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Rodent Anterior Ischemic Optic Neuropathy (rAION) Induces Regional Retinal Ganglion Cell Apoptosis with a Unique Temporal Pattern

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Abstract

Purpose—Nonarteritic anterior ischemic optic neuropathy (NAION) results in optic nerve damage with retinal ganglion cell (RGC) loss. An NAION model, rodent anterior ischemic optic neuropathy (rAION), was used to determine AION-associated mechanisms of RGC death and associated regional retinal changes.

Methods—rAION was induced in male Wistar rats, and the retinas analyzed at various times after induction. RGCs were positively identified by both retrograde fluorogold labeling and brain-expressed X-linked protein-1/2 (Bex1/2) immunoreactivity. RGC death was analyzed by fluorescein-tagged annexin-V labeling (FITC-annexin-V), as well as by terminal nucleotide nick-end labeling (TUNEL). Retinal flatmount preparations enabled regional retinal analysis of labeled dying cells. Apoptosis pathway activation was confirmed by Western analysis, with an antibody that recognizes cleaved caspase-3.

Results—Post-rAION, RGCs die by apoptosis over a longer period than previously recognized. Cleaved caspase-3 immunoreactivity was greatest between 11 and 15 days. rAION-induced RGC death occurs regionally, with sparing of large contiguous regions of RGCs.

Conclusions—rAION results in later RGC death than in traumatic optic nerve damage models. Apoptosis, measured by FITC-annexin, occurs maximally in the second to third week after infarct. Cleaved caspase-3 activation confirms that after rAION, RGCs undergo apoptosis by the caspase activation pathway. The regional pattern in dying RGCs after rAION implies that a measure of retinotopic organization occurs in the rodent optic nerve. The prolonged period from insult to death suggests that the window for successful treatment after ON infarct may be longer than previously recognized.

Retinal ganglion cells (RGCs) die by apoptosis after optic nerve (ON) axonal damage that includes ischemia and axotomy.^{1,2} Apoptosis is a stepwise cellular program ultimately

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resulting in cellular removal with minimal disruption to the surrounding tissues.³ Evaluation of apoptotic cells in the retina and central nervous system (CNS) has traditionally relied on terminal deoxynucleotide nick-end labeling (TUNEL).⁴ Using this method to evaluate regional retinal apoptosis throughout the entire retina typically requires serial sectioning, a tedious, labor-intensive method requiring extrapolation of a small number of positive apoptotic cells from many analyzed sections. The use of serial sections makes it expensive to evaluate multiple retinas. Serial section analysis also makes interpretation of regional apoptosis patterns difficult. These disadvantages have provided the impetus to evaluate apoptosis by other methods. In situ labeling of the whole retina can enable easier, rapid analysis of multiple retinas, providing a useful tool in studying retinal diseases and dysfunction.

During early stages of apoptosis, phosphatidylserine residues normally present on the inner surface of the cell membrane accumulate on the outside of the lipid bilayer. Apoptotic cells thus are “marked” by a high concentration of phosphatidylserine residues on the external surface of their cellular membranes.⁵ The protein annexin-V has a high affinity for phosphatidylserine and binds to this moiety, providing the basis for an early test for apoptosis.⁵ When annexin-V is conjugated to a fluorescent dye such as FITC, annexin-V marked apoptotic cells fluoresce, allowing rapid analysis of early apoptotic cells.^{5,6}

Nonarteritic anterior ischemic optic neuropathy (NAION) is an optic nerve infarct,^{7,8} and the most common cause of sudden optic nerve-related vision loss in the developed world.^{9,10} In NAION, RGC loss occurs due to axonal ischemia.⁸ Although there is a dearth of NAION-associated clinical material, a single previous report suggests that NAION-affected RGCs die by apoptosis.² The lack of appropriate clinical material makes it relevant to study the mechanism whereby NAION causes RGC death.

We recently generated a new model of optic nerve infarct, rodent anterior ischemic optic neuropathy (rAION).^{11,12} This method produces direct damage to optic nerve capillaries supplying RGC axons, resulting in axonal ischemia and damage, followed by regional RGC loss.¹³ Retinal vascular imaging after rAION has shown that retinal vessels remain patent throughout the postinduction period (see Figs. 2A, 2C in Ref. 10; additional data not shown).

We wanted to evaluate the mechanism(s), timing, and overall progression of rAION-induced RGC death. Since rAION, like clinical NAION, results in regional RGC loss, we wanted to be able to estimate the degree of regional RGC damage. We decided to identify positively the dying cells in the RGC layer by using FITC-conjugated annexin-V labeling. We confirmed RGC identity by two methods: fluorogold retrograde labeling and immunolabeling with an antibody to brain-expressed X-linked protein-1/2 (Bex-1/2), a protein that is differentially expressed at high levels in RGCs.¹⁴ The use of labeled annexin-V in a retinal flatmount regional analysis enables rapid identification of early-stage apoptosis, as well as allowing an estimate of the relative number of annexin-V-positive cells within the RGC layer. In addition, this approach allows identification of the topographical pattern of RGC apoptotic cell death after optic nerve stroke. Because apoptosis is commonly considered to activate caspase pathways,¹⁵ we also evaluated whether caspase 3 cleavage, which occurs during the early apoptotic stage, follows rAION induction.

Materials and Methods

A FITC-labeled in situ cell detection kit from Roche (Indianapolis, IN) was used for the TUNEL assay. Male Wistar rats were purchased from Charles River Laboratory (Boston, MA). FITC-conjugated annexin-V was obtained from Invitrogen (Carlsbad, CA). Rabbit anti-Bex1/2 antibody was a kind gift of Frank Margolis and Jae Hyung, University of Maryland at Baltimore (UMAB) Department of Anatomy and Neurobiology. Secondary tagged fluorescent antibodies

were obtained from Jackson ImmunoResearch (West Grove, PA). A rabbit polyclonal antibody that recognizes intact and cleaved caspase-3 (Asp 175) was purchased from Cell Signaling Technology (Beverly, MA) and was used at a 1:1000 dilution.

All animal protocols were approved by the UMAB institutional animal care university committee (IACUC) and adhered to guidelines recommended by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were given food and water *ad libitum*.

Fluorogold Retrograde Labeling

Two weeks before induction, male Wistar rats (130-150 g), were anesthetized with 10% chloral hydrate (3.5 mL/kg), and the skull skin infiltrated with 1% lidocaine. After skull exposure, 2 μ L of 2% fluoro-gold (Fluorogold; Invitrogen-Molecular Probes, Eugene, OR) in 0.9% saline was stereotactically injected into each superior colliculus, by using a stereotactic frame with a digital readout (Stoelting Corp., Wood Dale, IL).

rAION Induction and Annexin-V Administration

The animals were anesthetized with ketamine/xylazine (80 mg/4 mg/kg). rAION was induced by frequency doubled yttrium-aluminum garnet (YAG; Iridex, Mountain View, CA) light (532 nm) after intravenous tail vein injection of rose Bengal (90% purity; Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS), as previously described.¹¹ Induction time was 12 seconds. This procedure routinely yields an average 60% to 70% cell loss in the RGC layer.¹¹ The retinas were examined in lightly anesthetized animals and photographed with a digital camera (D1X; Nikon, Tokyo, Japan; 2.3 megapixels, ASA 800, incandescent). The retinas were evaluated before- and 3 days after induction, except that the retinas of animals used for 1-day experiments were photographed before euthanatization. Animals were eliminated from the study if significant intraretinal vessel dilation/tortuosity or intraretinal edema, both of which can suggest central retinal vein occlusion or branch retinal vein occlusion, were noted.

Two hours before euthanatization, rats used for annexin-V labeling were reanesthetized with ketamine/xylazine (80 mg/4mg/kg). A paracentesis was performed in the anterior chamber of each eye, to reduce intraocular pressure and improve posterior chamber injection efficiency. The posterior chamber of each eye then received 2 μ L of labeled annexin-V. The animals were allowed to recover and then were terminally anesthetized with pentobarbital and euthanatized by intra-cardiac perfusion with 4% paraformaldehyde-phosphate-buffered saline (PF-PBS).

Annexin-labeled tissue was obtained from four retinas at each time point. Times analyzed were 0 (naïve preinduction), 1, 3, 7, 10, 13, 15, 18, 21, and 31 days after rAION induction. The retinas were postfixed in 4% PF-PBS at 4°C. After isolation, they were incubated in hyaluronidase (Wydase; Wyeth Pharmaceuticals, Collegeville, PA) at 1:1000 in PBS for 1 hour before further tissue preparation, to remove excess vitreous material that can result in artifactual annexin-V staining. After hyaluronidase digestion, the retinas were partially bisected to within 1 mm of the optic nerve, and then each of the two retinal leaves was further bisected to yield a maltese cross pattern, with roughly equivalent leaf size.

TUNEL Analysis

We confirmed RGC apoptosis with the TUNEL assay. Briefly, 10- μ m-thick, paraffin-embedded retinal sections were dewaxed and rehydrated in PBS. Positive controls were generated with DNase 1; 1:1000/30 minutes at 25°C in the DNase buffer supplied by the manufacturer. Sections were labeled with the commercially prepared labeling mix supplied by the manufacturer (1 hour/37°C). Slides were analyzed by confocal microscopy for fluorescent cells.

Caspase-3 Western Analysis

Two rAION-induced retinas from each time point (0 -15 days) were homogenized in SDS-lysis buffer (150 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.5% NP-40, 1% Triton X-100 [pH 7.4]) with protease inhibitors. Total retinal protein homogenate loading was normalized with the Bradford reagent (Sigma-Aldrich). Thirty micrograms of protein per lane was loaded onto a 4% to 12% polyacrylamide gel with the appropriate molecular weight standards. After transfer and immobilization onto polyvinylpyrrolidone fluoride (PVDF) membranes, protein loading was confirmed with antibody to β -actin (Sigma-Aldrich). Immunoreactivity was detected with peroxidase linked anti-rabbit IgG and the enhanced chemiluminescence (ECL) detection reagent from GE Healthcare (Piscataway, NJ). Controls for the uncleaved and cleaved forms of caspase-3 were generated with rat pheochromocytoma (PC-12) cells, grown in DMEM with 10% horse serum and 5% fetal bovine serum (Biofluids, Rockville, MD). The cleaved control homogenate was generated by exposing PC-12 cells for 18 hours to 100 nM staurosporine.¹⁶ Densitometric normalization was performed with the β -actin signal intensity.

Tissue Preparation and Bex1/2 Staining

Annexin-V FITC-labeled retinas were washed four times with PBS/0.5% Triton X-100, 15 minutes each. Retinas were then frozen on a microscope slide, face up at -70°C with a drop of PBS/0.5% Triton X-100 15 minutes and thawed at room temperature. This freeze-thaw procedure permeabilizes the inner limiting membrane, which can limit diffusion of enzymes and other reagents to the inner retina. Retinas were then immunostained with Bex1/2 antibody, which selectively recognizes RGCs and their axons, enabling verification of the nerve fiber and RGC layers.¹⁴ After overnight blocking in 2% normal donkey serum at 4°C, the retinas were reacted with Bex-1/2 antibody at a concentration of 1:1000 at 4°C. The tissues were then washed four times in PBS, and reacted with a Cy3 donkey anti-rabbit secondary antibody. Tissues were mounted with confocal tissue mounting medium and examined with a four-channel confocal microscope (Fluoview 400; Olympus, Tokyo, Japan) at the appropriate wavelengths.

Stereological Analysis of Annexin-V-Positive Cells

Initial characterization of annexin-V-labeled regions was performed with low-power (40 $\times$) examination of the total retina. Photocomposites were made, and individual retinal regions were evaluated at 200 $\times$ magnification with a confocal oil-immersion lens, which typically enables visualization of approximately 500 to 700 total RGCs/field.

Three fields were obtained from each of the four retinal regions. RGC fields, while not random, were sampled from radially adjacent peripapillary (0 - 0.8 mm), midperipheral (0.8 -1.6 mm), and peripheral (1.6 -2.4 mm) areas taken from the center of each retinal leaf. All annexin-V-positive cells were counted in each field. Obvious labeling artifacts (large clumps of fluorescence-positive material outside the RGC layer) were eliminated from the analysis. Data were plotted with commercial software (SigmaPlot; Systat Software, Inc., San Jose, CA).

To confirm that annexin-V-positive cells in the RGC layer were indeed RGCs, we retrograde labeled RGCs with fluorogold (Fluorogold; Invitrogen-Molecular Probes) before rAION induction. Two retinas each at 3, 11, and 28 days were used for confirmation. Fluorogold-labeled retinas were reacted with Bex1/2 antibody and the appropriate labeled secondary antibody and examined by confocal microscopy.

Results

rAION resulted in RGC apoptosis detectable by annexin-V labeling. Annexin-V-positive-Bex1/2 immunoreactive cells were detectable in the RGC layer after rAION (Fig. 1). A few

annexin-V-positive cells were discernible in the RGC layer in control retinas (Fig. 1A). Three days after rAION, there was a slight increase in annexin-V-labeled cells (Fig. 1B). These annexin-V-positive/Bex-1/2 immunopositive cells were noted to be at the level of the RGC layer at higher magnification (Fig. 1C, arrow), just below the nerve fiber layer (Fig. 1C; arrowhead). Ten days after induction, many annexin-V-positive cells were detectable in the RGC layer (Fig. 1D). The number of annexin-V-positive cells declined significantly by 3 weeks, but a few annexin-V-positive cells were still present 31 days after induction (Fig. 1E). Examination at higher magnification again revealed that these cells were present in the RGC layer, just below the NFL layer (Fig. 1F; arrowhead).

Fluorogold, Bex1/2, and Annexin-V Colocalization

To confirm that Bex-1/2(+) cells in the RGC layer undergoing apoptosis are RGCs, we performed retrograde fluorogold labeling before rAION induction. Fluorogold-labeled RGCs were easily demonstrable in the RGC layer in different regions of a flatmounted retina 28 days post-rAION induction (Fig. 2A; arrows). These cells were typically Bex-1/2 immunoreactive (Fig. 2B; arrows). Some cells in this layer also labeled with annexin-V (Fig. 2C). A number of these annexin-V-labeled cells were also fluorogold+Bex1/2 positive, revealing that they are RGCs (Fig. 2D; merged). Thus, these data show that RGCs undergo apoptosis after rAION.

Confirmation of RGC Apoptosis after rAION

Using TUNEL analysis, we detected TUNEL-positive cells in the RGC layer at time points between 7 and 15 days (Fig. 3B; RGC layer). Rare TUNEL-positive cells were also detected in the outer nuclear layer of the retinas of control and induced animals (Fig. 3A; ONL), but TUNEL-positive RGCs were detected only in rAION-induced retinas.

Although there are multiple forms of programmed cell death, apoptosis activates the caspase pathway.³ We confirmed that rAION induces caspase activated apoptosis, with cleaved caspase-3-Western analysis of retinal homogenates (Fig. 4). Control experiments included rat pheochromocytoma (PC-12) cells treated with staurosporine (Fig. 4; C and C+S), which induces cell death by caspase-associated apoptosis.¹⁶ Although total protein loading was similar in all retinal samples (Fig. 4; B-actin), total retinal caspase 3 expression was apparently up-regulated after rAION induction (Fig. 4, compare the upper [35-kDa] band from 11 to 15 days with days 1-3). PC12 cells expressed high levels of uncleaved caspase 3 and a smaller amount of the cleaved product (Fig. 4; C). Staurosporine administration resulted in PC12 apoptosis, with increased amounts of cleaved caspase 3 product (Fig. 4; C+S). Cleaved caspase 3 increased significantly in retinal homogenates between 11 and 15 days after induction (Fig. 4; 17 kDa). This suggests that after rAION induction, RGCs die by a caspase-associated cleavage mechanism, at least up to 15 days after induction.

rAION and Regional RGC Apoptosis

rAION has been shown to induce RGC-layer loss without detectable cell loss in other retinal cell layers.^{11,13} rAION-induced RGC loss has also been shown to be regional within the retina.^{14,17} RGC death is therefore likely to occur in regional areas of the retina as well, as was demonstrated by RGC-layer annexin-V labeling in rAION-induced rat retinas (Fig. 5). Annexin-V labeling was apparent in whole retinal regions 10 days after induction (Fig. 5A), whereas another region of the same retina revealed no annexin-V labeling (Fig. 5B).

Temporal Pattern of rAION-Induced RGC Apoptosis

We analyzed the mean number of annexin-V-positive RGCs occurring over a 31-day period after rAION induction (Fig. 6). A few annexin-V-positive cells were typically present in the control retinas (0 days), probably because of a minimal amount of RGC apoptosis occurring

in normal young rodents.¹⁸ The number of annexin-V-positive cells increased from 1 to 10 days after induction, reaching a peak by 10 days after induction (Fig. 6A; 10d). The number of annexin-V-positive cells then began to decline, but there were still a few annexin-V-positive cells in the RGC layer at 31 days after induction (Fig. 6).

Because rAION-induced RGC loss with the above induction parameters was regional, some retinal areas did not have many affected RGCs. Including data from areas without affected RGCs generated a large standard error for quantified means from each time point (Fig. 6A). To resolve this phenomenon, we reanalyzed the data with counts of annexin-V-positive cells from the three contiguous retinal regions with the greatest number of annexin-V-positive cells (Fig. 6B). This approach yielded results with considerably smaller standard errors, again emphasizing the regional nature of post-rAION-associated RGC death (Fig. 6B; compare days 0-31). Thus, rAION in rats results in prolonged, regional RGC apoptosis.

Discussion

In the present study, there was a regional loss of RGCs by apoptosis after rAION induction. These results are consistent with the histologic findings seen in a single early clinical case of human NAION, which demonstrated TUNEL(+) cells in the RGC layer by 30 days after the event,² as well as in our previously published work.^{11-14,17} Although some RGCs die soon after induction, apoptosis is demonstrable for nearly an entire month after induction. The apoptotic peak occurs between 7 and 15 days. The time course in the present study is more similar to the prolonged RGC loss after intracranial axotomy,¹ than from models of intraorbital traumatic optic neuropathy.¹⁸ Because we evaluated only the relative course of RGC death, it is not possible from this study to give an absolute quantification of the total number of RGCs undergoing apoptosis at any time point.

Increased levels of cleaved caspase-3 are evident in retinas 11 to 15 days after rAION induction. Although some caspase-3 cleavage likely occurs at earlier times than demonstrable by the Western analysis, the cleaved caspase-3 activity at times <6 days is lower than the current detection limits set by our Western assay. For this reason, we used only cleaved caspase-3 as a confirmation of the type of cell death, rather than relying on it for detection and quantification of the complete time series. Because annexin-V-associated membrane changes occur early in the timeline of apoptosis-related events,⁵ it is not surprising that increased cleaved caspase-3 immunoreactivity occurs somewhat later than annexin-V reactivity.¹⁹

In the RGC layer, the number of annexin-V-positive cells were greatest between 7 to 15 days. However, a significant number of apoptotic cells were also still apparent in the RGC layer at 18 to 21 days after induction. Annexin-V-associated apoptotic signal is largely complete by 31 days after induction. This suggests that, in the rAION model, a longer time is required from induction until quantification of cell loss to determine potential long-term therapeutic effects. Several possibilities may account for the extended period of apoptosis. Late apoptosis may be present in other models, but at levels so low that it was not recognized. Another possibility is that late RGC death may occur in neurons with partially compromised axons or loss of specific retrograde/anterograde signaling to the CNS. This may correspond to the wave of late RGC death found in partial ON transection.²⁰ Alternatively, different RGC populations may have relative resistance or sensitivity to axonal ischemia.²¹ These interesting possibilities must be evaluated in a future study.

Primate optic nerve is stereotopically organized with a defined region corresponding to the high-acuity macula,^{22,23} but, until now, the rodent ON was not believed to be regionally organized, because rodents do not have a macula. However, rAION produces regional RGC loss.¹⁷ After rAION, apoptotic cells are concentrated in individual retinal regions, but some

apoptotic RGCs occur diffusely throughout the retina. This pattern of cell death is similar to the pattern of rAION-induced RGC loss reported previously.^{13,14,17} The current results, coupled with the previous identification of ON regional damage and other recent reports, suggest that a degree of rodent optic nerve stereotopic organization exists, although probably less pronounced than in primates. Thus, focal damage to individual rat ON regions will produce regional RGC death.

Although trauma models are routinely used for evaluating the timing of RGC death, degeneration, and mechanisms of axonal regeneration, these techniques disrupt normal retinal function and optic nerve architecture, which may result in accelerated RGC death.²⁴ The preservation of underlying structures after optic nerve ischemia may increase local antiapoptotic and survival factors. Our current data suggest that isolated axonal ischemia results in RGC death later than that in ON trauma. Because rAION, like clinical NAION is ischemic, therapeutic strategies used to evaluate rAION are likely to be more relevant to treatment of optic nerve ischemia in general. The extended period of cell death after rAION induction suggests that there may be an extended time period after sudden axonal ischemia during which RGCs may still be responsive to neuroprotective strategies. The prolonged period of RGC death after axonal ischemia implies that the window of treatment opportunity after optic nerve stroke may be longer than previously believed.

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Figure 1.

Annexin-V-positive labeling of cells in the RGC layer. (A) Control. Few annexin-V-positive cells were apparent. (B) Three days after induction. More annexin-V-positive cells were present within the RGC layer (*arrows*). (C) Three days after induction, high-power view. Annexin-V-positive cells (*arrow*) were present in the NFL plane. (D) Ten days after induction. Numerous annexin-V-positive cells were scattered throughout the retinal region. (E) Thirty-one days after induction. Annexin-V-positive cells were still visible, although much diminished in number. (F) Thirty-one days after induction, high-power view. Annexin-V-positive cells (*arrow*) were still present at the level of RGC axons. *Double-tailed arrowheads*: RGC axons in the NFL; *arrowheads*: Bex1/2-positive cells; VS, retinal vessels. Magnification, $\times 200$. Scale bar (D for A, B, E) 200 μm ; (F for C) 100 μm .

Figure 2.

RGC colocalization of fluorogold, Bex1/2, and annexin V, 28 days after rAION induction. (A) RGCs were retrograde fluorogold labeled. (B) Bex1/2 staining showed a consistent overlap with fluorogold retrograde labeling. (C) Several annexin-positive cells were visible in this field and appeared outlined by annexin V binding. (D) Merged image. Bex1/2 and fluorogold colocalized. Most annexin-V-positive cells were also fluorogold and Bex1/2 positive. Arrows: RGCs that were fluorogold, Bex1/2, and annexin positive. Scale bar: 50 μ m.

Figure 3. rAION results in RGC layer apoptosis. Ten- μ m-thick sections of rat retina were analyzed by TUNEL assay. **(A)** In negative control (uninduced) retinas, no TUNEL-positive cells were apparent. **(B)** Nine days after rAION induction, scattered TUNEL-positive cells were present in the RGC layer (*arrows*). There was artifactual light staining surrounding the cells in the inner and outer nuclear layers. **(C)** Positive control. TUNEL-control retina (DNase-1 treated). All nuclear layers were TUNEL positive. RGC, RGC layer; INL, inner nuclear layer; ONL, outer nuclear layer; Prc, photoreceptors. Scale bar, 100 μ m.

Figure 4.

Evaluation of caspase 3 (35 kDa) and cleaved caspase-3 (17 kDa) fragment in retinas and PC-12 cells after rAION induction. Controls included total protein homogenates from uninduced PC-12 cells (C) and PC-12 cells treated with 100 ng/mL staurosporine (C+S). Protein-transferred membranes were reacted with anti cleaved caspase 3. Signal was developed with ECL reagents. Protein loading was confirmed by reprobing the stripped membrane with antibody to β -actin. β -Actin-normalized values of cleaved caspase-3 expression are shown.

Figure 5. rAION resulted in regional RGC apoptosis. Different retinal regions of the same 10-day post-rAION flatmounted retina. *Red*: immunolabeling with Bex-1/2 (*red*). *Green*: FITC-annexin-V positivity. (A) Positive region. Images obtained from the peripheral region of one retinal quadrant. Numerous annexin-V-positive cells were seen in the RGC layer. (B) Negative region. Photograph obtained from the contralateral side of the same retina. No annexin-V-positive cells were seen. Scale bar, 100 μ m.

Figure 6.

Time course of RGC death after rAION induction. **(A)** Total retina. The y-axis shows the mean number of annexin-V-positive cells/retina ($n = 12$ fields retina; 3 fields/region). Each time point is the mean average of four retinas. The x-axis is the number of days after infarct. Two peaks are apparent: one at 10 days, and a second at 21 days. **(B)** Regional analysis. The y-axis shows the mean number of annexin-V-positive cells in three contiguous fields. The x-axis is the number of days after infarct. RGC apoptosis was greatest at 10 days after induction, but the SE was greatly reduced at nearly all time points, suggesting that rAION-induced RGC apoptosis occurs regionally. All data are the mean $\pm$ SEM.