Effect of Glatiramer Acetate on Primary and Secondary Degeneration of Retinal Ganglion Cells in the Rat

Michael Blair, Mary Ellen Pease, John Hammond, Danielle Valenta, Jennifer Kielczewski, Hana Levkovitch-Verbin, and Harry Quigley

Purpose. After crush injury to the optic nerve, elevated intraocular pressure, and glutamate toxicity, the immune modulator glatiramer acetate (GA, Cop-1; Copaxone; Teva Pharmaceutical Industries, Pitach Tikva, Israel) has been shown to reduce the delayed cell death of retinal ganglion cells (RGCs). This study was undertaken to confirm the protective effect of GA on secondary degeneration of RGCs in the rat, by using a spatial, rather than temporal, model.

Methods. A total of 131 Wistar rats divided into 10 groups underwent bilateral stereotactic injection of fluorescent tracer (Fluorogold; Fluorochrome, Denver, CO) into the superior colliculus to label RGCs. They received a concurrent subcutaneously injection of (1) GA mixed with complete Freund's adjuvant (CFA), (2) CFA alone, or (3) saline. One week later, the superior one third of the left optic nerve was transected in animals in the six partial transection groups. Optic nerves in four additional groups underwent full transection. Rats were killed and retinas harvested from both eyes 1 or 4 weeks after partial transection and 1 or 2 weeks after full transection. RGC densities were calculated from retinal wholemounts, and differences between right (control) and left (transected) eyes were compared across treatment groups.

Results. Among the partial transection groups, differences in the mean percentage of RGC loss in the inferior retinas were not significant at 1 or 4 weeks (ANOVA; $P = 0.20$, $P = 0.12$, respectively). After full transection, there was significantly more RGC loss in the GA group than in the CFA group when comparing whole retinas at 1 week, but not at 2 weeks (two-tailed *t*-test; $P = 0.04$, $P = 0.36$, respectively).

Conclusions. There is no evidence that GA has a neuroprotective effect after optic nerve transection, either for primarily injured or secondarily involved RGC. (*Invest Ophthalmol Vis Sci.* 2005;46:884–890) DOI:10.1167/iovs.04-0731

Controlled clinical trials have demonstrated that the immune modulator, glatiramer acetate (GA; Cop-1; Copaxone; Teva Pharmaceutical Industries, Pitach Tikva, Israel), reduces the relapse rate and progression of disability in patients with multiple sclerosis (MS).[1–3] It also decreases the number of gadolinium-enhancing lesions and T_1 hypointense lesions on magnetic resonance imaging in patients with MS.[4] This drug was originally designed to mimic myelin basic protein, which is thought to have a central role in the pathogenesis of MS and is a synthetic copolymer of four amino acids—glutamic acid, lysine, alanine, and tyrosine—with variable sequence but fixed molar ratio.[3] The mechanism of action is not clear, but may be to induce immune tolerance by inducing helper T-cell type 2 predominance[5,6] and to increase the secretion of neurotrophins,[7] including brain-derived neurotrophic factor.[8] Recent studies have demonstrated an inhibitory effect of GA on degeneration of retinal ganglion cells (RGCs) from glutamate toxicity in mice,[9] and optic nerve crush injury in mice[9] and rats,[10] and ocular hypertension in rats.[9,11]

Neural degeneration includes the death of cells from the primary injury, such as ischemia or trauma, and death from events that are secondary to the primary damage.[12] Thus, secondary degeneration can be defined as cell death not directly attributable to the primary injury, but rather from proximity to dead or dying cells, probably through the release of toxic substances. Secondary degeneration is thought to occur in neural tissue surrounding infarcts and trauma, and it may contribute to RGC loss in glaucoma as well.[12–15] In a recent study, RGCs survived optic nerve crush better after treatment with GA, a result attributed by the authors to the inhibition of secondary degeneration.[10] However, it is impossible to distinguish between primary and secondary degeneration in animal models such as crush injury, glutamate exposure, or experimental glaucoma, because all neurons are exposed to the primary insult. Investigators have speculated that RGCs that die at longer times after primary injury are more likely to undergo secondary degeneration. Yet, this conclusion is problematic, because some cells may simply undergo primary degeneration more slowly than others.[16,17]

A recently published technique of partial optic nerve transection in the rat allows improved separation of primary and secondary RGC degeneration.[17] The superior one third of the optic nerve is transected, leaving the inferior one third undisturbed. Because RGCs residing in the superior and inferior retina send axons into the optic nerve in general topographic orientation, a decline of RGC density in the inferior retina can be attributed to secondary degeneration, rather than delayed primary degeneration.[17]

Using this partial transection model, we investigated the effect of GA on secondary degeneration of rat RGCs and also compared the effect of GA on primary degeneration in animals with complete optic nerve transection. If the potential benefits of GA in preventing primary or secondary RGC degeneration are confirmed, it may be useful therapeutically in ischemic, traumatic, or glaucomatous retinal and optic nerve diseases.

Methods

Animals

Male Wistar rats, weighing between 400 and 450 g, were treated under protocols adherent to the ARVO Statement for the Use of Animals in

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Corresponding author: Harry Quigley, Wilmer B-122, Johns Hopkins Hospital, 600 N Wolfe St, Baltimore, MD 21287; hquigley@jhmi.edu.

Ophthalmic and Vision Research and approved by the Johns Hopkins School of Medicine Animal Care Committee. They were housed under a 14-hour light and 10-hour dark cycle and fed standard chow and water ad libitum.

Anesthesia

Before RGC labeling, optic nerve transection, and death, rats were anesthetized by intraperitoneal administration of ketamine (50–75 mg/kg) and xylazine (5 mg/kg).

RGC Labeling

RGC were back-filled with 5% fluorescent tracer (Fluorogold; Fluorochrome, Inc., Denver, CO) in 0.9% normal saline by injection into the superior colliculus bilaterally. After induction of anesthesia, the scalp was treated with povidone iodine (Betadine; Purdue Frederick, Norwalk, CT), and the eyes were protected by lubricating ophthalmic ointment. Rats were positioned in a stereotaxic device (Stoelting, Inc., Wood Dale, IL) and a sagittal midline incision was created through the superior scalp to the skull. Using cranial suture landmarks and standard coordinates of the Wistar rat brain,[18] we determined the location of each superior colliculus. A burr hole was drilled through the cranium above each colliculus with a 0.75 mm bit (Dremel, Inc., Racine, WI). A blunt 30-gauge needle attached to a 50-μL syringe (Hamilton Co., Reno, NV) was advanced to depths of 2.7, 3.2, and 3.6 mm from the surface of the cortex, where 1.5 μL of the fluorescent tracer was injected at 1 μL/min using a microprocessor controlled pump (Stoelting, Inc.). After injections at each depth, the needle was left in place for 2 minutes. The skin was then closed with staples.

Immunization

At the conclusion of each RGC labeling procedure, each rat received a 0.1-mL subcutaneous injection at the base of the tail. Rats in the experimental group were immunized with a 1:1 mixture of GA (200 μg) in complete Freund's adjuvant (CFA). CFA was prepared by combining 10 mg crushed, killed *Mycobacterium tuberculosis* H37 RA with 20 mL incomplete Freund's adjuvant (Difco-BD Biosciences, distributed by Cardinal Health, Columbia, MD). CFA potentiates and prolongs the cellular and humoral response to the immunogen with which it is injected. Rats in one control group were injected with a 1:1 mixture of CFA and phosphate-buffered saline. In a second control group, rats received injections of saline.

Partial or Complete Transection of the Optic Nerve

Unilateral partial or complete transections were performed 1 week after the labeling procedure. After the administration of general anesthesia, rats received topical 1% proparacaine as additional corneal anesthesia. A limbal incision was made in the superior conjunctiva, and the eye was gently retracted outward with fine forceps, exposing the optic nerve. An incision was then made through the superior nerve approximately 1.5-mm posterior to the sclera with a modified radial keratotomy–style diamond blade, under direct visualization.[17] Typically, the blade shaft was advanced 50 μm past a metal guard to cut 10% to 30% of the superior nerve (partial transection). For complete transection, the blade was set to extend 250 μm beyond the guard. Care was taken during transection procedures to avoid damaging the central retinal artery and vein, which are adjacent to the inferior aspect of the nerve sheath. The conjunctival incision was self-closing. The eye was inspected after surgery to ensure patency of its blood flow, with a standard indirect ophthalmoscope and 90-D condensing lens (Volk Optical Inc., Mentor, OH). Topical 5% erythromycin ointment was applied at the end of surgery.

Retina Wholemount Preparation

After induction of anesthesia, rats were killed by exsanguination at 1 or 4 weeks after partial transection or at 1 or 2 weeks after full transection. These time points were chosen because primary degeneration is expected to be substantial by 2 weeks, whereas secondary degeneration is expected to be at its maximum by week 4 in a partial-transection model.[17] Fixation was achieved with systemic perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), at a rate of 20 mL/min for 20 minutes. Both eyes were then enucleated after the limbus was marked at the 12 o'clock position with cautery. The optic nerve was removed flush from the sclera and postfixed in additional fixative for later processing into epoxy resin. The cornea was removed, and relaxing incisions were made at the superotemporal and superonasal aspect of the retina and eyewall. The retina was then removed with a blunt spatula and flatmounted onto slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) after two more peripheral relaxing incisions were created 180° from the initial incisions. The mounted retinas were coverslipped with an antifade mounting medium (Vectashield; Vector Laboratories, Burlingame, CA).

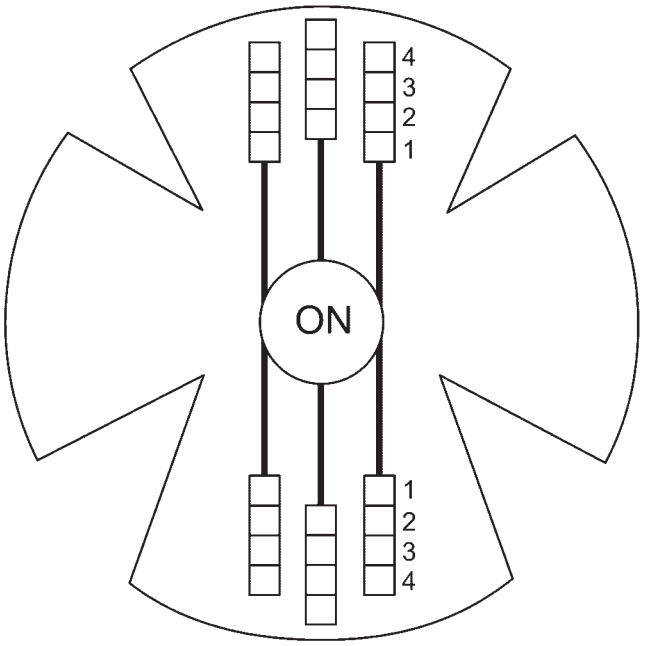

Figure 1. Fields sampled in retina wholemounts. Each of three parallel lines extending superiorly and inferiorly from the former location of the optic nerve head (ON) contains four consecutive fields.

Counting RGCs

Images of the wholemounts were captured with a fluorescence microscope (Axioskop; Carl Zeiss Meditec, Inc., Thornwood, NY) with a fluorescent tracer filter set (Fluorogold; Chroma Technology Corp., Brattleboro, VT) in combination with a camera (Sensys; Roper Scientific, Trenton, NJ) and image analysis software (MetaMorph; Universal Imaging Corp, Downingtown, PA). Four consecutive 40× fields, beginning 0.81 mm from the former position of the optic nerve head, were captured along each of three parallel lines extending superiorly from the optic nerve and along each of three parallel lines extending inferiorly (Fig. 1). The area of each field sampled was 0.1 mm^2, yielding a 4.5% sample of the Wistar rat retina which has an average area of 54 mm^2. Each image series was masked at the time of image capture. Fluorescent tracer–labeled RGCs were counted, and the field area was measured to determine the density per field. To determine whether there was significant interobserver or intraobserver variability, both observers (DV, JH) counted a series of 11 masked fields as well as counting a subset of four fields an additional two times. The correlation coefficient for the observers was 0.99, and the mean coefficient of variation for each was 1.95% and 2.31%, indicating that the data from both observers were interchangeable in the statistical analysis. While

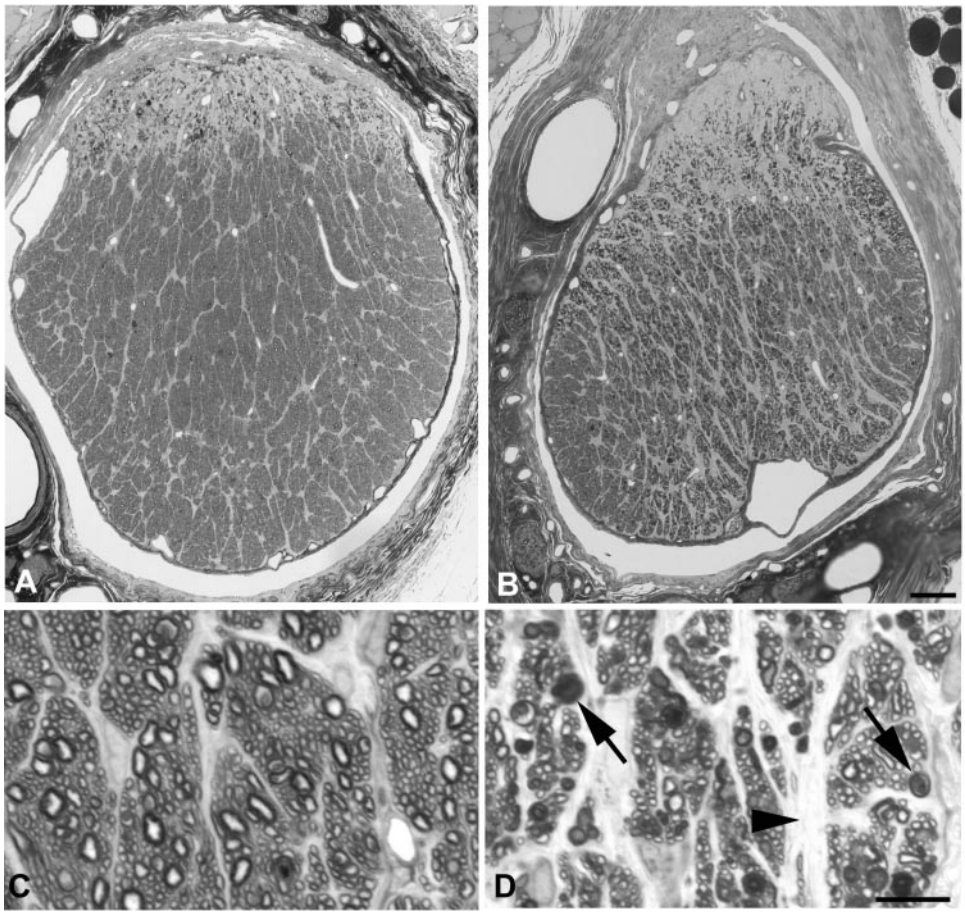

FIGURE 2. (**A**, **C**) Toluidine blue–stained optic nerve cross section, 1 week after partial transection, from a GA-treated rat. This section demonstrates injury confined to a discrete zone of the superior nerve, where darker, degenerating axons are present. (**B**, **D**) Optic nerve cross section, 4 weeks after partial transection, from a GA-treated rat. Diffuse degeneration is present. *Arrows*: degenerating axons; *arrowhead*: thickened septa resulting from nerve degeneration. Bar: (**A**, **B**) 50 μm; (**C**, **D**) 10 μm.

counting RGCs we were careful to distinguish them from glial cells that have engulfed dead RGCs and their fluorescent tracer. RGCs have a diffuse fluorescence, whereas glia have a bright fluorescence and a fusiform shape, as illustrated in a previous publication.[17]

Statistical Comparison

Because RGC density declines with distance from the optic nerve, the percentage of decline in RGC density was estimated by the following method. For each half retina of the control and experimental eyes of each rat, the mean density of the three fields equidistant from the disc was calculated. These areas were designated levels 1 to 4, where level 1 was closest to the disc and level 4 was most peripheral (Fig. 1). Mean densities in each level of the superior and inferior control retinas were pooled by averaging the data from each rat within a given treatment group at that level location. For each rat, the mean densities for levels in the superior and inferior experimental retinas were compared with the pooled control densities at the same level location. From these, the absolute number of cells lost and the proportion of cell loss per experimental level were calculated. The results were used to calculate an average proportion lost in the inferior, superior and whole retinas of each rat. The data were then combined for an average percentage lost in the inferior, superior, and whole retinas in a given treatment group.

RGC mean density differences between pooled control and treatment eyes were tested for significance in whole, superior, and inferior retinas. To verify that significant degeneration occurred after partial transection of the superior 10% to 30% of the retina, the mean ($\pm$SD) percentage of RGC loss was tested for whole, superior, and inferior retinas at each time point of each experimental group, by using the null hypothesis of no loss (one-sample, two-tailed *t*-test). Differences in change in RGC densities were compared among the three experimental groups in the whole, superior, and inferior retinas, at both the 1- and 4-week time points in the partial transection groups (one-way analysis of variance; ANOVA), and at the 1- and 2-week time points in the full transection groups (unpaired two-tailed *t*-test). Significance was set as $P < 0.05$. Statistical analysis, including power calculations, was performed on computer (InStat ver. 3.01 for Windows 95/NT; GraphPad Software, San Diego, CA, www.graphpad.com).

Processing of Optic Nerves for Histologic Evaluation

To confirm that the depth of initial injury during partial transection was appropriate, each treated optic nerve was evaluated histologically. Optic nerve samples were fixed by perfusion in paraformaldehyde, and then postfixed in buffered 1% osmium tetroxide. Tissues were washed in 0.1 M phosphate buffer (pH 7.2) followed by dehydration in increasing gradients of ethanol. Infiltration was performed with propylene oxide washes followed by epoxy resin in a 1:1 ratio with propylene oxide overnight. Tissues were transferred into 100% resin and placed in a vacuum for 4 hours before embedding with fresh resin. After they were polymerized for 48 hours, the blocks were sectioned to prepare 1-μm sections, which were stained with 1% toluidine blue before mounting. Cross sections of each nerve were evaluated for degeneration by light microscopy. For 1-week partial-transection nerves, serial sections were cut until the lesion was located, and eyes with damage from incision or trauma that extended through more than one half of the nerve were not included in the analysis. It was not possible to distinguish primary from secondary degeneration in the nerves evaluated 4 weeks after partial transection (Fig. 2), and thus nerves could not be excluded on the basis of degree of primary insult.

RESULTS

RGC Density

The mean density of RGCs in the control eyes of all groups combined ($N = 132$) was 1426.4 ± 225.8 cells/mm^2 (all results are expressed as the mean ± SD). This density is within the range of reported RGC densities in the Wistar rat (1400–2200 cells/mm^2).[17,19] RGC density probably varies by species, age, and sampling locations.[19–21] Fluorescent tracer back-labeling of RGCs in our study was concluded to be adequate (Figs. 3A, 3B). All cells labeled with the fluorescent tracer were not RGCs, as the phagocytosis of dead RGC leads to typical dense labeling of glia that must be carefully distinguished from RGCs in counting (Fig. 3B). Although there may be some difficulty in distinguishing RGCs from glial cells, our RGC counts were precise, as judged by inter- and intraobserver variability. The correlation coefficient for the two observers was 0.99 and the mean coefficient of variation for each of the two observers was 1.95% and 2.31%.

Partial Transection Model

One week after partial transection, the mean loss of RGC in all retinas was 17.4% ± 17.6% (Table 1). Although there was variability among animals, the mean loss was significantly different from control values ($P < 0.0001$; one sample *t*-test). Mean RGC loss across all three experimental groups in the inferior retina at 1 week was 70% of that in the superior retina, but the difference was not statistically significant (two sample *t*-test; $P = 0.25$). There was no significant effect of treatment with GA, compared with the two control groups (CFA or saline injection; two-sided ANOVA; Table 1).

Four weeks after partial transection, mean RGC loss had increased to 45.0% in all retinas. There was a significantly greater loss in the superior retina overall compared with the inferior retina ($P = 0.01$; two sample *t*-test). Treatment by GA did not show a significant difference from the two control groups, either in superior or inferior RGC loss (Table 1).

Post hoc analysis demonstrated that the powers of the ANOVAs (two-tailed, $\alpha = 0.05$) comparing means within the whole, superior, and inferior retinas were 0.75, 0.63, and 0.82, respectively. Post hoc analysis of the 4-week comparisons yielded powers of >0.90 for the whole and superior retina ANOVA and 0.60 for the inferior retina ANOVA.

Two-tailed *t*-tests, used to compare means of percentage of inferior RGC loss between the GA group and the CFA group (the sole control in some prior studies),[10,11] also demonstrated no significant difference ($P = 0.13$ at the 1-week and $P = 0.08$ at the 4-week time points). The GA group had more RGC loss than did the CFA group at 4 weeks ($P = 0.08$; two-tailed *t*-test). There was a 99% power to detect a 25% difference and 80% power to detect a 16% difference between the 1-week groups and a 99% power to detect a 51% difference and an 80% power to detect a 34% difference between the 4-week groups (two-tailed *t*-tests).

Complete Nerve Transection

One week after full nerve transection, there was a mean RGC loss across the retina of 35.3% ± 5.8%. There was no significant overall difference between superior and inferior RGC loss ($P = 0.24$, two sample *t*-test). Unexpectedly, the GA group had a somewhat greater mean RGC loss than did the CFA control at 1 week ($P = 0.04$; Table 2), but the differences were not significant when stratified by region (superior retina, $P = 0.06$; inferior retina, $P = 0.10$; two-sample *t*-tests).

At 2 weeks after full transection, 85.1% ± 4.6% of RGCs were dead overall. There was no significant effect of GA treatment compared with CFA injection either in whole retina or in the superior compared with the inferior retina (Table 2). There was a slight loss of inferior RGCs compared with superior RGCs ($P = 0.05$; two tailed *t*-test). This superior–inferior difference was not statistically significant in the GA treatment group ($P = 0.37$; two-tailed *t*-test), but it was significant in the CFA group ($P = 0.003$; two tailed *t*-test). Although these upper/lower retinal differences have no interpretable explanation, they illustrate the power of the data to detect small differences with the large sample of rats studied.

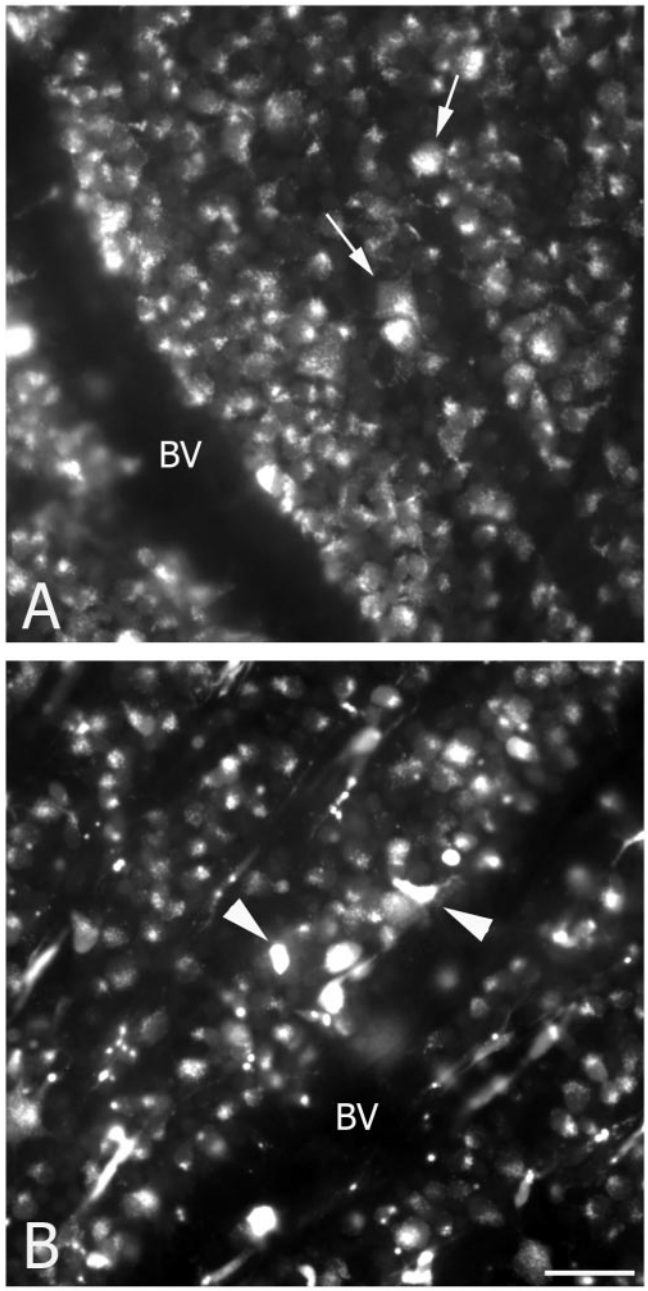

FIGURE 3. (**A**) Image of a fluorescent gold–labeled retina wholemount of the control eye of a rat in the 1-week partial transection saline group. *Arrows*: typical RGC morphology. BV indicates a blood vessel and thus an absence of cells in that area. (**B**) Image of the inferior retina from the experimental eye of the same rat. *Arrowheads*: glial components that have phagocytosed fluorescent tracer from degenerating RGCs. It is important to note that this cell type (bright, small, round or fusiform cells) was not counted as RGCs. Magnification, ×40; bar, 50 μm.

TABLE 1. Percentage of RGC Loss after Partial Transection

		RGC Loss (%)		
	n	**Whole Retina**	**Superior**	**Inferior**
Partial transection, 1 week				
All animals	51	17.4 ± 17.6	19.9 ± 24.9	15.0 ± 17.6
Glatiramer	19	18.1 ± 16.2	23.7 ± 20.1	12.9 ± 17.5
CFA control	16	18.7 ± 17.9	15.8 ± 29.5	21.6 ± 15.3
Saline control	16	15.3 ± 19.7	19.4 ± 25.9	11.1 ± 19.1
P (two-sided ANOVA)*		0.85	0.65	0.20
Partial transection, 4 weeks				
All animals	43	45.0 ± 31.0	54.4 ± 31.9	35.6 ± 33.5
Glatiramer	16	49.7 ± 33.9	59.8 ± 35.0	40.0 ± 36.1
CFA control	11	33.3 ± 20.2	48.4 ± 24.9	17.9 ± 20.0
Saline control	16	48.3 ± 33.6	53.0 ± 33.6	43.2 ± 35.6
P (two-sided ANOVA)*		0.36	0.66	0.12

Data are expressed as the mean ± SD.
* Comparing means of glatiramer, CFA Control, and Saline Control Groups.

Power analysis of whole retina RGC loss in the GA group compared with the control at the 2-week time point disclosed a 99% power to detect a relative difference of 10%, or 9% absolute difference in RGC death ($\alpha = 0.05$, two tailed *t*-test). For the 1-week time point, comparing whole retinas there was 99% power to exclude an absolute 13% difference, and 80% power to exclude an absolute 9% difference.

Optic Nerve Cross Sections

Within the partial-transection groups, all 54 optic nerves were evaluated to assess depth of incision (Fig. 2). Optic nerves from two rats had diffuse damage, probably from trauma during the partial-transection procedure, and the depth of transection of an additional optic nerve was judged to be too deep, because it extended more than half of he diameter of the nerve. These three rats were excluded from statistical analysis. Table 3 shows the depth of initial damage for 1-week partial transactions. Overall, 70% of evaluated nerves had the desired depth between 10% to 30% of the nerve. The distribution of depth of optic nerve damage was not significantly different by treatment group (<10% compared with 10%–50% depth of damage, χ^2, $P = 0.20$). It was not possible to evaluate the depth of initial damage in the 4-week groups, because, by that time, diffuse secondary degeneration had begun to occur, which obscured the disappearance of intact axons that had been caused by the incision alone (Fig. 2B, 2D).

RGC Density Outliers

Because our result was seemingly contrary to those in prior experiments with GA, we repeated the statistical analysis of the partial-transection group after eliminating outlying data, to see whether the conclusion might be different. Outlying data were defined as either those that had a counterintuitive result, with more RGC loss inferiorly than superiorly, or those with too little RGC death after partial transection (less than a 20% decline in RGC density superiorly). Animals that met these criteria comprised 27 rats from the 1-week partial-transection groups and 20 rats in the 4-week partial-transection groups. The elimination of these outliers had no effect on study outcomes in these groups (Table 4).

DISCUSSION

We have confirmed that transection of optic nerve axons in the orbit leads rapidly to death of RGC bodies in the retina.[17,22] Within 1 week, one third of RGCs were no longer visible as back-filled neurons, and by 2 weeks after injury, >85% were dead. Ultimately, all RGC with transected axons died. It is important to point out that the dead RGCs and the fluorescent tracer that they contained could still be seen in presumed macrophagic cells in the retina in these specimens.[17] We carefully differentiated between cells containing fluorescent

TABLE 2. Percentage of RGC Loss after Full Transection

		RGC Loss (%)		
	n	**Whole Retina**	**Superior**	**Inferior**
Full transection, 1 week				
All animals	16	35.3 ± 5.8	36.6 ± 6.3	33.9 ± 6.4
Glatiramer	5	39.5 ± 5.3	41.1 ± 6.1	37.9 ± 4.7
CFA control	11	33.4 ± 5.1	34.6 ± 5.6	32.1 ± 6.5
P (two-tailed Student's *t*-test)*		0.04	0.06	0.10
Full transection, 2 weeks				
All animals	21	85.1 ± 4.6	83.6 ± 5.1	86.7 ± 4.7
Glatiramer	12	84.3 ± 5.8	83.2 ± 6.3	85.5 ± 5.9
CFA control	9	86.2 ± 2.2	84.1 ± 3.3	88.4 ± 1.5
P (two-tailed Student's *t*-test)*		0.36	0.73	0.16

Data are expressed as the mean ± SD.
* Comparing means of glatiramer and CFA control groups.

TABLE 3. Depth of Damage of Partial Transections at 1 Week

		Depth of Damage Given as Percentage Penetration of Optic Nerve Diameter*			
	n	<10%	10%–30%	30%–50%	% of Total Nerves (*n*) Unable to Evaluate†
All animals	51	22% (9)	70% (31)	9% (4)	14% (7)
Glatiramer	19	33% (6)	67% (12)	0% (0)	5% (1)
Freund's Adjuvant control	16	8% (1)	75% (9)	17% (2)	25% (4)
Saline control	16	14% (2)	72% (10)	14% (2)	13% (2)

χ^2 test for independence, $P = 0.20$, comparing the <10% column with the combined 10%–30% and 30%–50% columns because of small numbers in the 30%–50% column.

* Percent of evaluated nerves with stated depth of penetration (*n*).

† Table does not include three additional optic nerves excluded for initial damage over 50% depth, one in the CFA group, and two in the saline group.

tracer that have neuronal morphology and the distinctly different macrophages.

In addition, we have reproduced the model of partial optic nerve transection originally reported.[17] One and 4 weeks after partial injury, the number of remaining RGCs was about twice the number in complete transections. There was a significantly greater loss of RGCs in the superior retina overall at 4 weeks, showing that the topographic relationship between axon position in the optic nerve and RGC body location in the retina is generally preserved at the site of partial transection. The preservation of the general topographic relationship of RGCs in the retina and their axons in the optic nerve was more fully explored in our prior description of the model.[17] Briefly, 1 week after superior transection of the rat optic nerve, an additional full transection was created closer to the globe, and the cut end was labeled with rhodamine-dextran. Animals were killed 24 hours later. Superior peripheral, but not superior central or inferior, retina had significantly greater RGC loss than control subjects. Our prior report also further elucidated the time course of cell death in the superior and inferior retina after partial transection of the optic nerve. RGC loss in the superior retina was substantial at 1 week, whereas that in the inferior retina remained low at 1 week but increased by week 4.[17] These temporal and spatial differences in degree of RGC loss in our model, along with the relative topographic preservation of RGC in the retina and optic nerve, should allow clear distinction between primarily and secondarily injured RGCs. It should be noted that sham surgery leads to a modest loss of RGCs.[17] In addition, there may be technical difficulties in the precision of nerve cutting or in histologic tissue orientation that prevent perfect separation of primary and secondary degeneration. However, data from only 3 of 54 rats in the 1-week groups had to be excluded because of damage to the inferior retina during the transection procedure. In addition, 70% of the remaining optic nerves evaluated had transection depths between the desired 10% and 30% thickness. This suggests that the depth of initial damage in the 4-week groups was precise as well.

We found no protective effect by GA among RGCs that were presumed to be undergoing either primary or secondary degeneration in our models. There are several possible explanations. First, our model may have greater variability in RGC death than other models. This explanation is unlikely, because our data have sufficient statistical power to detect effects of the size observed in prior studies, certainly for optic nerve injury from glutamate and experimental glaucoma in which the approximate protective effect ranged from 24% to 33% absolute reduction.[9,11] Neither the percentage of RGC survival or density in uninjured retina that is necessary to calculate the percentage of survival were reported in the rat optic nerve crush injury model.[10] We did have and equal or larger number of rats in several of the protocols (51 at 1-week and 43 at 4-week partial transection), compared with the number of animals in that crush injury report ($n = 20$–24).[10] We had expected the prevention of secondary degeneration to be the greatest in the inferior retina of the 4-week partial transection groups,[17] but

TABLE 4. Percentage of RGC Loss after Partial Transection, with Outliers Removed*

		Percentage of RGC Loss (%)		
	n	Whole Retina	Superior	Inferior
Partial transection, 1 week				
All animals	24	25.5 ± 9.9	37.9 ± 11.0	13.5 ± 14.0
Glatiramer	9	25.9 ± 14.7	38.2 ± 17.6	14.5 ± 14.3
CFA control	7	23.7 ± 9.7	32.7 ± 7.8	14.6 ± 13.0
Saline control	8	18.5 ± 8.0	29.1 ± 6.3	7.9 ± 11.0
P (2-sided ANOVA)†		0.42	0.32	0.50
Partial transection, 4 weeks				
All animals	23	44.4 ± 23.2	59.2 ± 23.9	29.5 ± 26.1
Glatiramer	8	47.0 ± 21.9	66.8 ± 18.6	28.3 ± 25.8
CFA control	9	39.3 ± 16.7	55.4 ± 21.6	23.3 ± 17.5
Saline control	6	55.4 ± 22.5	68.3 ± 23.2	41.5 ± 25.0
P (2-sided ANOVA)†		0.34	0.42	0.33

Data are the mean ± SD.

* More RGC loss inferiorly than superiorly or less than a 20% decline in RGC density superiorly.

† Comparing means of glatiramer, CFA control, and saline control groups.

were able to exclude a difference 'in means greater than 34% (80% power, two sided *t*-test). Animals in the 4-week partial-transection GA group actually had greater mean RGC loss than those in the CFA group. Even, after eliminating outliers in terms of RGC damage, the effect of GA was unchanged. In particular, the complete transection data are quite definitive—our data have a 99% power to detect a 9% protective effect of GA had it been present.

Second, we may have achieved different results with GA in our study compared to previous reports due to differences in dosage or delivery of the agent to RGCs. However, for several reasons, we believe this is not the explanation. We obtained GA from the same source as prior reports and mixed and injected it according to specific methods described therein. The emulsification formed with GA with CFA was continuously resuspended due to a tendency to settle. The dose of 200 μg per animal is comparable to doses in the literature for rats (200 μg) and mice (75 μg) in the ocular hypertensive and glutamate toxicity models.[9] However, rats in crush injury experiments received an initial 100-μg injection of GA immediately after the injury, followed by a supplemental 5-day course of oral GA, 1 mg per day.[10] Rats in our experiment did not receive this oral supplement. We chose our immunization regimen based on prior experiments on glutamate toxicity and GA, in which a greater neuroprotective effect of GA was found when immunization occurred 10 days before injury rather than on the day of injury or 48 hours later.[9] Vaccination occurred 7 days before injury in our study, near to this more effective time.

Third, it is possible the lack of GA effect occurred because transection injury differs in mechanism from the crush injury, glutamate toxicity, and experimental glaucoma (all of which appear to show GA benefit). Ultimately, cell death in all these models is associated with apoptosis,[7,16,23,24] but there may be broad differences in the specifics of the initiation and progress of cell injury and death. It is conceivable that axonal transection is so severe an injury that the potential benefit of GA cannot take effect. It is curious, however, that insults that act to damage the axon at very similar locations would respond selectively to a therapy. These results should stimulate more careful examination of the differences in the cell death process in RGCs with respect to types of injury and types of treatments. We will investigate in more detail the similarities and differences between primary and secondary degeneration in this model. At present, we conclude that there is no evidence that GA has a neuroprotective effect after optic nerve transection, either for primarily injured or secondarily involved RGCs.

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