Optic Nerve Transection in Monkeys May Result in Secondary Degeneration of Retinal Ganglion Cells

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Purpose. Interest in neuroprotection for optic neuropathies is, in part, based on the assumption that retinal ganglion cells (RGCs) die, not only as a result of direct (primary) injury, but also indirectly as a result of negative effects from neighboring dying RGCs (secondary degeneration). This experiment was designed to test whether secondary RGC degeneration occurs after orbital optic nerve injury in monkeys.

Methods. The superior one third of the orbital optic nerve on one side was transected in eight cynomolgus monkeys (*Macaca fascicularis*). Twelve weeks after the partial transection, the number of RGC bodies in the superior and inferior halves of the retina of the experimental and control eyes and the number and diameter of axons in the optic nerve were compared by detailed histomorphometry. Vitreous was obtained for amino acid analysis. A sham operation was performed in three additional monkeys.

Results. Transection caused loss of 55% ± 13% of RGC bodies in the superior retina of experimental compared with fellow control eyes (mean ± SD, *t*-test, $P < 0.00{,}001$, $n = 7$). Inferior RGCs, not directly injured by transection, decreased by 22% ± 10% ($P = 0.002$). The loss of superior optic nerve axons was 83% ± 12% (mean ± SD, *t*-test, $P = 0.0008$, $n = 5$) whereas, the inferior loss was 34% ± 20% ($P = 0.02$, $n = 5$). Intravitreal levels of glutamate and other amino acids in eyes with transected nerves were not different from levels in control eyes 12 weeks after injury. Fundus examination, fluorescein angiography, and histologic evaluation confirmed that there was no vascular compromise to retinal tissues by the transection procedure.

Conclusions. This experiment suggests that primary RGC death due to optic nerve injury is associated with secondary death of surrounding RGCs that are not directly injured. (*Invest Ophthalmol Vis Sci.* 2001;42:975–982)

In the central nervous system (CNS), injury from various primary lesions, such as ischemia and trauma, can lead to widespread damage to neurons beyond the initial injury site.[1–6] This phenomenon is known as secondary degeneration and can result in greater loss of tissue than that caused by the initial disorder. Moreover, it may continue for an extended period after termination of the primary event. The secondary death of neighboring neurons and glia is believed to occur by apoptosis. A variety of mechanisms for this secondary degeneration have been proposed, including alteration of extracellular ion concentration, release of oxygen free radicals, and high levels of excitatory neurotransmitters.[4–9]

Because the optic nerve is part of the CNS, we decided to investigate whether secondary degeneration occurs also in the optic nerve. Damage to the optic nerve by diseases or trauma is one of the most frequent causes of blindness in the world. Axonal injury within the optic nerve leads inevitably to retrograde degeneration of retinal ganglion cells (RGCs) whose axons make up the optic nerve.[10–13]

The most common optic neuropathy is glaucoma. Glaucoma is the second leading cause of visual loss worldwide, with loss of peripheral vision due to the death of RGCs.[14] Studies of human and experimental glaucomatous eyes point to the optic nerve head as a major site of injury to RGCs.[15–17] At this location, RGC axons show morphologic and physiological indications of obstructed axonal transport. This may act as a functional transection of the axon at this site. It is not known whether there is additional secondary degeneration of RGCs that are not primarily injured in glaucoma or in other clinical or experimental optic neuropathies. If present, secondary degeneration could be a substantial additive factor in glaucoma damage, and its therapy could represent an important new avenue of treatment. Yoles and Schwartz[1] suggest that secondary degeneration occurs in RGCs after crush injury to the rat optic nerve, based on the fact that some RGCs die over a protracted period after the insult. However, in their paradigm, all axons may have been subjected to direct insult, making separation of primary and secondary degeneration difficult.

Experimental glaucoma models using intraocular pressure elevation cannot be used to test for secondary degeneration, because all RGCs are presumably exposed to the primary insult. There are, to our knowledge, no methods of identification that distinguish primary from secondary RGC death. To attempt to identify secondary degeneration in RGCs more clearly, we exploited the known topographic separation of RGCs in the primate retina and optic nerve. RGC bodies are separated into upper and lower retinal zones, divided by a horizontal raphe. RGCs with cell bodies quite close together above and below the raphe send their axons into the upper and lower poles of the optic nerve, where they are widely separated.[18] We performed partial transections of the upper third of the intraorbital nerve in monkeys, causing primary degeneration of upper RGCs. Our assumption was that RGCs of the inferior retina would be unaffected unless secondary degeneration occurred. If secondary degeneration were detected, its magnitude, distribution, and selectivity by RGC size class could then be estimated.

Materials and Methods

Eleven cynomolgus monkeys (*Macaca fascicularis*) were included in experiments, which were approved and supervised by the Animal Care Committee of the Johns Hopkins University School of Medicine and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Before surgery, color photographs of the optic disc, black and white photographs of the retinal nerve fiber layer, and

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fluorescein angiograms were obtained with the monkeys under intramuscular ketamine sedation (15 mg/kg) followed by Fluothane anesthesia (Wyeth–Ayerst, Philadelphia, PA) delivered by endotracheal intubation.

Eight monkeys underwent partial transection of one optic nerve, and three monkeys underwent only a unilateral sham operation. Partial optic nerve transection was performed under Fluothane anesthesia by a transorbital approach. The side to be operated on was chosen randomly. In brief, a conjunctival peritomy was performed and sutures placed under the four rectus muscles to control the position of the eye. The pupil was dilated with 1% tropicamide eye drops. After sterile preparation of the skin, a curved incision following the eyebrow and lateral orbital rim was made through skin and muscle. The lateral orbital wall was removed for approximately 5 mm with a Stryker saw and rongeurs. The optic nerve was identified after retraction of orbital fat with cotton pledgets. The dura of the superior nerve was focally incised with Vannas scissors at least 5 mm posterior to the globe. A sharp blade was used to transect the upper one third of the nerve. Animals in the sham operation group underwent the same procedure including the dural incision, but the optic nerve was not transected.

The retinal and choroidal circulations were inspected immediately after transection. None of the eyes had any detectable difference between the surgical and nonsurgical sides in blood flow or retinal color. The muscle sutures were removed and the peritomy closed with interrupted Vicryl sutures (Ethicon, Piscataway, NJ). The facial muscle incision was closed with 4-0 gut, and the skin was closed with interrupted 8-0 silk sutures. The eye was dressed with antibiotic ointment.

Animals were evaluated daily for 1 week for signs of distress and pain. None had any indication of postoperative complication. Fluorescein angiography was repeated 2 weeks after surgery, and nerve fiber layer and optic disc photographs were repeated 1 and 3 months after transection.

Twelve weeks after optic nerve surgery, the animals were killed by exsanguination under Fluothane anesthesia. The eyes were rapidly enucleated, and a slit was made in the pars plana with a razor blade. One milliliter of vitreous humor was aspirated from the midvitreous and immediately frozen. Aliquots of vitreous from transection and control eyes underwent amino acid analysis by a method identical with that used by Dreyer et al. (Bioresource Center, Cornell University, Ithaca, NY).[19]

The anterior segment was removed, and the posterior globe was fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer. After brief fixation, the retina was separated from the choroid and optic nerve head, and relaxing incisions were made in five to six areas to allow flat preparation, with one incision directly on the horizontal raphe. The vitreous was carefully removed from the retinal surface. The retina was stained with 0.05% cresyl violet and mounted with photoreceptors against the slide.

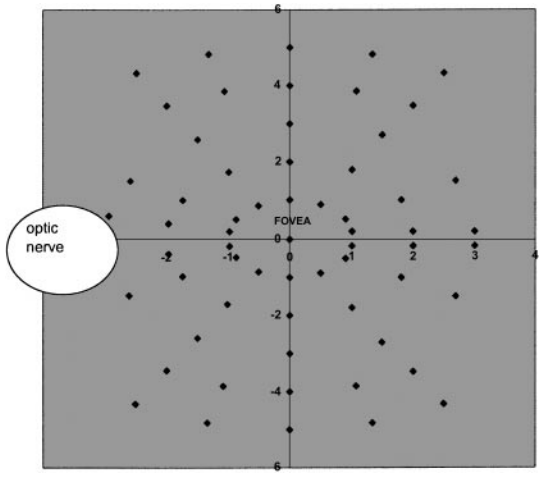

FIGURE 1. Diagram shows the 60 positions at which RGC density was evaluated along circles 1 mm apart, centered on the fovea, and divided by a horizontal line from the disc through the fovea. The fovea is shown but was not a sample location.

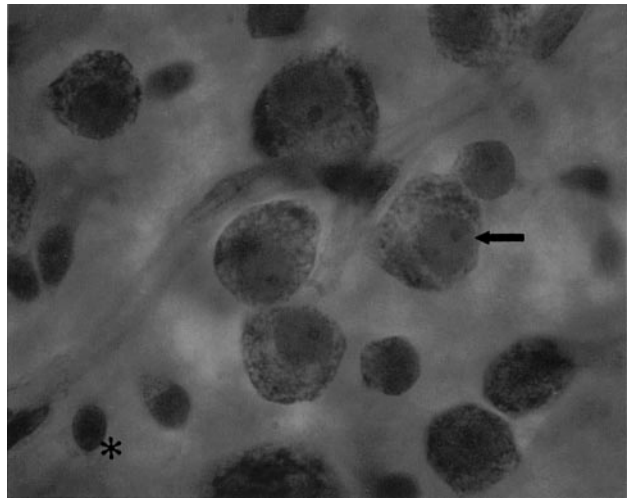

FIGURE 2. Retinal wholemount from a monkey eye at the level of the RGC layer. RGCs have large nuclei, frequently with visible nucleoli and with basophilic cytoplasm (*arrow*). Other smaller cells are probably glial cells or amacrine cells (*).

After 2 hours of immersion in fixative, a 1-mm-thick portion of the optic nerve was removed 1 to 3 mm from the globe, with razor slits marking the superior (one slit) and nasal (two slits) meridians for orientation after sectioning. The specimens were postfixed in 1% osmium tetroxide, dehydrated in alcohol, and embedded in epoxy resin. One-micrometer sections were stained with 1% toluidine blue.

One observer, masked to the procedure for each eye, used the retinal wholemounts to quantify RGC density. At ×1000 magnification and using a camera lucida and planimeter, 60 locations with an area of 0.022 mm^2 were identified along five circles from 1 to 5 mm in radius centered on the fovea (Fig. 1). An equal number of locations was counted in the superior and the inferior half of each retina. The total area sampled per retina was estimated to be 0.5% of total retinal area. RGCs were identified by their presence in the innermost nuclear layer and by cell and nuclear morphology. They exhibited large, round-to-oval nuclei, frequently with visible nucleoli and with basophilic cytoplasm (Fig. 2). Their diameter was most often greater than 7 μm. Glial cells and vascular endothelium and pericytes were easily distinguished from RGCs. It is possible that a small proportion of the cells identified were amacrine cells present in the RGC layer, although our previous investigations show that amacrines are rarely larger than 7 μm in diameter.[20,21]

As a second independent approach to counting RGCs, we quantified the number of RGC axons in the optic nerve cross section by a published method.[22] Briefly, the nerve area was determined by outlining it on a calibrated planimeter, and the area of connective tissues was excluded by planimetry. The nerve was divided into 16 segments by four radial and one centroperipheral division, and the area occupied by nerve bundles was measured planimetrically in each segment. Sixty-four areas were sampled for axon density (four per segment) with an image analysis system (Vidas; Carl Zeiss, Thornwood, NY). The total number of axons in each segment and in the entire nerve was calculated by multiplying the nerve bundle area of each segment by its density of RGC axons. This method samples approximately 4% to 5% of the total fibers in the normal monkey optic nerve. The analytic system measures axon diameter within the myelin sheath, providing the minimum diameter of each axon. Those performing the optic nerve counting were also masked as to the procedure performed on each nerve.

The estimated number of RGC bodies in the retina and axons in the nerve was compared between the overall nerves of transected and fellow eyes, as well as in subdivisions of retina and nerve, including

TABLE 1. Vitreal Glutamate Levels of Eyes with Partially Transected Nerves and the Controls

Subject	Transected	Control
M987	3.08	2.62
M988	3.20	4.38
M989	3.60	3.40
M990	3.40	3.10
M991	2.42	3.36
M992	1.00	4.84
M993	3.46	5.06
M994	3.22	3.06
Mean ± SD	2.92 ± 0.85	3.72 ± 0.9

Data are expressed in picomoles per microliter. $P = 0.16$.

superior and inferior halves. Because all retinas were treated similarly during preparation and because our primary data compare the right and left eyes of each animal, we did not correct any data for shrinkage.

Statistical significance of differences was evaluated with paired *t*-tests, Wilcoxon rank test, and linear regression analysis.

RESULTS

Fluorescein Angiography and Retinal Anatomy

The surgery produced no detectable difference in fluorescein angiograms in eyes with transected nerves and control eyes before and after the surgery. Similarly, no microscopic evidence of inner retinal ischemic atrophy was seen in any of the wholemount retinal specimens.

Nerve Fiber Layer Photography

Nerve fiber layer photographs were normal before transection in seven of the eight eyes with transected nerves, and in all the sham-transected eyes. In one animal, masked review of black and white photographs showed that there was pre-existing loss of the nerve fiber layer in both eyes temporally. The optic nerve cross sections of both the surgical and control eyes confirmed that central temporal areas of both nerves had axon loss. By analogy to human clinical entities with similar patterns of RGC loss, this animal appeared to have had RGC loss compatible with a toxic, nutritional, demyelinative, or genetic disorder. Because such an animal would be inappropriate as a subject in the experiment, its data were excluded.

Nerve fiber layer photographs were inspected in a masked manner. In the remaining 10 animals, nerve fiber layer photographs were entirely normal before surgery. For the seven animals with transected optic nerves, we detected loss of superior nerve fiber layer at 1 and 3 months after transection. This clinical atrophy progressed to severe loss of superior fibers in each partially transected nerve between the two postoperative observation points. Inferior fiber loss was also evident in five of seven animals at 1 month after surgery and was clearly seen in all at 3 months.

Amino Acid Analysis

The intravitreal levels of amino acids were the same in surgical and fellow control eyes 3 months after injury. In all animals, these levels were within expected values ($P > 0.05$, paired *t*-test, $n = 7$). Specifically, intravitreal glutamate levels are shown in Table 1.

RGC Density

The RGC density estimates for the 30 sampled areas (Fig. 1) of the superior retina for each experimental eye and for each control eye were pooled into a mean average, and a similar mean was constructed for the inferior retina. The transected superior retinas had 55% ± 13% lower density than their fellow control eyes ($P = 0.00005$, paired *t*-test, $n = 7$ animals; Table 2). The mean density of RGCs for the 30 inferior retinal areas was also significantly lower in eyes with transected nerves than in control eyes by 22% ± 10% ($P = 0.002$, paired *t*-test, $n = 7$ animals; Table 2).

The retinal data were further divided by the horizontal raphe and by a vertical line through the fovea into four areas. The mean decreases in density between surgical and fellow eyes were: superiotemporally (60% ± 12%), superionasally (48% ± 17%), inferonasally (26% ± 13%), and inferotemporally (19% ± 20%). There was no statistically significant correlation between the magnitude of the inferior retinal density loss to that superiorly in each pair of eyes ($P > 0.05$, $R^2 = 0.1$, linear regression, $n = 7$). Furthermore, in the inferior retina there was no statistically significant correlation between the distance from the horizontal raphe and the loss of RGC bodies ($P > 0.05$, $R^2 = 0.53$, linear regression, $n = 7$, Fig. 3)

Optic Nerve Axon Number

Of the 14 nerves in seven animals with transected nerves for which retinal data are presented, two optic nerves of two animals were unsuitable for optic nerve fiber counting because of processing errors. Thus, the nerve data consist of five partially transected and control nerve pairs. Although our retinal data consisted only of density values, for the optic nerves we have estimates of the density within segments, number of axons in a segment, region or whole nerve, as well as the diameter distribution of axons. In seven fellow control nerves, the estimated number of fibers in the whole nerve was 1,018,352 ± 183,706, similar to our previous estimates for this monkey species.[22]

TABLE 2. Percentage of Cells and Axons Lost in Eyes with Partially Transected Nerves

	RGC Body Loss		Axonal Loss	
Subject	Superior	Inferior	Superior	Inferior
987	62.4	15.1	68.3	−1.5
988	50.3	33.0	100	50.3
990	44.7	6.0	72.3	37
991	69.2	21.2	88.0	41
992	44.7	29.8	84.5	43.9
993	72.0	32.8	NA	NA
994	39.4	15.1	NA	NA
Mean ± SD	55 ± 13	22 ± 10	83 ± 13	34 ± 20

Percentage loss = 100 − 100 · RGC density of transected area/RGC density of control area.

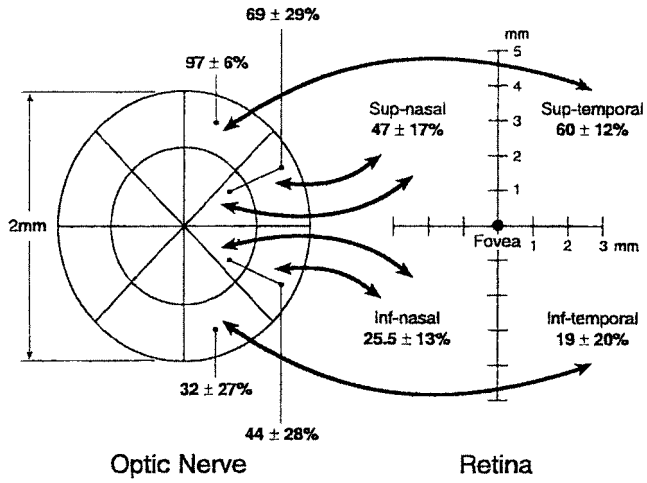

FIGURE 3. Comparison of RGC density loss in transection eyes for four areas of the retina (*right*) with the corresponding zones of the optic nerve into which axons of each group of RGC project (*arrows*). There is a general correlation between RGC density decrease and axon loss, greatest in the superotemporal retina (upper temporal nerve) and lowest in the inferotemporal retina (lower temporal nerve). Data are presented to represent a left eye.

The distribution of axonal loss in each segment of the retina in surgically altered eyes is shown in Figure 4A. Comparing the superior eight segments of the transected nerves with the fellow control nerves, we found an axonal loss of 83% ± 13% ($P = 0.0008$, paired *t*-test, $n = 5$ animals; Table 2). The inferior eight segments of the transected nerves had 34% ± 20% fewer axons than the fellow nerves ($P = 0.02$, paired *t*-test, $n = 5$). Cross sections of the transected optic nerves showed that the superior nerve had total axon loss in most areas of the upper one third, corresponding to the surgical incision. Loss in the remainder of the nerves was subtler by direct inspection and was diffusely distributed throughout the nerve (Fig. 5).

The loss in the inferior optic nerve was clearly present in four of the five nerves, with differences from control of 36% to 50%. In one nerve, there was no detectable loss inferiorly, compared with its control. This nerve appears to have had the lowest axonal loss in the superior half of the nerve. Similar to the retinal density estimates, we found no statistically significant relationship between degree of inferior optic nerve axon loss and the severity of superior axon loss. However, the power to estimate such a relationship was limited by the modest number of animals and the relatively uniform superior loss in those affected ($P > 0.05$, linear regression, $n = 5$).

The loss in RGC density was compared with loss of optic nerve axons in regions of the retina and optic nerve that were thought to contain the cell bodies and the axons of the same RGCs (Fig. 3). In general, the greater the RGC density loss, the more the axonal loss in the segment of the nerve that contains the fibers of those cells of origin, although this was statistically significant only for the superotemporal area of the retina and its corresponding superior pole of the nerve ($P = 0.0018$, paired *t*-test, $n = 5$).

Sham Operation

In eyes subjected to sham operation, the superior retinas had 2% ± 13% higher density of RGC bodies than their fellow control eyes ($P = 0.85$, paired *t*-test, $n = 3$; Table 3), whereas the mean RGC density for the inferior retina was higher in the sham transection group than in controls by 7% ± 14% ($P = 0.4$, paired *t*-test, $n = 3$). In contrast to these RGC retinal density estimates, there was mild loss of optic nerve axons in the animals that underwent sham operation (Fig. 4B). The superior eight segments of the sham-transected nerves had 19% ± 15% fewer axons than their intact fellow nerves ($P = 0.16$, paired *t*-test, $n = 3$; Table 3), whereas the inferior eight segments of the sham-transected nerves had 15% ± 9% fewer fibers than their fellow nerves ($P = 0.07$, paired *t*-test, $n = 3$). In cross sections of the nerves in eyes that underwent sham surgery, there were small, peripheral areas of axon loss in the superior or superotemporal nerve (Fig. 6). The degree of axon loss in the nerves in the sham transection group was significantly less than that in transected nerves, with 4.4 times fewer axons lost superiorly and 2.4 times fewer inferiorly.

Selectivity by RGC Size

There was no selective loss of any axonal diameter group in the superior halves of the transected nerves, although it should be noted that the loss superiorly was severe. When axon loss as a proportion of normal was calculated at each axon diameter, the loss superiorly was uniformly severe across all diameters ($P = 0.36$, $R^2 = 0.28$, linear regression, $n = 5$, Fig. 7A). By contrast, in the inferior area there was a relatively greater loss of small-diameter axons ($P = 0.017$, $R^2 = 0.89$, linear regression, $n = 5$, Fig. 7B). These findings did not result from alteration of all axons before death, because there was no shift

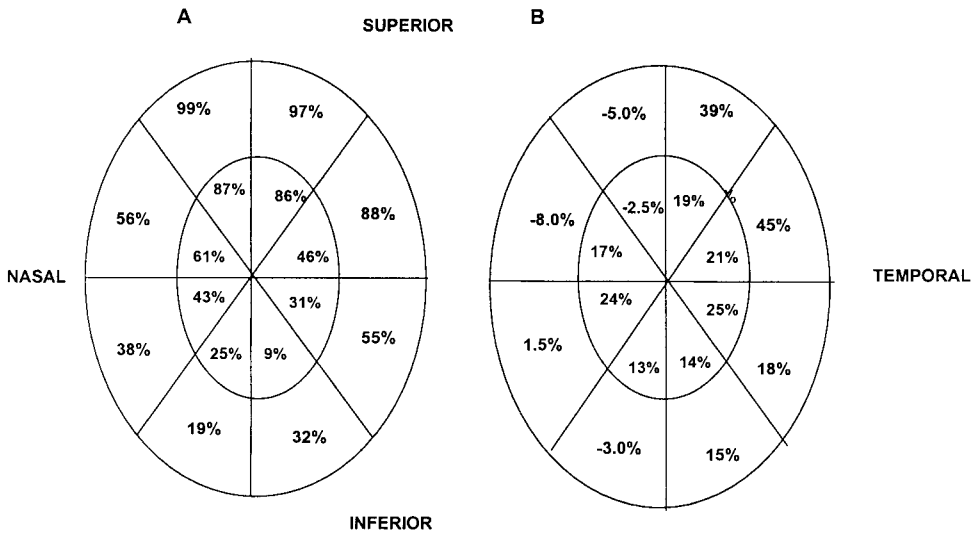

FIGURE 4. (**A**) Cross section of optic nerve (presented as a left nerve) shows the mean axonal loss in each of the 16 segments for the five pairs of eyes that underwent superior nerve transection. The loss was greater superiorly, and there was total loss in the two upper segments. The loss in the inferior area was significant and diffusely distributed. (**B**) Cross section of sham-transected optic nerves shows the mean axon loss in each of the 16 segments in the three pairs of eyes. There was loss in the superotemporal area where the meningeal sheath was opened and almost no visible loss in the peripheral segments that were far away from the surgical manipulation.

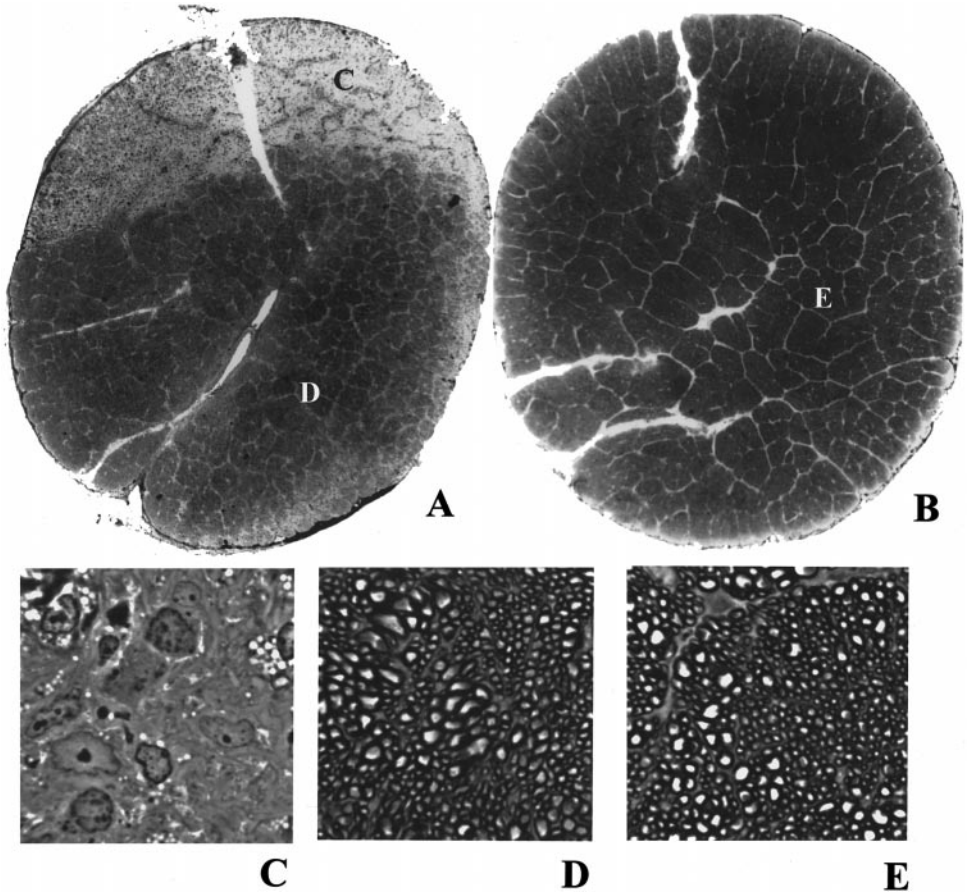

FIGURE 5. Cross sections of optic nerves from a monkey 3 months after the superior one third of the right optic nerve had been transected. (**A**) Partially transected optic nerve shows total atrophy (light area) in the superior segment; (**B**) control optic nerve. (**C**, **D**, and **E**) Higher magnification of corresponding areas in (**A**) and (**B**): (**C**) Area of total atrophy in the superior optic nerve tissue; (**D**) tissue from the inferior area showing relatively intact axons with diffuse loss and predominance of large-diameter axons. (**E**) Normal optic nerve tissue. Staining: 1% toluidine blue; magnification, (**A**, **B**) ×35; (**C**, **D**, and **E**) ×1670.

toward either shrinkage or swelling in the overall axon diameter distribution.

DISCUSSION

This study found a substantial loss of RGCs and their axons in the inferior retina and optic nerve of monkeys whose optic nerves had only been transected in the superior half. In the superior optic nerve, the areas subjected to transection were almost completely devoid of axons 3 months after injury, with 97% to 99% loss in the two most peripheral superior segments of the nerve (Fig. 4A). The nerve area that was evaluated is closer to the eye than the transection site; therefore, the measures of atrophy presented here represent the effects of retrograde degeneration. There was a 22% loss of RGC density in the inferior retina and a 34% loss in inferior optic nerve axons, comprising highly significant damage. The marked histologic loss of inferior RGCs was confirmed by masked photographic detection of nerve fiber layer atrophy in the inferior retina. The loss of inferior RGCs in this experiment could have occurred either from primary injury to inferior nerve axons during the surgery or from secondary degeneration. We will detail below the evidence that supports secondary degeneration as the more likely cause.

Primary injury to inferior RGCs and their axons hypothetically occurred for one or more of the following reasons: the transection extended beyond the middle half of the optic nerve; the manipulations during surgery primarily injured inferior RGCs or their axons; some inferior RGC axons that passed into the superior optic nerve were cut by transection; and transection of blood vessels in the superior nerve decreased blood supply to the inferior nerve or cause bleeding that could lead to axon degeneration. We are certain that extension of transection into the inferior nerve did not occur. The monkey nerve is 3 mm in diameter in the orbit, and under direct observation with the operating microscope, making a cut 1 mm deep into the tissue is a straightforward procedure. Because transection leads to nearly complete loss of all axons in

TABLE 3. Percentage of Cells and Axons Lost in Eyes with Sham-Transected Nerves

	RGC Body Loss		Axonal Loss	
Subject	**Superior**	**Inferior**	**Superior**	**Inferior**
995	−16.9	−23.4	3.7	7.7
997	9.8	3.6	19.5	12.4
998	1.0	−1.5	34.4	24.6
Mean ± SD	−2 ± 13	−7 ± 14	19 ± 15	15 ± 9

Percentage loss = 100 − 100 · RGC density of transected area/RGC density of control area. Negative value means higher density in operated eye compared with control.

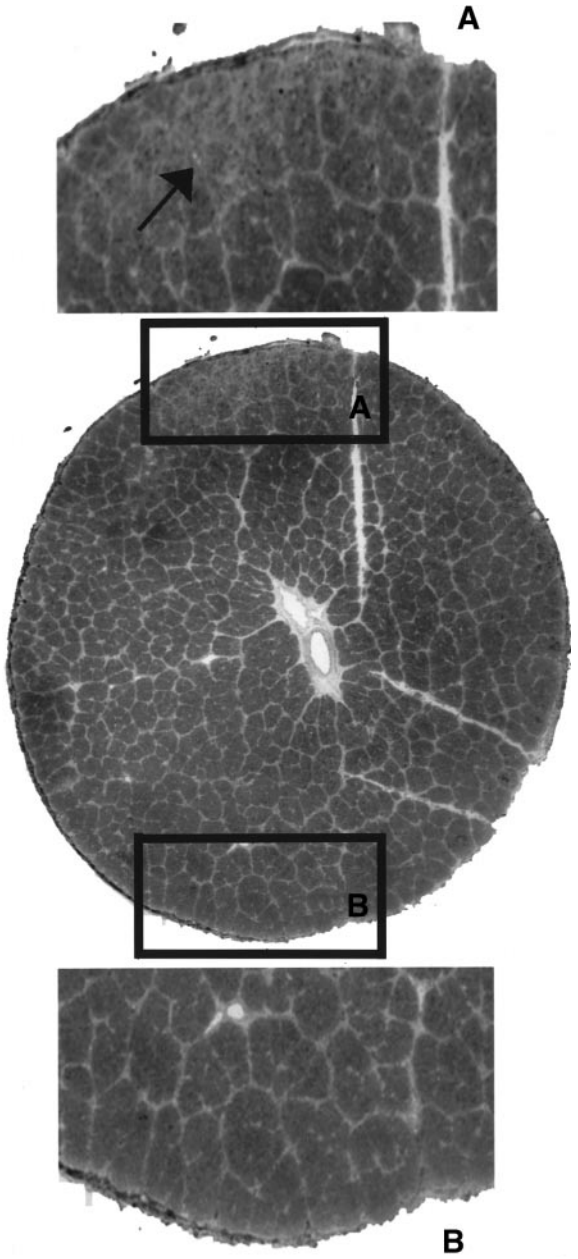

FIGURE 6. Cross section of the sham-transected optic nerve. *Arrow*: Wedge-shaped loss of axons in the superotemporal area. (**A**) and (**B**) correspond to boxed sections: (**A**) Superior atrophic area and (**B**) inferior area that looks grossly normal. Staining: 1% toluidine blue; magnification, ×35.

the cut zone, nerves with a transection that is too extensive would have 98% loss of axons in their inferior halves. This was not observed in any of the specimens. The percentage loss of axons was never greater than 70% in any individual inferior segment.

To evaluate whether inadvertent primary injury happened to inferior RGCs, we performed a number of examinations, as well as conducting sham operations. Clinical retinal examination and fluorescein angiography provided no evidence that the surgery had compromised the blood supply to the retina or choroid. If retinal ischemia had occurred during or immediately after surgery, retinal edema would have been detected, and nerve fiber layer loss would have occurred promptly. Neither was observed. Histologic retinal examination showed no loss of the middle or outer retinal layers that would have resulted from vascular occlusion.

In eyes subjected to sham operation, some primary injury to the optic nerve occurred, even when no direct nerve cutting was performed. In each case, the most prominent atrophic zone in sham-transected nerves was a wedge of peripheral loss superiorly, in the area where the meninges had been opened, comprising much of the 19% axon loss in the upper nerve. The retinal zones in which cell bodies were counted were not affected at all by sham surgery. Therefore, this effect cannot be the explanation for the decline in inferior RGCs in retinal counts in eyes with partially transected nerves. We presume that axons were injured during sham (and actual) transection in the far peripheral optic nerve through the trauma of opening the meninges or by interruption of their blood supply, which enters the nerve through penetrating vessels in the meninges.

The diffuse loss of 14% of inferior axons is less easily explained as direct primary trauma, although the globe was rotated and the orbital vessels were presumably compressed during the surgery. It is clear that the additional transection of the nerve in seven eyes produced more than twice as much inferior RGC loss in the optic nerve as did the sham surgery. The RGC density estimates in eyes with sham-transected nerves showed no statistically significant decrease; however, the sampling proportion in retinal counts was lower than in the nerve, and its extent was limited to the central retina. Likewise, the clinical nerve layer photographs showed no atrophy in eyes with sham-transected nerves, expressing the mild level of damage caused by surgery without transection.

The third manner in which primary degeneration could explain loss of inferior RGCs in our transection experiment is random topographic representation of RGCs in the optic nerve. Although there have been scattered reports that axons

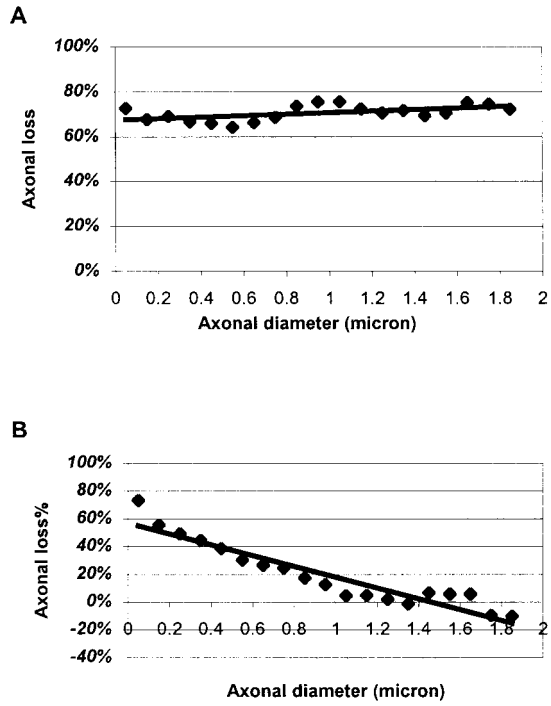

FIGURE 7. (**A**) Diameter distribution curve shows that the axonal loss in the superior area of the optic nerve is uniformly severe and is similar across all diameters ($P = 0.36$, $R^2 = 0.28$, linear regression, $n = 5$). (**B**) A selectively greater loss of small diameter axons was observed in the inferior halves of the optic nerves, with 60% loss of small axons and almost no loss of axons larger than 1.0 μm ($P = 0.017$, $R^2 = 0.89$, linear regression, $n = 5$).

from RGCs are not precisely ordered in the nerve fiber layer of the retina or the optic nerve,[23] the preponderance of evidence strongly supports an orderly arrangement.[18,24,25] Clinical–pathologic correlations of ocular disease with atrophy in optic nerve cross sections confirm such regional organization.[26] If we had evaluated only RGC density in the retina, our evidence against topographic wandering would be much less convincing; however, we sampled optic nerve cross sections 1 to 3 mm behind the globe, with a transection that was performed at 5 to 6 mm behind the globe. For the nerve sections to have loss of inferior axons that were misrouted to the superior nerve (and cut there), more than 20% of inferior fibers would have to be in the inferior nerve at 1 to 3 mm and move to the upper half of the nerve in less than a 2-mm distance along the nerve. This appears so unlikely that we have concluded that topographic considerations do not explain our data.

We propose that our data indicate that much of the inferior RGC loss after superior nerve transection was due to indirect, or secondary, mechanisms. The magnitude of the secondary degeneration was impressive. As many as one third of RGCs in the inferior retina died 3 months after injury. We do not know how much damage occurred soon after the superior nerve transection and how much might continue beyond the time of our observations. Certainly, we observed progressive loss of the inferior retinal nerve fiber layer between the 1-month and 3-month clinical photographs. The death and disappearance of primarily injured RGCs after transection is believed to be complete at 3 months.[13] However, it is reasonable to speculate that secondary RGC degeneration could continue beyond the actual loss of the primarily injured RGCs. Neufeld[27] detected activated microglia in the optic nerve heads of human glaucomatous eyes. It could be that initial RGC death begins a cascade of toxic processes mediated by such effector cells.

It is interesting to hypothesize where the effects of primary injury act to initiate secondary degeneration. The most obvious site would be at the position of primary injury. In traumatized spinal cord, the primary lesion is known to expand over time rostrally and caudally. Crowe et al.[2] found apoptotic death of neurons and oligodendrocytes in white matter tracts distant from the lesion site, leading to demyelination of axons spared by initial injury. Similar findings have been reported after transection of the optic nerve in developing and adult rats.[28] In a variety of CNS disorders, primary injury leads to secondary damage by creating a hostile environment in the surrounding tissues.[29–31] In the present experiment, the hostile environment may be in the orbital optic nerve, leading to injury to axons of nontransected RGCs and retrograde degeneration of their cell bodies. It is known that there is a time lag of weeks between axonal injury and cell body death for RGCs.[12,32] Our data suggested a greater proportionate loss of optic nerve fibers than RGC bodies at 3 months after injury. It is conceivable that this is an expression of axons that are already injured and uncountable, whose cell bodies have not yet died. In this regard, it would be evidence for the optic nerve as the site of secondary degeneration. However, the differences in our retinal and optic nerve counts may be a result of differences in methodology or distribution of RGC loss.

It is equally likely that secondary degeneration occurs by toxic effects of primarily dying RGC bodies in the retina. Dreyer et al.[19] reported abnormal levels of glutamate in the vitreous humor of experimental monkey and human eyes with glaucoma. Perhaps the death of a large number of RGCs intraretinally disturbs the normal mechanisms for limiting the extracellular concentration of glutamate. Excitotoxic cell injury would result from stimulation of *N*-methyl-D-aspartate receptors.[33–39] We did not find any difference in glutamate levels in the vitreous at 3 months after injury between eyes with partially transected nerves and control eyes although it is possible that glutamate levels were high earlier than the time we measured. We should stress that our experimental model was transection, which may differ significantly from glaucoma, although both involve optic nerve injury. More detailed sampling may be indicated at different time points after transection. Such sampling was not undertaken in this study to avoid the probable intraocular alterations that would have resulted from multiple penetrations of the globe.

If the source of toxic effect for secondary degeneration is in the retina, then greater death of RGC might be expected nearest to the primarily dying RGCs. Our data do not provide definitive information about this point. The regional data from the optic nerve also do not show a clear trend for axons to die more often when they are closer to the partial transection.

We investigated not only whether some RGCs die by secondary degeneration, but whether particular RGCs were more likely to die. We found no size selectivity in the superior optic nerve, but the near total loss of axons in many superior areas made it unlikely that this would be productive. There was, however, a significant trend for smaller diameter axons to die preferentially in the inferior nerve. It is intriguing that this apparent tendency toward small axon loss is different from the susceptibility of the larger axons apparent in human glaucoma.[40]

The present result suggests that secondary degeneration can occur after optic nerve injury. Although partial transection is different from glaucomatous optic neuropathy, we still might consider the possibility that secondary degeneration occurs in glaucoma. If this is true, blocking secondary degeneration may provide additive protection in glaucoma. Neuroprotection is a potential therapy that would intervene in the death of neurons in novel ways.[8,9,41–44] If secondary degeneration occurs in human optic nerve diseases, as we believe occurred in this experimental setting, then neuroprotective approaches might be devised to block the effector sequences that emanate from primary injury and lead to secondary death.

In summary, we suggest that secondary degeneration may cause substantial loss of RGCs as an indirect effect of the injury and death of the transected RGCs. This study points to the need for further investigations to explore the mechanisms of secondary degeneration, to identify markers that distinguish it from primary degeneration, and to prevent it by specific therapy.

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