustrating time course of fast transport inhibition in optic nerves treated with ET-1 (**A**, **C**) or saline as control (**B**). At 2 hours, HRP labeling is reduced in the ET-1-treated nerve (**A**) and distal to the ET-1 application site (*arrow*). At 6 hours, HRP transport recovered completely (**C**). The saline control animal showed no blockade (**B**). The direction of the optic chiasm is on the *left* side of each section. Scale bars, 500 μm.

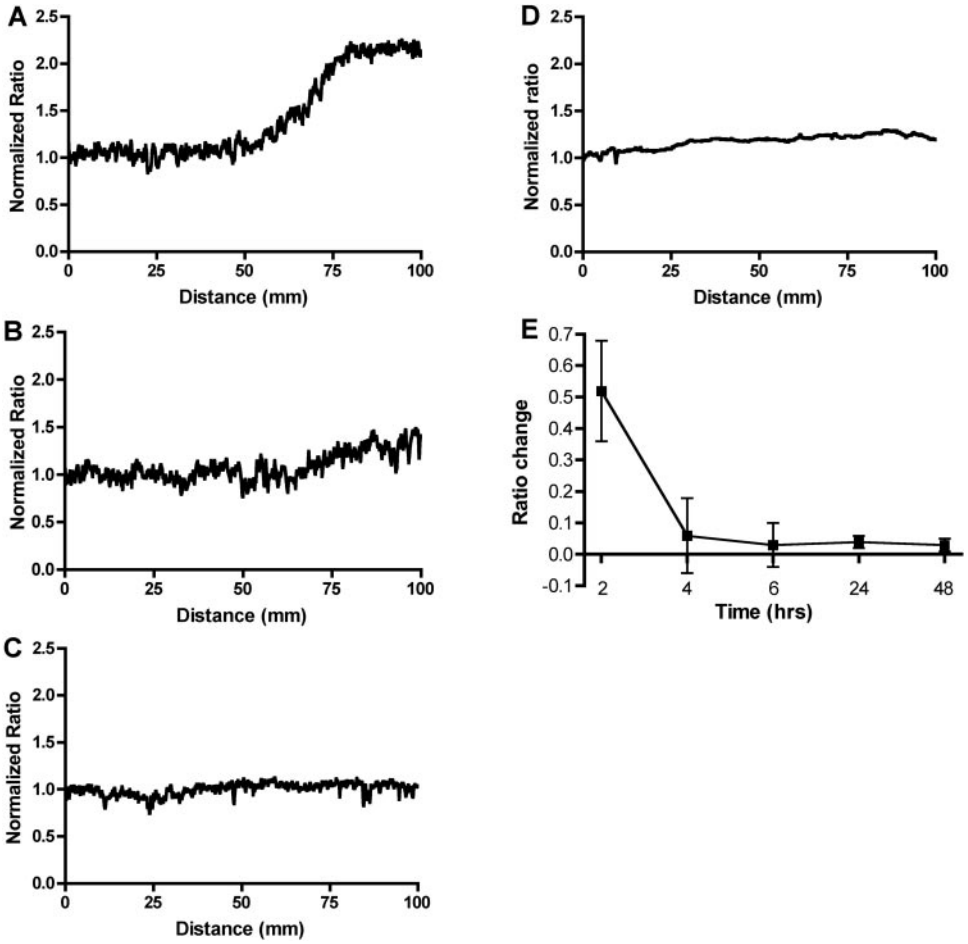

Figure 4. Representative densitometry of HRP labeling along the optic nerve at 2 (**A**), 4 (**B**) and 6 (**C**) hours after a 1-hour application of ET-1. There was an abrupt ratio change at 2 hours, indicating blockade of HRP. Saline-treated control nerve (**D**). Mean ratio (± SD) change at all time points (**E**) in all animals. The direction of the optic chiasm is on the *left* side of (**A**–**D**).

In the retinas corresponding to the ET-1-treated nerves, HRP-labeled RGCs were first observed at 6 hours (Fig. 5A). The number of HRP-positive cells was less than that in the contralateral retina in all animals. The ratio of the counts in the experimental to fellow untreated eyes in the ET-1 group was lower than that in the naive control group (0.66 ± 0.13 [mean ± SD] and 1.02 ± 0.28, respectively). This difference reached borderline statistical significance ($P = 0.059$; group *t*-test; Fig. 6). At 24 hours, the number of HRP-labeled cells increased significantly, although it was still less when compared with the number in the contralateral retina. The ratio of the counts in the ET-1 group at this time point was 0.67 ± 0.17 and was significantly lower than that in the naive control group (1.05 ± 0.13, $P = 0.011$; Figs. 5B, 5C, 6). At 48 hours, the number of HRP-labeled RGCs increased further, and there was no significant difference in the ratio of counts in the two eyes when compared with the naive control group ($P = 0.728$; Fig. 6).

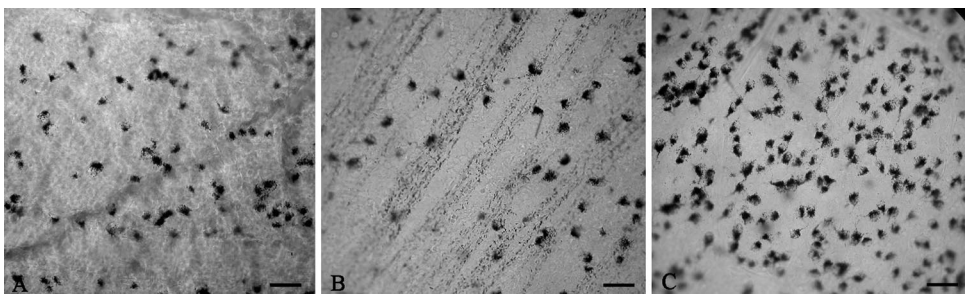

Figure 5. Photomicrographs showing HRP labeled RGCs and axons in wholemount retinas in ET-1 treated (**A**, **B**) and the fellow untreated eye (**C**) at 6 (**A**) and 24 (**B**, **C**) hours after HRP application to the SC. The density of HRP-labeled RGCs is less in the ET-1 treated side when compared to the fellow untreated one. Scale bars, 50 μm.

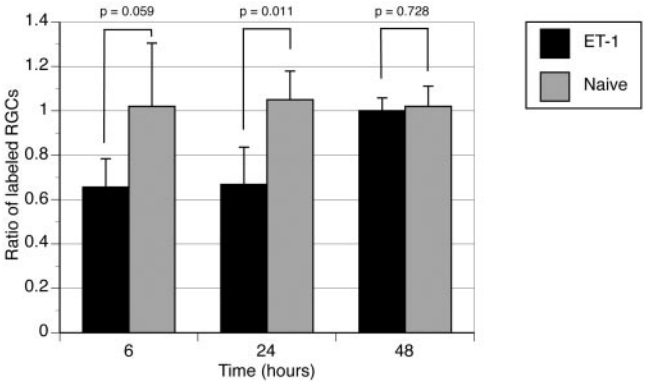

FIGURE 6. Mean ratio (experimental to fellow control retina) of HRP-labeled RGCs at 6, 24, and 48 hours after HRP application to the SC. HRP was applied immediately after nerves were treated with 1 nm ET-1 for 1 hour. Naive control data are shown for comparison. Error bars: ± SD.

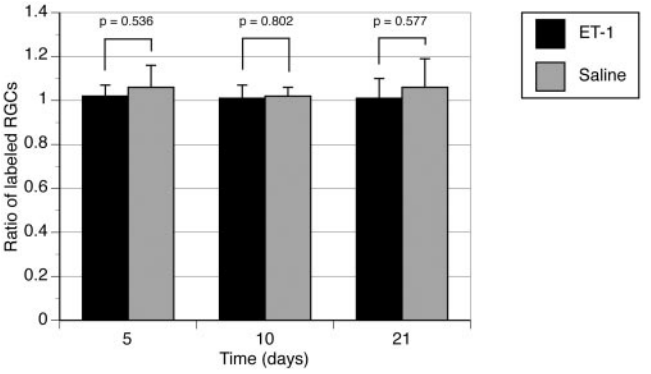

FIGURE 8. Mean ratio (experimental to fellow control retina) of FG-labeled RGCs at 5, 10, and 21 days after FG application to the SC. FG was applied immediately after nerves were treated with 1 nm ET-1 or saline for 1 hour. Error bars: ± SD.

RGC Survival

No FG-positive cells that were obviously microglia, macrophages, or fragments of FG debris, typically seen when RGCs die,[30] were observed in the retinas of saline-treated, ET-1-treated, or fellow untreated eyes at each time point (Fig. 7). Quantification of FG-positive RGCs indicated there was no RGC loss in the ET-1 or saline-treated retinas when compared with the fellow untreated eyes. The proportion of surviving RGCs is shown in Figure 8. The mean ± SD ratio of RGC survival in the ET-1 treated animals at 5, 10, and 21 days was 1.02 ± 0.05 (n = 4), 1.0 ± 0.06 (n = 4) and 1.01 ± 0.09 (n = 4), respectively. There was no significant difference from the ratios in the saline control groups, which were 1.06 ± 0.10, 1.02 ± 0.04, and 1.06 ± 0.13, respectively (P = 0.536, 0.802, and 0.577, respectively).

DISCUSSION

In this study, we systematically examined the time course of retrograde transport of HRP in optic nerves of adult Brown Norway rats. We also used HRP to study fast axonal transport after acute application of 1 nM ET-1 to the optic nerve. HRP has been used widely as a neural tracer in the visual system for over 30 years but most of the previous studies focused on visual system development, RGC labeling, and axonal projections in the visual cortex.[39–41] As a fast axonal transport tracer, HRP undergoes endocytosis into lysosomes and is retrogradely transported in the axon at a rate of 100 to 200 mm/day.[42] Its uptake and transport are influenced by the state of neural activity, possibly controlled by neuronal and hormonal systems, and so its movement is highly dependent on ATP and calcium.[38,42,43] To investigate axonal transport in optic nerve, most researchers have used tracers or radiolabeled proteins applied to the retina or different segments of the optic nerve.[32,44–46] In our work in naive and ET-1-treated animals, HRP was clearly an ideal tracer for assessing fast axonal transport in the optic nerve. The advantage of this method is that it can provide a direct visualization of the location of blockade and its longitudinal extent. The disadvantage of this method is that there can be differences in the density of staining in individual reactions. We attempted to minimize this with our densitometric technique.

A very short-term and focal application of 1 nM ET-1 to the optic nerve rapidly arrested retrograde transport of HRP, but the blockade was transient and was restored shortly after removal of ET-1. Saline applied in the identical manner did not arrest axonal transport. Our findings suggest that ET-1 application induces a temporal disorder of neural activity on fast axonal transport. Although other researchers have recently shown that intravitreal injection of ET-1 causes blockade of anterograde transport,[46,47] the mechanism by which ET-1 causes axonal dysfunction remains to be elucidated. We showed axonal transport blockade only at the distal portion of the optic nerve near the site of ET-1 application, suggesting that the effect of ET-1 is restricted to its application site. We showed in our previous studies that the concentration of 1 nM does not cause measurable reduction in blood flow,[30] suggesting that at this concentration ET-1 may not affect axonal transport via ischemia. However, we cannot rule out that ET-1 produces small focal areas of ischemia that are not measurable. On the other hand, local ET-1 application could alter other features of the microenvironment that are necessary for axoplasmic transport.[48] ET-1 has been shown to induce elevation

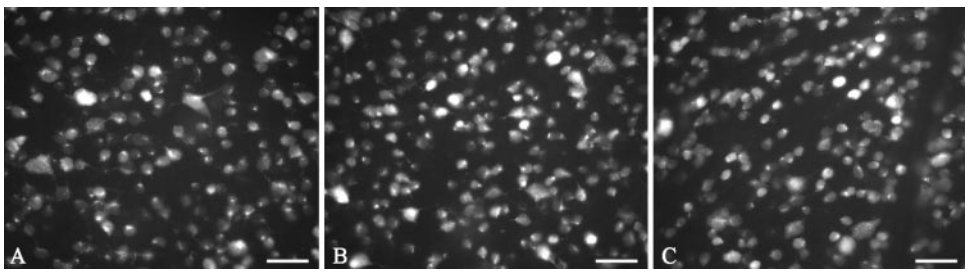

FIGURE 7. Fluorescence photomicrographs showing FG-labeled retinal wholemounts at 21 days after 1 hour treatment of the optic nerve with saline (**A**), 1 nM ET-1 (**B**), and the fellow untreated control (**C**). Neither a reduced number of RGCs nor signs of obvious damage were observed in the retina of saline- or ET-1-treated eyes, compared with the fellow untreated eye. Scale bars, 40 μm.

of intracellular calcium[49,50] and to reduce anterograde transport of mitochondria,[46] suggesting a possible disorder of energy supply in the optic nerve. Also, increased activity of astrocytes and matrix metalloproteinases in the optic nerve in the presence of ET-1 indicates the involvement of optic nerve glial cells in the neuropathic processes induced by ET-1 (LeVatte TL et al. *IOVS* 2007;48:ARVO E-Abstract 3289).[49,51]

In a prior study, we showed that low concentrations of ET-1 (including 1 nM ET-1) delivered chronically to the optic nerve leads to RGC loss (Archibald ML et al. *IOVS* 2005;46:ARVO E-Abstract 1239). In the present study, however, no RGC loss was observed after acute application of 1 nM ET-1. However, RGC loss may be observed if the duration of ET-1 application or its dose is increased, secondary to axonal injury caused by application of ET-1.

In summary, we have described a model of local and acute ET-1 delivery to the optic nerve and examined fast axonal transport in the optic nerve using HRP. The present study showed that local acute application of 1 nM ET-1 produced a reversible blockade of rapid axonal transport in the optic nerve, and that the application of this concentration and duration did not cause RGC loss. Further work is now under way to determine whether there is a critical period of axonal transport blockade that leads to RGC loss and mechanisms of ET-1-induced axonal transport blockade.

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