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Inflammatory Demyelination Induces Axonal Injury and Retinal Ganglion Cell Apoptosis in Experimental Optic Neuritis

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Abstract

Optic neuritis is an inflammatory disease of the optic nerve that often occurs in patients with multiple sclerosis and leads to permanent visual loss mediated by retinal ganglion cell (RGC) damage. Optic neuritis occurs with high frequency in relapsing-remitting experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, with significant loss of RGCs. In the current study, mechanisms of RGC loss in this model were examined to determine whether inflammation-induced axonal injury mediates apoptotic death of RGCs. RGCs were retrogradely labeled by injection of fluorogold into superior colliculi of 6–7 week old female SJL/J mice. EAE was induced one week later by immunization with proteolipid protein peptide. Optic neuritis was detected by inflammatory cell infiltration on histological examination as early as 9 days after immunization, with peak incidence by day 12. Demyelination occurred 1–2 days after inflammation began. Loss of RGC axons was detected following demyelination, with significant axonal loss occurring by day 13 post-immunization. Axonal loss occurred prior to loss of RGC bodies at day 14. Apoptotic cells were also observed at day 14 in the ganglion cell layer of eyes with optic neuritis, but not control eyes. Together these results suggest that inflammatory cell infiltration mediates demyelination and leads to direct axonal injury in this model of experimental optic neuritis. RGCs die by an apoptotic mechanism triggered by axonal injury. Potential neuroprotective therapies to prevent permanent RGC loss from optic neuritis will likely need to be initiated prior to axonal injury to preserve neuronal function.

Keywords

optic neuritis; EAE; demyelination; axonal loss; retinal ganglion cell

1. Introduction

Optic neuritis manifests as an acute, self-limited episode of optic nerve inflammation with decreased vision that recovers over several weeks in the majority of patients (Arnold, 2005). However, some level of permanent vision loss occurred in approximately 40% of patients in the Optic Neuritis Treatment Trial (Beck et al., 1992), and subsequent studies have shown that

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retinal nerve fiber layer thinning, used as a surrogate marker for RGC axonal loss, correlates with vision loss after an episode of optic neuritis (Steel and Waldock, 1998; Parisi et al., 1999; Trip et al., 2005; Costello et al., 2006). Patients with multiple sclerosis also have retinal nerve fiber layer thinning correlating to decreased vision even without a documented episode of optic neuritis (Fisher et al., 2006), suggesting that RGC axonal loss mediates visual loss in this disease. Together these ophthalmologic findings are consistent with studies suggesting that axonal injury and loss of neurons mediates permanent neurological disability in multiple sclerosis and EAE (Davie et al., 1995; Losseff et al., 1996a and 1996b; Trapp et al., 1998; Kornek et al., 2000; Wujek et al., 2002).

Understanding the timing and mechanisms of axonal injury and neuronal cell death is important for developing potential neuroprotective therapies to prevent permanent vision loss from optic neuritis. Several experimental optic neuritis models have been reported, including optic neuritis in relapsing-remitting EAE (Potter and Bigazzi, 1992; Shindler et al., 2006), chronic EAE (Meyer et al., 2001; Shao et al., 2004; Guyton et al., 2005; Qi et al., 2007a), and isolated optic neuritis in myelin oligodendrocyte glycoprotein (MOG)-specific T cell transgenic mice (Bettelli et al., 2003; Guan et al., 2006). We previously found that two-thirds of eyes from relapsing-remitting EAE mice develop optic neuritis by day 11 after immunization, but significant RGC loss is not detected until day 14 (Shindler et al, 2006), suggesting that RGC loss occurs secondary to inflammation. In other EAE models, however, some RGC loss occurs prior to histological optic neuritis (Hobom et al., 2004), and cell damaging reactive oxygen species are produced in EAE optic nerves just 3–6 days after immunization (Qi et al., 2007a), suggesting that the course of optic neuritis may vary depending on the specific autoimmune disease course.

In the current study we examined the timing and extent of several parameters (inflammation, demyelination, axonal injury, and RGC loss) of optic nerve damage during acute optic neuritis in EAE mice with relapsing-remitting disease, the most common disease course in multiple sclerosis patients (Noseworthy et al., 2000), and studies examined whether RGC death occurs by an apoptotic mechanism. Results suggest that inflammatory cells infiltrating the optic nerve induce demyelination and an axonopathy that mediates apoptotic cell death of RGCs.

2. Materials and methods

2.1 Animals

6–7 week old SJL/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Housing and treatment of animals conformed to Institutional Animal Care and Use Committee Guidelines at Thomas Jefferson University.

2.2 Induction of EAE

EAE was induced by immunization with proteolipid protein peptide139–151 (PLP; purchased from the Protein Chemistry Laboratory, University of Pennsylvania, Philadelphia, PA, USA) as described previously (Shindler et al., 2006). Briefly, mice were anesthetized with 0.2 ml solution containing 10 mg/ml ketamine and 1 mg/ml xylazine by intraperitoneal injection. Mice were immunized by subcutaneous injection in two sites on the back with 0.1 ml PLP emulsified in complete Freund's adjuvant (CFA; Difco, Detroit, MI, USA). 200 ng pertussis toxin (List Biological, Campbell, CA, USA) was injected on day 0 (day of immunization) and again on day 2. Mice were scored daily for clinical EAE using a 5-point scale (Gran et al., 2002). For all experiments, control mice were sham-immunized with subcutaneous injections of PBS in CFA at the time of immunization and sacrificed at the same time as EAE mice. Age-matched control mice for axonal studies were sacrificed and processed separately.

2.3 Optic nerve histopathology

Following sacrifice 7–14 days after immunization, 5 μm longitudinal paraffin embedded sections were prepared from isolated optic nerves. Optic neuritis was detected by presence of inflammatory cell infiltration on hematoxylin and eosin (H & E) staining using a 0 – 4 point scale as described previously (Shindler et al., 2006). Demyelination was detected by luxol fast blue staining and was scored by a blinded investigator on a relative scale similar to prior studies of demyelination in EAE spinal cords (Gran et al., 2002): 0 = no demyelination; 1 = mild; 2 = moderate; and 3 = severe demyelination.

2.4 Quantification of axonal area

Longitudinal optic nerve sections described above (2.3) were stained by Bielschowsky silver impregnation (Czasch et al., 2006). Three photographs were taken at 40X magnification of each stained nerve (one each of the proximal, central, and distal portion of the optic nerve) using a Nikon Eclipse 80i microscope with a Retiga 2000R camera (Nikon Instruments, Melville, NY, USA). The area of staining was calculated using ImagePro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA) software. The total area photographed in three fields/nerve was 38,500 μm^2, and data shown represent the cumulative area of positive staining/nerve.

2.5 RGC labeling and quantification

RGCs were labeled by retrograde transport of fluorogold (hydroxystilbamidine; Invitrogen, Carlsbad, CA, USA) injected stereotactically into the superior colliculi and counted by fluorescent microscopy of isolated retinas following sacrifice as in previous studies (Shindler et al., 2006). Briefly, eyes were removed, fixed in 4% paraformaldehyde, and retinal whole mounts were prepared. Photographs of RGCs were taken in 12 standardized fields (1/6, 3/6, and 5/6 of the retinal radius in each quadrant) at 20X magnification. Data shown represent total number of RGCs counted per retina, covering a combined total area of 0.74 mm^2.

2.6 TUNEL labeling

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL), a marker of apoptosis, was used to identify dying RGCs similar to prior studies (Guan et al., 2006). Briefly, eyes were fixed in 4% paraformaldehyde, embedded in paraffin, and cut in 6 μm sagittal sections. TUNEL label was performed using the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. At least 3 sections/eye were examined by a blinded investigator to determine whether any TUNEL-positive apoptotic cells were present in the RGC layer.

2.7 Statistics

Comparisons of RGC numbers and of areas of axonal staining between control, optic neuritis and non-optic neuritis eyes were analyzed by one way ANOVA and Bonferroni's Multiple Comparison test using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1 Demyelination develops after optic nerve inflammation begins in EAE mice

Sections of isolated optic nerves from EAE and control mice were evaluated histologically for presence of inflammation and demyelination. Similar to prior studies (Shindler et al., 2006), inflammatory cell infiltration was detected in some optic nerves as early as 9 days after immunization, with peak incidence of two thirds of eyes developing inflammation by day 12 (Fig. 1A–C, Fig. 2A). Demyelination, detected as areas of reduced luxol fast blue staining within optic nerves, occurred in optic nerves from EAE mice but not controls (Fig. 1D–F). Demyelination was first detected at day 11, with increasing incidence through day 14 (Fig.

2A). The onset of demyelination was delayed by 1–2 days as compared to the onset of optic nerve inflammation (Fig. 2A). The average severity of demyelination per affected nerve also increased with time (Fig. 2B).

3.2 Inflammation induces significant axonal loss within EAE optic nerves

Axons stained by Bielschowsky's silver impregnation in sections of EAE and control optic nerves from mice 14 days after immunization were analyzed as a measure of axonal area. EAE optic nerves that developed optic neuritis had significantly less axonal staining than control optic nerves or optic nerves from EAE mice that did not develop optic neuritis (Fig. 3). Truncation of axons with terminal axonal swelling (axonal ovoids), markers of axonal damage reported in brain and spinal cord lesions of multiple sclerosis (Trapp et al., 1998), were observed in EAE optic neuritis nerve sections (Fig. 3). A similar pattern of axonal loss was observed in three separate experiments (Table 1).

3.3 Axonal degeneration precedes RGC loss

The area of axonal staining progressively decreased in EAE optic neuritis nerves, with statistically significant axonal loss first measured at day 13 (Fig. 4A). Axonal loss occurred after inflammation and demyelination began (Fig. 2A), but one day prior to the significant loss of RGCs detected at day 14 (Fig. 4B), which occurred with a similar time course to prior studies (Shindler et al., 2006). A non-significant trend toward decreased axonal area was observed in control nerves from mice sham-immunized with CFA and EAE nerves that did not develop optic neuritis, as compared to control nerves from age-matched normal mice not exposed to CFA sacrificed separately (Fig. 4A).

3.4 RGC death occurs by apoptosis

Sections of whole eyes from EAE and control mice removed on day 14 post-immunization were processed by TUNEL labeling to identify cells undergoing active apoptosis. No TUNEL positive cells were observed within the retinal ganglion cell layer of control eyes, whereas TUNEL positive cells were present in the ganglion cell layer of EAE optic neuritis eyes (Fig. 5). 70% (7 of 10) of EAE eyes with optic neuritis had TUNEL positive RGCs.

4. Discussion

The timing of axonal degeneration and RGC loss, following development of inflammation and demyelination, in relapsing-remitting EAE mice with optic neuritis suggests that inflammation itself induces demyelination and axonal injury that then leads to RGC death by apoptosis. The extent of axonal and neuronal loss in this model of optic neuritis is notable along with other studies indicating that this type of neuronal damage correlates closely with permanent vision loss and neurological dysfunction (Davie et al., 1995; Losseff et al., 1996a and 1996b; Trapp et al., 1998; Fisher et al., 2006) in multiple sclerosis. While optic neuritis and multiple sclerosis have long been recognized as autoimmune diseases, symptoms previously were felt to largely be due to inflammation and demyelination alone, with relative sparing of axons. Recent results, including the current study, demonstrate the importance of understanding the sequence and mechanisms of neuronal damage in this disease.

While the current results suggest that one mechanism of neuronal damage may be induced by optic nerve inflammation in relapsing-remitting disease, additional mechanisms leading to neuronal damage may also occur in optic neuritis. In a chronic EAE model, some RGC loss occurs prior to detectable optic nerve inflammation (Hobom et al., 2004), and consistent with those findings, metabolic changes with accumulation of reactive oxygen species can occur just days after immunization to induce EAE (Qi et al., 2007a). It is possible that adjuvant induced changes may contribute to axonal loss independent of inflammation, as we also saw a trend

toward decreased axonal staining in non-optic neuritis eyes as well as sham-immunized control eyes. Likely, both inflammation dependent and independent mechanisms of RGC damage are possible in different disease courses, and indeed, even in the chronic EAE model there is a second wave of significant RGC loss that occurs after inflammation develops (Hobom et al., 2004). Together with our results, these observations suggest that there is likely an important window of time after patients present with symptoms from optic nerve inflammation, but before axonal damage has occurred, in which therapies can be initiated to prevent permanent loss of RGCs and vision.

Several potential therapeutic strategies to prevent RGC loss in experimental optic neuritis have already been reported, including reduction of reactive oxygen species (Qi et al., 2007a and 2007b), and treatment with erythropoietin (Sattler et al., 2004) or glatiramer acetate (Maier et al., 2006). Some of these therapies (e.g. glatiramer acetate), however, only prevent RGC loss when administered before or concurrent with the induction of EAE (Maier et al., 2006), before inflammation develops. Since optic neuritis patients do not present with symptoms until inflammation begins, the observed effects of glatiramer acetate in experimental optic neuritis may have limited clinical application, and consistent with this, immunomodulatory therapies like glatiramer acetate and interferon-β, the most widely used medications for multiple sclerosis, have only limited ability to prevent long term neurodegeneration and neurological disability (Parry et al., 2003). In the relapsing-remitting model, we have previously found that activation of the SIRT1 deacetylase prevents RGC loss during acute optic neuritis, and these effects occur when SIRT1 activators are administered at day 11, after inflammation has begun (Shindler et al., 2007). In support of the current observations that axonal injury mediates RGC loss, SIRT1 activation was found to preserve functional axons following optic neuritis (Shindler et al., 2007).

Our results suggest that autoimmune inflammation and demyelination are disease-specific triggers of neuronal damage in experimental optic neuritis, but the downstream mechanisms of axonal truncation and apoptosis are processes that are known to occur in other optic nerve disorders as well. Direct axonal injury by mechanically crushing or transecting the optic nerve as a model of traumatic optic neuropathy, for example, also leads to axonal degeneration and RGC apoptosis (Berkelaar et al., 1994), suggesting that this is a common final pathway leading to RGC loss in response to multiple different insults. Therapies that prevent axonal injury or inhibit apoptosis in one model are therefore likely to have effects in other optic nerve conditions as well. To prevent RGC loss and permanent vision loss from optic neuritis, a combination of anti-inflammatory medications to reduce active inflammation with neuroprotective agents to prevent axonal injury are likely to have additive effects. The current results demonstrate a time period in which such potential therapies can be evaluated in experimental optic neuritis.

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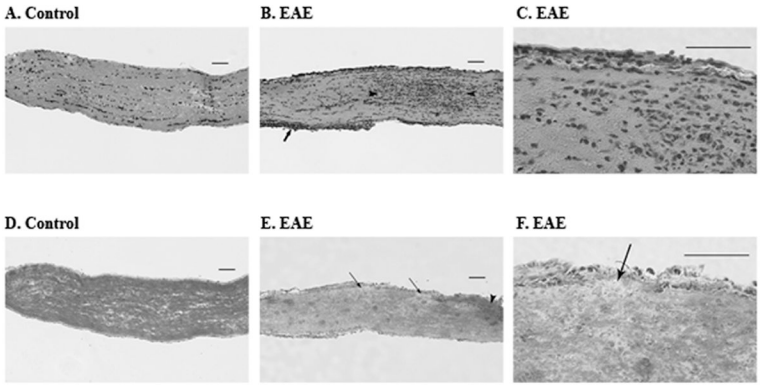

Fig. 1.

EAE optic nerves develop demyelination. (A) H & E stained section of optic nerve from a day 14 control mouse showing normal histology. (B) Inflammatory cell infiltrates observed in the optic nerve (between arrowheads) and optic nerve sheath (arrow) from a day 14 EAE mouse with optic neuritis. (C) Higher magnification view of inflammatory cells in the nerve shown in B. (D) Luxol fast blue staining in the control optic nerve shown in A demonstrates the normal myelinated nerve. (E) The EAE optic nerve shown in B contains an area of normal myelin (arrowhead) along with mild diffuse reduction in staining and focal areas of complete demyelination (arrows). (F) Higher magnification view of an area of demyelination (arrow). All scale bars = 50 μm.

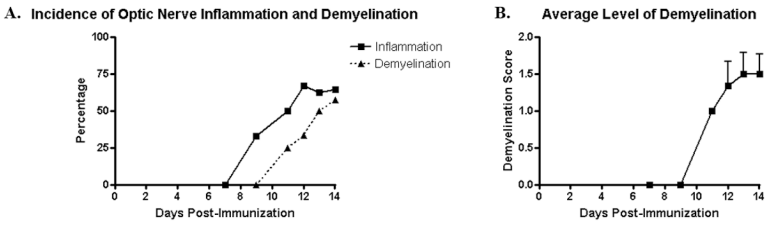

Fig. 2.

Optic nerve demyelination begins after onset of inflammation and progresses over several days. (A) The earliest time optic nerve inflammation was detected in EAE eyes was 9 days post-immunization. 33.3% incidence of inflammation at day 9 increased through day 12 and remained steady at 64.3% (9 of 14 eyes) through day 14. In contrast, no demyelination was detected until day 11, and the incidence of demyelination increased to 57.2% (8 of 14 eyes) by day 14. (B) The severity of demyelination, scored on a 0–3 point scale, also increased over time. The average score ± SEM per eye with demyelination is shown. One representative experiment of three is shown.

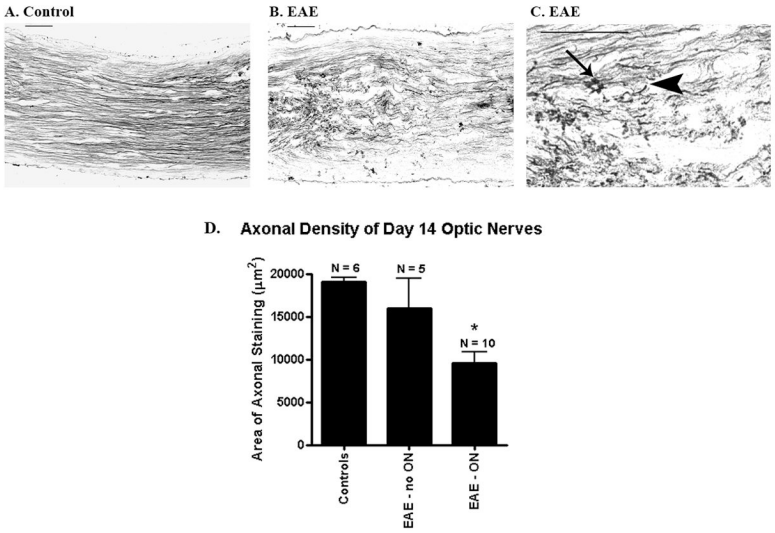

Fig. 3.

Injury and loss of axons in day 14 EAE optic nerves. (A) Normal density of RGC axons observed with silver staining in a control optic nerve. (B) An optic nerve from a day 14 EAE mouse demonstrates reduced axonal staining and disorganization from axonal injury. (C) Higher magnification view of injured nerve in B with truncated axon (arrowhead) and terminal axonal ovoid swelling (arrow). (D) EAE eyes that developed optic neuritis (10 of 15 eyes) had a significant reduction in the area of axonal staining (9558 ± 1357 μm^2) compared with control eyes from sham-immunized mice (19104 ± 471; *$p \leq 0.01$). EAE eyes that did not develop optic neuritis had no significant decrease in axonal area (15998 ± 3489) compared to controls. Data represent the mean ± SEM. One representative experiment of three is shown. Scale bars (A–C) = 50 μm.

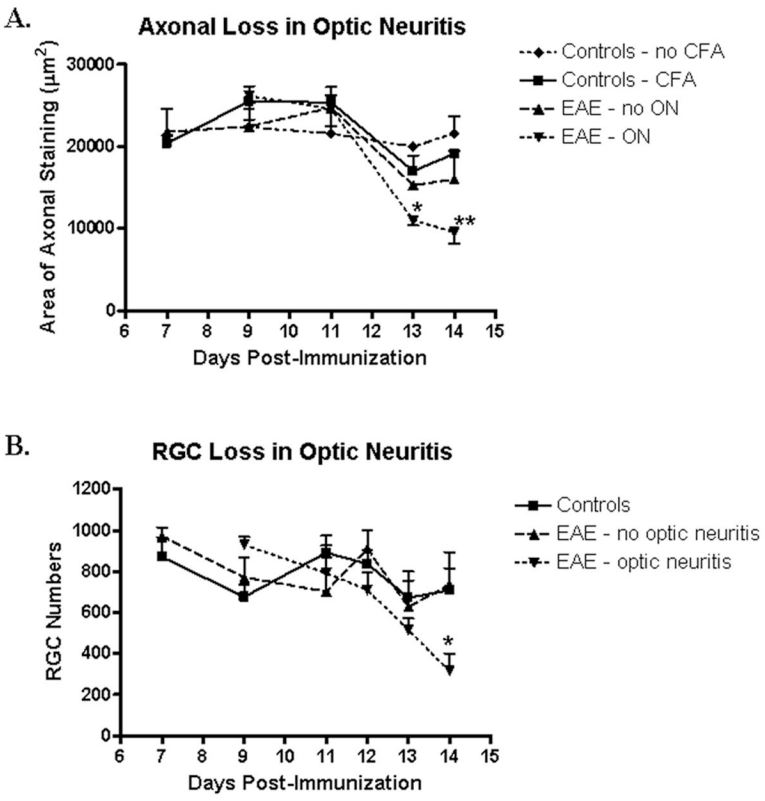

Fig. 4.

Axonal loss precedes RGC loss. (A) The average axonal area (mean ± SEM) measured from days 7 –14 in control and EAE optic nerves with or without optic neuritis is shown. Control nerves from age-matched normal mice (no CFA) and from mice sham-immunized with CFA were used. Significant reduction in axonal staining in optic neuritis vs. control (+/− CFA) eyes is first detected at day 13 (*$p \leq 0.05$) and persists at day 14 (**$p \leq 0.01$). 5 – 10 nerves were examined per group. (B) The number of surviving RGCs (mean ± SEM) in the retina of EAE eyes with optic neuritis declines slowly, with no significant difference compared to control eye RGC numbers detected until day 14 (*$p \leq 0.05$). Retinas of 5 – 6 eyes were counted per group. Data represent results from one of three experiments.

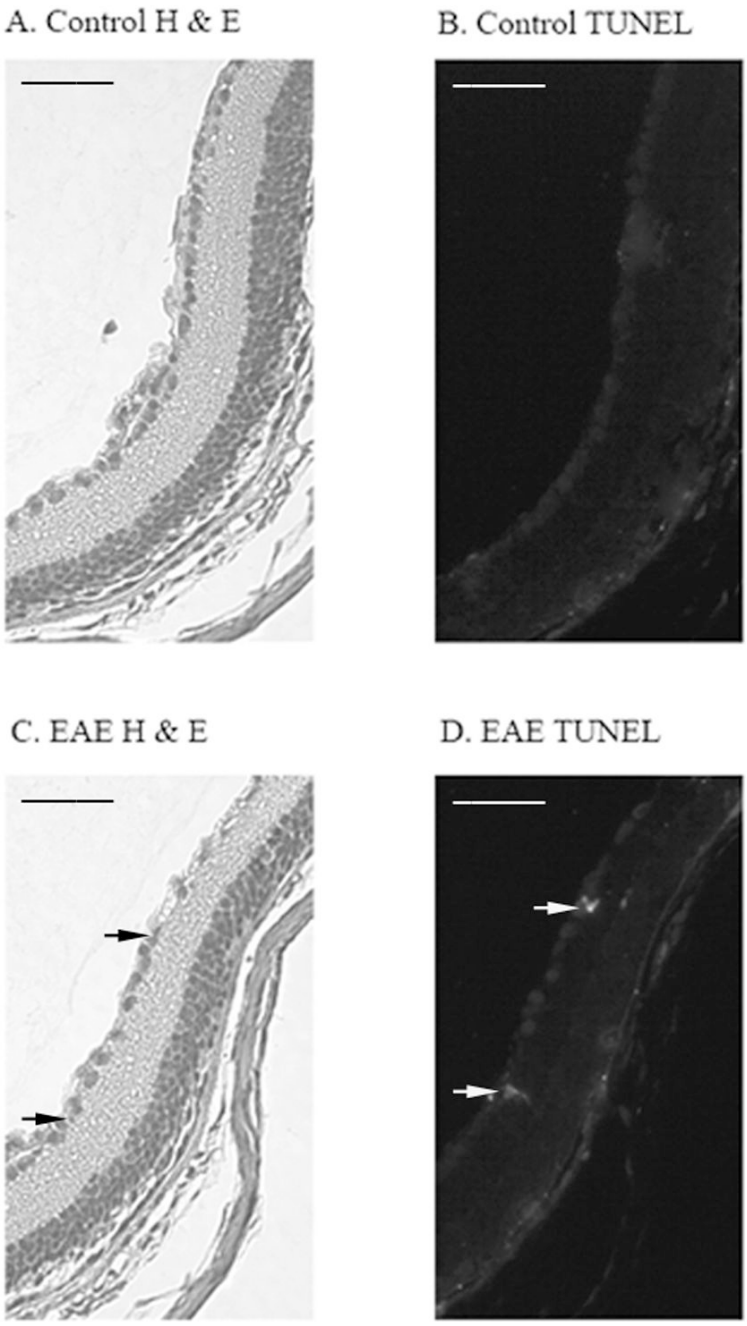

Fig. 5.

Apoptosis of RGCs in optic neuritis eyes. (A) H & E stained section through a control eye shows the normal single layer of RGC nuclei in the ganglion cell layer. (B) No TUNEL positive cells were detected in the control eye shown in A. (C,D) Arrows indicate two RGC nuclei with condensed chromatin that were undergoing apoptosis as indicated by positive TUNEL labeling. Scale bars = 50 μm.

Table 1

Reduced axonal area was detected in EAE optic nerves with optic neuritis 14 days post-immunization in three separate experiments. Data represent the mean ± SEM area of axonal silver staining (μm^2). The number of nerves examined in each group is indicated in parentheses.

Exp.#	Controls	EAE – no ON	EAE - ON
1	19104.9 ± 470.7 (6)	15998.0 ± 3488.9 (5)	9558.3 ± 1357.4 (10)*
2	20825.7 ± 1292.0 (4)	18229.8 ± 858.8 (4)	13618.4 ± 1256.8 (7)**
3	20878.6 ± 928.9 (6)	18286.9 ± 965.1 (5)	12275.9 ± 1389.7 (6)**

* $p \leq 0.01$

** $p \leq 0.05$ vs. controls.