HE3286 Reduces Axonal Loss and Preserves Retinal Ganglion Cell Function in Experimental Optic Neuritis

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Purpose. Optic nerve inflammation, demyelination, and axonal loss are all prominent features of optic neuritis. While corticosteroids hasten visual recovery in optic neuritis, no treatment improves final visual outcomes. HE3286 (17α-ethynyl-5-androstene-3β,7β,17β-triol), a synthetic derivative of a natural steroid, β-AET (5-androstene-3β,7β,17β-triol), exerts anti-inflammatory effects in several disease models and has purported neuroprotective effects as well. HE3286's ability to suppress optic neuritis was examined in experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis.

Methods. Experimental autoimmune encephalomyelitis was induced in C57/BL6 mice. Mice were treated daily with intraperitoneal vehicle or 40 mg/kg HE3286. Visual function was assessed by optokinetic responses (OKR) at baseline and every 10 days until euthanasia at 40 days post immunization. Retinas and optic nerves were isolated. Inflammation (hematoxylin and eosin and Iba1 staining), demyelination (Luxol fast blue staining), and axonal loss (neurofilament staining) were assessed in optic nerve sections. Retinal ganglion cells (RGCs) were immunolabeled with Brn3a antibodies to quantify RGC survival.

Results. Progressive decreases in OKR occurred in vehicle-treated EAE mice, and HE3286 treatment reduced the level of this vision loss. HE3286 also attenuated the degree of inflammation, demyelination, and axonal loss in EAE optic nerves as compared to nerves from vehicle-treated EAE mice. Retinal ganglion cell loss that occurred in both vehicle- and HE3286-treated EAE mice was reduced in the temporal retinal quadrant of HE3286-treated mice.

Conclusions. HE3286 suppresses inflammation, reduces demyelination and axonal loss, and promotes RGC survival during experimental optic neuritis. Importantly, HE3286 treatment also preserves some RGC function. Results suggest that HE3286 is a potential novel treatment for optic neuritis.

Keywords: optic neuritis, EAE, HE3286, neuroprotection, multiple sclerosis

Multiple sclerosis (MS) is a chronic and disabling autoimmune-mediated neurodegenerative disease of the central nervous system with characteristic inflammatory demyelination in tissues including the optic nerve. Experimental autoimmune encephalomyelitis (EAE) is a commonly used model of MS that can be induced in mice by immunization with myelin antigens.[1] The first clinical presentation in many MS patients is optic neuritis,[2] an acute, self-limited episode of optic nerve inflammation that results in demyelination and can lead to temporary or permanent loss of vision.[2,3] Neuronal loss, including retinal ganglion cell (RGC) apoptosis in eyes with optic neuritis, also occurs in EAE.[4] Current medications available for MS are partially effective as they specifically target the inflammatory phase, but not the neurodegenerative phase, and therefore have limited effects on long-term disability.[5–8] Thus, new agents that target both inflammatory and neurodegenerative phases of MS and optic neuritis may be particularly beneficial.

HE3286 (17α-ethynyl-5-androstene-3β,7β,17β-triol) is a synthetic derivative of the adrenal steroid β-AET (5-androstene-3β,7β,17β-triol),[9] which itself is a pharmacologically active metabolite of dehydroepiandrosterone (DHEA), a major product of the adrenal gland.[10–12] HE3286 is pharmacologically unrelated to glucocorticoids and sex steroids, and although anti-inflammatory, it is not immunosuppressive[13] and has not been found to be toxic at pharmacologically relevant exposures in rodents and canines.[14] HE3286 is effective in the treatment of a variety of inflammatory disease models, including collagen-induced and adjuvant-induced arthritis[15–17] and autoimmune models of diabetes and insulin resistance.[18,19] There are limited data on the effects of HE3286 in EAE. In one report, HE3286 reduced clinical disease scores, suggestive of reduced spinal cord inflammation, in a relapsing EAE model,[20] but effects on neuronal loss were not examined. The ability of HE3286 to suppress more chronic inflammation and to suppress optic neuritis is unknown. Recently, it was shown that HE3286 has direct neuroprotective effects, preserving function and preventing neuronal loss, in a Parkinson's disease model.[21] Thus, we hypothesized that HE3286 has both anti-inflammatory and neuroprotective properties that will suppress optic neuritis and improve visual outcomes. In the present study, we investigated the ability of HE3286 to prevent inflammation, demyelination, neuronal degeneration, and vision loss in experimental optic neuritis using a chronic EAE model.

Methods

Mice

Six-week-old female C57/BL6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed at the University of Pennsylvania in accordance with university and National Institutes of Health guidelines. All procedures were conducted according to protocols approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania, and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction and Scoring of EAE

Experimental autoimmune encephalomyelitis was induced as in prior studies.[4] Briefly, at 8 weeks of age, mice ($n = 14$) were anesthetized by isoflurane, and a total of 200 μg myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}; Genscript, Piscataway, NJ, USA) emulsified in Complete Freund's Adjuvant (CFA; Difco, Detroit, MI, USA), containing 2.5 mg/mL mycobacterium tuberculosis (Difco), was injected subcutaneously at two sites on the back. Control mice ($n = 5$) were injected with an equal volume of phosphate-buffered saline (PBS) and CFA. In addition, each animal received 200 ng pertussis toxin (List Biological, Campbell, CA, USA) in 0.1 mL PBS by intraperitoneal (IP) injection at 0 and 48 hours post immunization. Severity of EAE was scored using a previously published[22] 5-point scale: no disease = 0; partial tail paralysis = 0.5; tail paralysis or waddling gait = 1.0; partial tail paralysis and waddling gait = 1.5; tail paralysis and waddling gait = 2.0; partial limb paralysis = 2.5; paralysis of one limb = 3.0; paralysis of one limb and partial paralysis of another = 3.5; paralysis of two limbs = 4.0; moribund state = 4.5; death = 5.0.

HE3286 Treatment

Mice were treated daily with vehicle (PBS) ($n = 7$) or 40 mg/kg HE3286 ($n = 7$) (Harbor Therapeutics, Inc., San Diego, CA, USA) by IP injection, starting from day 1 (24 hours post immunization) and continuing until euthanasia at day 40 post immunization. This dose was found previously to be most effective at suppressing paralysis in a relapsing EAE model.[20]

Histopathologic Evaluation of Optic Nerves

Forty days after the EAE induction, optic nerves were isolated, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm longitudinal sections. For histological analysis, sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy. Presence of inflammatory cell infiltration in the optic nerves was assessed by a blinded investigator according to previously published criteria[22,23]: no infiltration = 0; mild cellular infiltration of the optic nerve or optic nerve sheath = 1; moderate infiltration = 2; severe infiltration = 3; massive infiltration = 4. To detect demyelination, sections of the optic nerve were stained with Luxol fast blue (LFB).[22,23] Photographs were taken of three fields/nerve (one each at the distal, central, and proximal regions of the longitudinal optic nerve section) by a blinded investigator using a standard exposure, and staining was quantified by calculating the optical density using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Histologic analysis and all subsequent analyses were performed using both eyes from each mouse as individual data points because prior studies demonstrated that optic neuritis can occur either bilaterally or unilaterally,[4,22,23] suggesting it is an independent event even in the same EAE mouse—similar to the typical unilateral presentation in MS patients.

Immunohistochemistry: Quantification of Inflammation and Axonal Area

Neurofilament (axons) and Iba1 (macrophage/microglia) staining was done according to a previously published protocol.[24] Briefly, sections of optic nerve were deparaffinized and rehydrated. For neurofilament staining, sections were also permeabilized with 0.5% Tween 20 in PBS. Antigen retrieval for Iba1 staining was done by heating sections at 95°C in Vector antigen unmasking solution (Vector Laboratories, Burlingame, CA, USA) for 15 minutes. Nonspecific binding was blocked with blocking reagent from Vectastain Elite Avidin/Biotin Complex kit (ABC; Vector Laboratories), and sections were then incubated in rabbit anti-neurofilament antibody 1:100 (Abcam, Cambridge, MA, USA) or rabbit anti-Iba1 antibody 1:200 (WAKO, Richmond, VA, USA) at 4°C overnight. Sections were washed three times with PBS and incubated with goat biotinylated anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA, USA) for 2 hours at room temperature. Avidin/Biotin Complex detection was performed using the Vectastain Elite ABC kit and DAB (diaminobenzidine) substrate kit (Vector Laboratories) according to the manufacturer's instructions. Photographs were taken of three fields/nerve (one each at the distal, central, and proximal regions of the longitudinal optic nerve section) by a blinded investigator using a standard exposure. Neurofilament staining was quantified by calculating the optical density using ImageJ software (National Institutes of Health). For Iba1 staining quantification, the number of stained cells/field was counted by a blinded investigator.

Quantification of Retinal Ganglion Cells

Retinal ganglion cells were immunolabeled and counted as described previously.[24] Briefly, retinas were isolated, prepared as flattened whole mounts, washed, and permeabilized in 0.5% Triton X-100 in PBS by freezing for 15 minutes at −70°C. The specimens were then rinsed in PBS containing 0.5% Triton X-100 and incubated overnight at 4°C with goat anti-Brn3a antibody (Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:100 in blocking buffer (PBS, 2% bovine serum albumin, 2% Triton X-100). The retinas were washed three times in PBS, incubated for 2 hours at room temperature with anti-goat secondary antibody diluted 1:500 in blocking buffer, washed in PBS, and mounted vitreous side up on slides in antifading solution. Retinal ganglion cells were photographed at 40× magnification in 12 standard fields (1/6, 3/6, and 5/6 of the retinal radius from the center of the retina in each quadrant) and counted by a masked investigator using image analysis software (Image-Pro Plus 5.0; Media Cybernetics, Silver Spring, MD, USA). Total cell counts in all 12 fields, as well as RGC numbers counted in the three fields from each retinal quadrant, were recorded.

Measurement of Optokinetic Responses (OKR)

Visual function was assessed by OKR using OptoMotry software and apparatus (Cerebral Mechanics, Inc., Lethbride, AB, Canada), as described previously.[4,25] Optokinetic response function is determined by the highest spatial frequency at which mice track a 100% contrast grating projected at varying spatial frequencies, and data are reported as cycles per degree.

Statistics

Data are expressed as means ± SEM. Differences in inflammation scores, immunostaining, RGC numbers, and OKR respons-

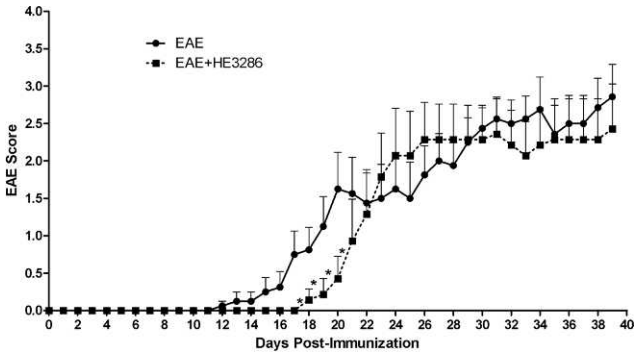

FIGURE 1. HE3286 delays the onset of EAE. Experimental autoimmune encephalomyelitis was induced in C57/BL6 mice by immunization with MOG peptide, and mice were treated daily with 40 mg/kg HE3286 ($n = 7$) or equal volume of vehicle ($n = 7$). HE3286-treated mice show a significant (*$P < 0.05$) delay in onset of EAE paralysis (days 17–20 post inoculation) but no significant difference during the chronic phase of EAE.

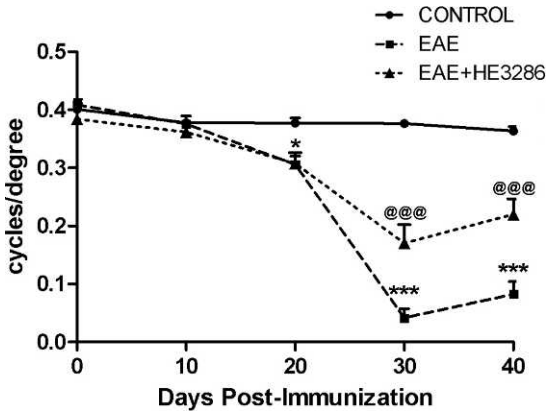

FIGURE 2. HE3286 preserves RGC function. Optokinetic responses (OKR) were measured to estimate visual function. Optokinetic responses significantly decrease (***$P < 0.001$) in both vehicle- and HE3286-treated EAE eyes ($n = 14$ eyes/group) compared to control eyes ($n = 10$ eyes), but OKR is significantly better preserved in HE3286 (40 mg/kg daily)-treated mice (@@@$P < 0.001$).

es were compared using one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

HE3286 Delays Onset of EAE

Experimental autoimmune encephalomyelitis was induced in 8-week-old female C57/BL6 mice by immunization with MOG peptide. Mice were treated daily with 40 mg/kg HE3286 ($n = 7$) or equal volume of PBS ($n = 7$) for 40 days IP. Mice treated with PBS developed a typical course of chronic EAE, marked by ascending paralysis beginning approximately 12 to 14 days after immunization, peaking several days later, and then persisting (Fig. 1). Treatment with 40 mg/kg HE3286 significantly delayed the onset of EAE, although by 22 to 25 days post immunization, EAE severity reached levels equivalent to those in PBS-treated mice (Fig. 1).

HE3286 Treatment Preserves Optokinetic Responses

Experimental autoimmune encephalomyelitis scores demonstrated only a temporary ability of HE3286 to preserve spinal cord function; but OKR, used as a marker of RGC function in the same EAE mice, showed persistent functional benefit. It has been shown previously that OKR responses decrease in EAE mice.[4] Optokinetic response was measured every 10 days post immunization. While OKR responses significantly decreased in eyes of both PBS- and HE3286-treated mice versus controls ($P < 0.001$) by day 20, treatment with HE3286 significantly attenuated this loss of OKR response at 30 to 40 days post immunization, maintaining responses significantly higher than those in PBS-treated EAE mice ($P < 0.001$; Fig. 2).

HE3286 Treatment Reduces Optic Nerve Inflammation

Experimental autoimmune encephalomyelitis mice treated with HE3286 ($n = 7$) or mock treated with PBS ($n = 7$) were euthanized on day 40 post immunization. Sections of optic nerves stained by H&E were examined for areas of inflammatory cell infiltration, and inflammation was graded on 0- to 4-point scale.[22,23] Control mice ($n = 10$ eyes of 5 mice) demonstrated normal histology without presence of inflammatory cell infiltrates (Fig. 3). Inflammatory cell infiltrates detected in most optic nerves from MOG-immunized EAE mice ($n = 14$) confirmed the presence of optic neuritis. Treatment with HE3286 induced a significant ($P < 0.05$) decrease in inflammation score compared to optic nerves from PBS-treated EAE mice (Fig. 3B). To further confirm inflammatory cell infiltration, optic nerve sections were stained with Iba1 antibody, a macrophage/microglia-specific marker. The number of Iba1-positive cells was significantly ($P < 0.001$) higher in optic nerves from MOG-immunized EAE mice compared with control, non-EAE mice. Treatment with HE3286 induced a significant ($P < 0.01$) decrease in Iba1-positive cell numbers compared to those in EAE mice (Figs. 3C, 3D).

HE3286 Treatment Reduces Optic Nerve Demyelination

Optic nerve demyelination begins after onset of inflammation and can be detected by LFB staining of myelin in EAE optic nerves.[22,23] To examine whether HE3286 can prevent demyelination, optic nerve sections from control, PBS-treated EAE, and HE3286-treated EAE mice were stained with LFB. Demyelination occurred in optic nerves from EAE mice, with a significant ($P < 0.001$) decrease in LFB staining compared to control optic nerves (Fig. 4). Optic nerves of EAE mice treated with HE3286 for 40 days showed significantly increased LFB staining ($P < 0.001$) compared with optic nerves of PBS-treated EAE mice (Fig. 4).

HE3286 Treatment Reduces Axonal Loss in Optic Nerve

To examine whether HE3286 can protect from axonal loss during EAE, optic nerve sections from control, PBS-treated EAE, and HE3286-treated EAE mice were stained with neurofilament antibody, and the amount of staining was used to quantify axon density as in prior studies.[24] Prior studies have shown that optic neuritis is associated with RGC axonal and neuronal loss in both relapsing and chronic EAE,[4,23,26] and the current results confirm this loss ($P < 0.001$; Fig. 5). In contrast, optic neuritis nerves from HE3286-treated EAE mice have

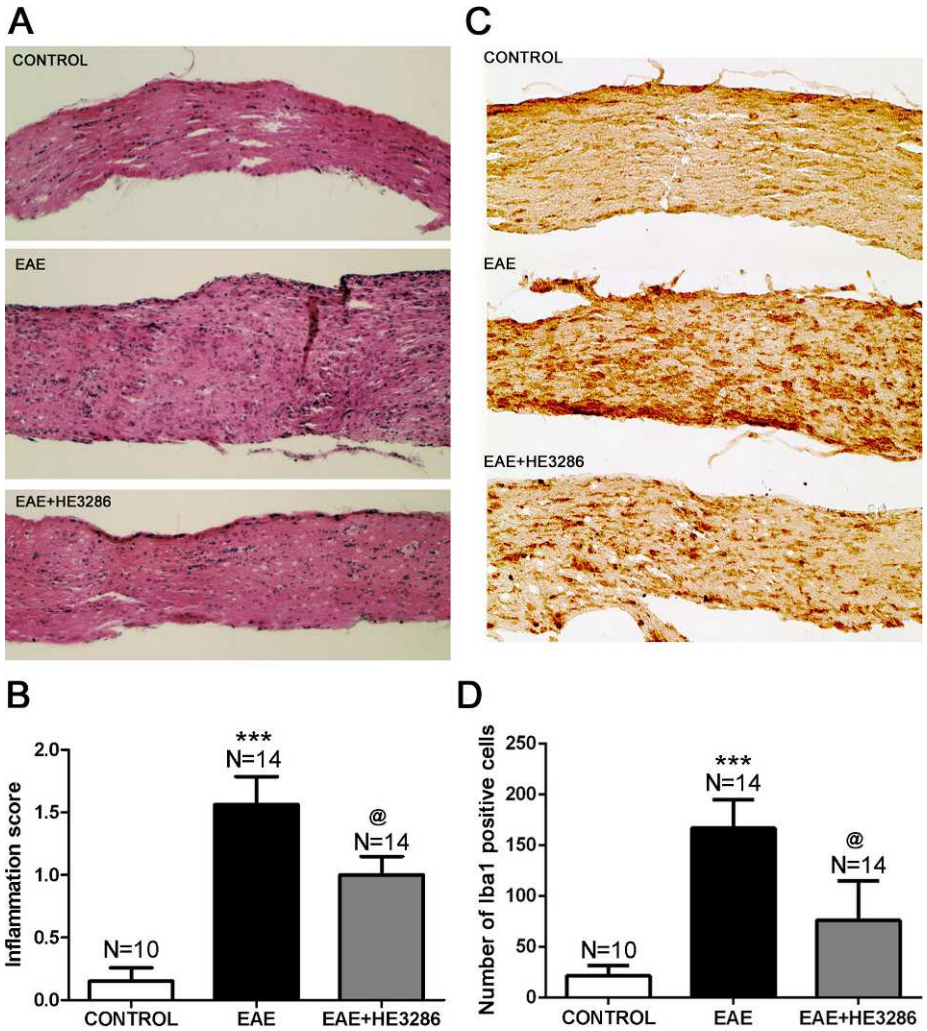

Figure 3. HE3286 attenuates optic nerve inflammation. Mice were immunized with MOG peptide and treated daily with 40 mg/kg HE3286 or equal volume of vehicle. After 40 days, mice were euthanized, and optic nerves were isolated and stained by H&E or immunostained for the macrophage/microglia marker Iba1. (**A**) A representative picture of optic nerve from each group shows that numerous inflammatory cells are evident in H&E-stained EAE optic nerves 40 days post immunization. Less inflammation is present in optic nerves from EAE mice treated with HE3286, and almost no inflammation is present in optic nerves from control mice. (**B**) Hematoxylin and eosin staining was graded on a 0- to 4-point scale. Inflammation was significantly higher in EAE optic nerves ($n = 14$) than in controls ($n = 10$) ($^{***}P < 0.001$). Treatment with HE3286 ($n = 14$ optic nerves) shows a significant ($^{@}P < 0.05$) decrease in inflammation score compared to EAE optic nerves. (**C**) A representative picture of optic nerve from each group shows numerous Iba1-positive macrophages/microglia in EAE optic nerves at 40 days post immunization. Fewer Iba1-positive cells are present in optic nerves from EAE mice treated with HE3286. (**D**) The number of Iba1-positive cells per high-powered field was counted. Optic nerves from EAE mice ($n = 14$) contain significantly more macrophages/microglia than controls ($n = 10$) ($^{***}P < 0.001$). Treatment with HE3286 ($n = 14$ optic nerves) shows a significant ($^{@}P < 0.05$) decrease in Iba1-positive cells compared to EAE optic nerves.

increased RGC axonal staining ($P < 0.01$) compared with PBS-treated EAE mouse optic nerves (Fig. 5).

HE3286 Treatment Reduces RGC Loss in the Temporal Retina

To examine whether HE3286 can also reduce RGC loss during EAE, retinas from control, PBS-treated EAE, and HE3286-treated EAE mice were stained with Brn3a antibody. Stained RGCs were counted in 12 standardized fields, three from each retinal quadrant (inferior, nasal, superior, temporal). Total RGC numbers (from all 12 fields) in optic neuritis eyes from PBS-treated EAE mice were significantly ($P < 0.001$) lower than in eyes from control mice. Treatment with HE3286 for 40 days showed just a trend toward increased RGC numbers (Fig. 6A). Analysis of RGC numbers by quadrant showed that RGC numbers in optic neuritis eyes from PBS-treated EAE mice were significantly ($P < 0.001$) lower than in eyes from control mice in all quadrants. Treatment with HE3286 for 40 days significantly ($P < 0.05$) increased RGC numbers in the temporal retinal quadrant compared to eyes from PBS-treated EAE mice, and led to a trend toward increased RGC survival in the other three retinal quadrants (Figs. 6B, 6C).

Discussion

The present studies examined potential anti-inflammatory and neuroprotective activities of HE3286 in chronic experimental optic neuritis. Data show that HE3286 provides clinical improvement in optic neuritis, marked by preservation of OKR responses, during EAE. This functional improvement is associated with improved histopathologic outcomes of reduced inflammation and demyelination in the optic nerve.

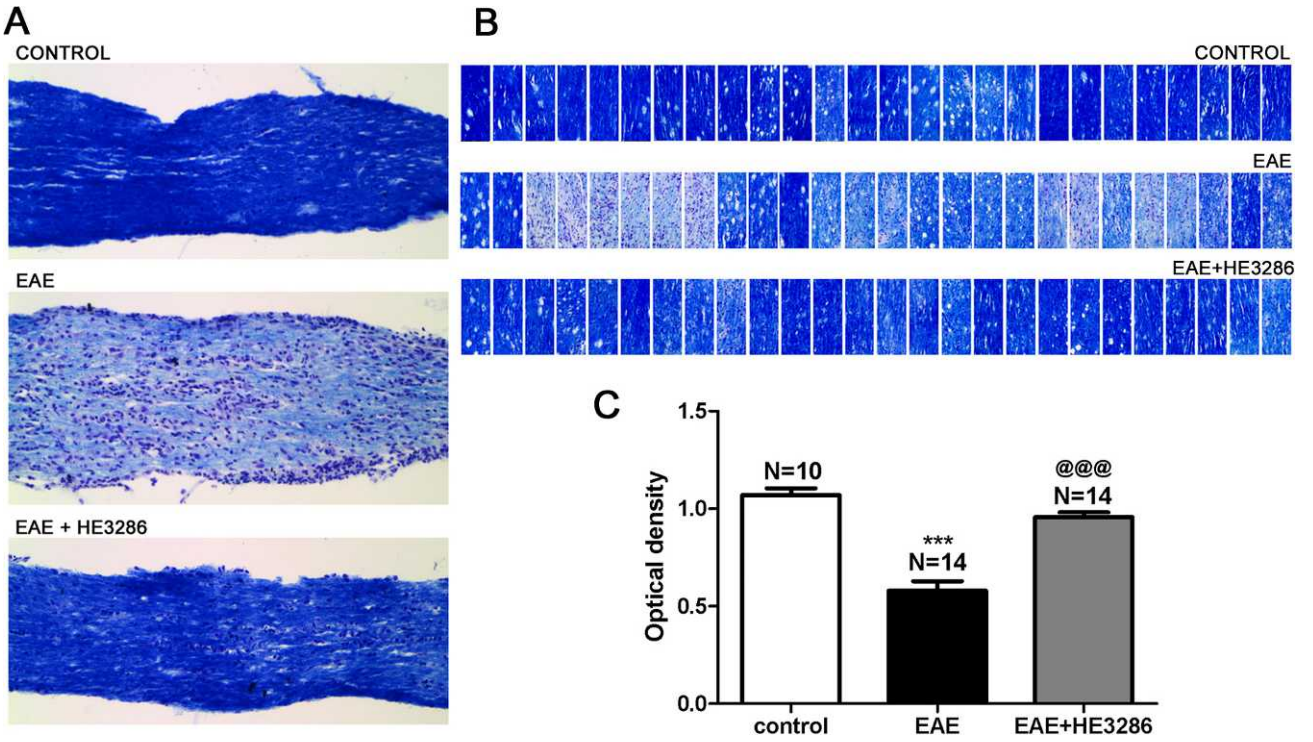

FIGURE 4. HE3286 attenuates demyelination in optic nerve during EAE. To examine whether HE3286 can protect from demyelination, optic nerves were isolated from EAE mice treated daily with 40 mg/kg HE3286 or equal volume of vehicle and stained with Luxol fast blue (LFB), which stains myelin. (**A**) An optic nerve from a PBS-treated EAE mouse shows loss of myelin at 40 days post immunization, whereas an optic nerve from an EAE mouse treated with HE3286 shows normal myelin staining. (**B**) A series of photographs of LFB staining in optic nerve sections from control, PBS-treated EAE, and HE3286-treated EAE mice shows normal myelin staining, numerous areas of myelin loss in PBS-treated EAE optic nerves, and less demyelination in HE3286-treated optic nerves. (**C**) The optical density of LFB staining was calculated from three equal-sized fields from each optic nerve using ImageJ software. Experimental autoimmune encephalomyelitis optic nerves ($n = 14$) have significantly lower ($^{***}P < 0.001$) optical density compared to control optic nerves ($n = 10$), whereas treatment with HE3286 significantly preserves ($^{@@@}P < 0.001$) the LFB staining density ($n = 14$).

Axonal loss in the optic nerve and RGC loss in the temporal retinal quadrant are also significantly attenuated, suggesting that HE3286 has neuroprotective properties as well, although this may be limited as RGC numbers showed only a trend toward increased survival in other retinal quadrants. Overall, results suggest that HE3286 preserves some aspects of visual function by reducing optic nerve inflammation and demyelination, although it remains to be determined whether partial neuroprotective effects are direct effects of HE3286 or alternatively are secondary to the reduced inflammation.

Although OKR is one tool used to estimate RGC function, discrepancies between RGC numbers and OKR have been observed previously.[27] More than a dozen different types of RGCs exist, with distinct structure and function,[28,29] and different modalities are conveyed in parallel to different sites in the brain by axons of different RGC types.[30] It has been reported that the direction of image motion is coded by direction-selective RGCs in the retina, which project axons specifically to terminal nuclei of the accessory optic system (AOS) responsible for OKR responses.[31,32] It is possible that HE3286 may provide relatively stronger neuroprotective effects on RGCs selectively responsible for OKR responses, possibly those RGCs protected in the temporal retina in the current study. Alternatively, reduced inflammation and demyelination may allow more efficient transmission of responses through the RGCs that are present in optic nerves of HE3286-treated EAE mice.

The observed anti-inflammatory effects of HE3286 in experimental optic neuritis are consistent with findings in a variety of other inflammatory disease models. HE3286 possesses significant anti-inflammatory activity in carrageenan- and lipopolysaccharide (LPS)-induced lung inflammation, rheumatoid and collagen-induced arthritis, ulcerative colitis, and autoimmune models of diabetes.[13–19] Interestingly, HE3286 appears to suppress inflammation without specifically modulating immune responses,[13,14,33] suggesting that it may offer benefits in optic neuritis and MS distinct from other approved medications, including interferon-beta, glatiramir acetate, and other newer agents that are classified primarily as immunomodulatory therapies.[5–8] The dose we used in the present study is consistent with previous studies.[20] No adverse effects on optic nerve or neurologic function were observed at this dose in the current study, consistent with prior studies[13] showing that this dose is well tolerated with no apparent systemic toxicity.

Experimental autoimmune encephalitis is a widely used animal model of MS and exhibits pathologies similar to the human disease. Like MS, which occurs most frequently in young adult women, age- and sex-related susceptibility is observed in the development of EAE,[34] so young adult female mice are used. While optic neuritis occurs in most eyes of EAE mice, we previously showed that it can be unilateral or bilateral and also does not always correlate with the degree of spinal cord disease in EAE mice.[4,22,23] This may explain why we see a different time course and pattern of functional benefit of HE3286 treatment between delayed onset of EAE paralysis at early time points, and improved OKR responses at later time points.

We have previously shown that experimental optic neuritis in EAE is a useful model to examine neuroprotective effects

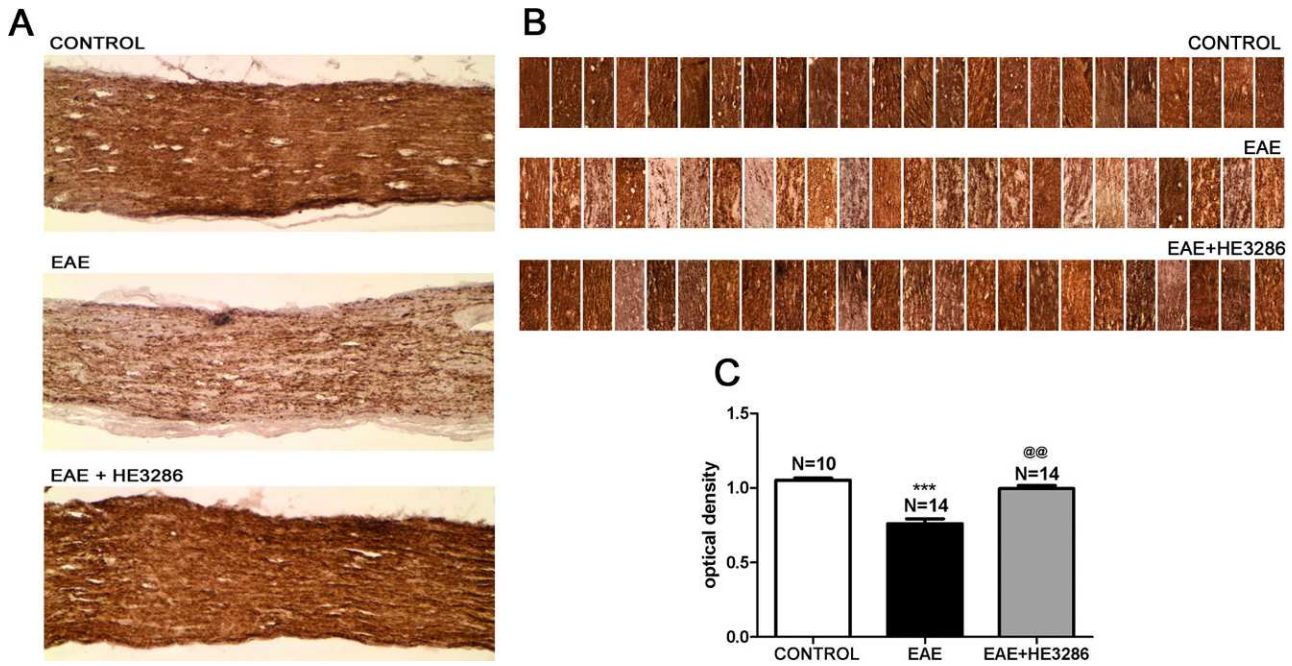

Figure 5. HE3286 reduces axonal loss in the optic nerve. Neuroprotective effects of HE3286 were evaluated by neurofilament staining. (**A**) Optic nerves from HE3286-treated EAE mice show increased RGC axonal staining compared to those from PBS-treated EAE mice. (**B**) A series of photographs of neurofilament staining in optic nerve sections from control, PBS-treated EAE, and HE3286-treated EAE mice shows normal axonal staining, focal areas of axon loss in PBS-treated EAE optic nerves, and less axon loss in HE3286-treated optic nerves. (**C**) Neurofilament staining optical density was calculated from three equal-sized fields from each optic nerve using ImageJ software. Experimental autoimmune encephalomyelitis optic nerves ($n = 14$) show a significant decrease ($P <$ ***0.001) in optical density compared to control optic nerves ($n = 10$). Treatment with HE3286 (40 mg/kg daily, $n = 14$) significantly increases ($^{@@}P < 0.01$) neurofilament stain optical density.

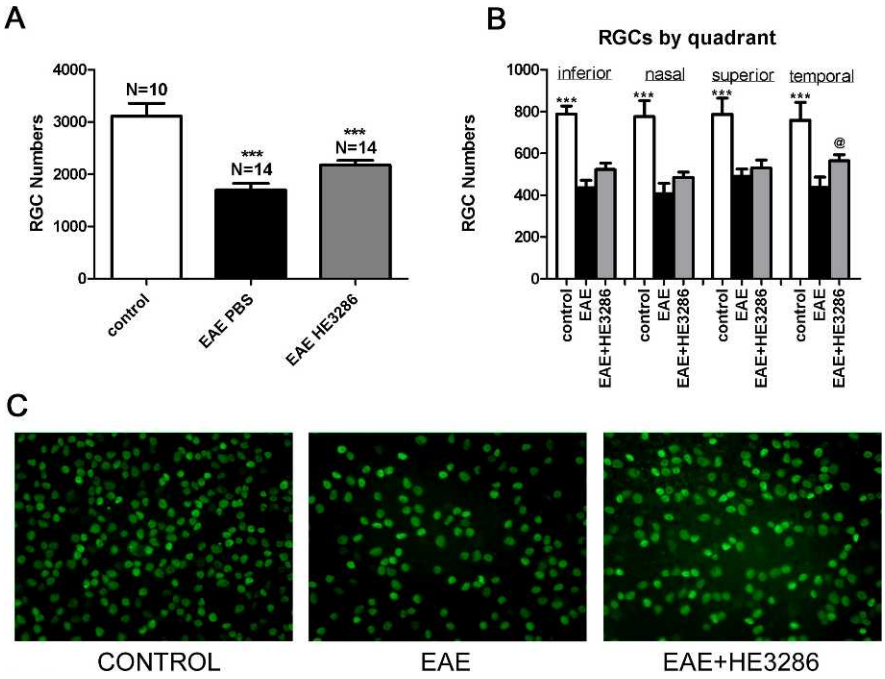

Figure 6. HE3286 reduces RGC loss in the temporal retina. Neuroprotective effects of HE3286 were evaluated by counting RGCs immunolabeled with Brn3a antibody in 12 standardized fields, three from each quadrant of the retina. (**A**) The average total number of RGCs counted in all 12 fields from each retina is shown. Experimental autoimmune encephalomyelitis optic neuritis eyes ($n = 14$) have significantly ($^{***}P < 0.001$) lower total RGC counts compared to control eyes ($n = 10$). Eyes from EAE mice treated with HE3286 (40 mg/kg daily, $n = 14$) for 40 days show similarly lower RGC counts, with just a small trend toward increased RGC survival. (**B**) The average number of RGCs counted in three fields from each quadrant of the retina is shown. In all quadrants, control eyes have significantly ($^{***}P < 0.001$) higher RGC numbers compared to eyes from EAE mice with or without HE3286 treatment. In the temporal quadrant, there are significantly ($^{@}P < 0.05$) more RGCs in EAE eyes from mice treated with HE3286 than in PBS-treated EAE eyes. (**C**) Representative photograph of Brn3a-labeled RGCs taken at 1/6 retinal radius in the temporal quadrant shows numerous RGCs in a control mouse eye, fewer RGCs in an EAE eye, and more RGCs in an EAE eye from a mouse treated with HE3286.

even in the absence of anti-inflammatory effects, as observed by treatment with compounds that activate the SIRT1 deacetylase.[26,35–37] HE3286 crosses the blood–brain barrier, decreases loss of motor coordination, and attenuates neuronal damage in a murine model of Parkinson's disease,[21] leading us to examine potential neuroprotective effects in conjunction with the anti-inflammatory effects found in experimental optic neuritis. Effects on RGC survival and the increased preservation of RGC axons suggest that neuroprotective effects may contribute to mechanisms of increased visual function. However, due to treatment beginning the day after immunization, the current study does not distinguish whether this neuroprotective effect of HE3286 is due to suppression of immune responses or whether it has a direct neuroprotective effect on RGCs. This may be addressed in future studies to examine effects of HE3286 in other, noninflammatory, optic neuropathies. It will also be important to determine whether HE3286 exerts similar neuroprotective effects when treatment is initiated after onset of optic nerve inflammation; however, even if later treatment is less effective, the present data still suggest a potential clinical use for HE3286. Because MS patients experience recurrent inflammatory episodes, HE3286 may have a role as a preventive therapy to suppress immune responses, thus reducing the potential of further neuronal damage occurring after a second inflammatory episode in MS/optic neuritis patients.

Other effects of HE3286 reported in the literature suggest potential mechanisms by which it may exert neuroprotective effects. HE3286 has the ability to bind to extracellular signal-regulated kinases (ERK)[33] and can also regulate ERK activation.[19] Extracellular signal-regulated kinase is an early central inflammatory signal mediator in neurodegenerative signaling cascades, and ERK hyperactivation through Toll-like receptors and receptor for advanced glycation end product (RAGE) has been linked to neuronal apoptosis and progressive neurodegeneration.[38] It is also reported to regulate inducible nitric oxide synthase (iNOS), TNFα, and IL-1β proteins, which play an important role in neurodegeneration through mitochondrial toxicity, N-methyl-D-aspartate (NMDA) receptor hyperactivation, and exitotoxicity.[21] These reports and our results suggest possible independent neuroprotective activity of HE3286, and further study of this compound in models of other optic neuropathies that involve varying degrees of inflammation may suggest further potential therapeutic uses.

Overall, the current studies demonstrate an ability of HE3286 to preserve significant levels of RGC function in experimental optic neuritis, with suppression of inflammation and demyelination in the optic nerve. More importantly, HE3286 preserves axons and RGCs. These neuroprotective effects may be independent of or secondary to anti-inflammatory effects; but by either mechanism, HE3286 represents a promising potential therapy that warrants further investigation for use in optic neuritis and other optic neuropathies.

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