

Neuroprotection by Sodium Channel Blockade with Phenytoin in an Experimental Model of Glaucoma

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PURPOSE. Sustained influx of intracellular sodium through voltage-gated sodium channels is an important event in the cascade leading to degeneration of axons. This study tested the hypothesis that sodium channel blockade with phenytoin would result in neuroprotection of retinal ganglion cells (RGCs) and optic nerve axons in an experimental model of glaucoma.

METHODS. Chronic elevation of rat intraocular pressure (IOP) leading to optic nerve damage was induced using the episcleral vein occlusion model. Before induction of glaucoma, a subset of animals was placed on phenytoin-containing chow; this treatment continued for 8 weeks. Quantitative counts of back-filled RGCs and optic nerve axons was performed to examine the effects of phenytoin on glaucoma-induced adverse neurodegeneration.

RESULTS. Elevated IOP resulted in a significant decrease in density of RGCs, as well as dropout of axons within the optic nerve at 8 weeks after induction. In phenytoin-treated animals, however, the loss of RGCs was significantly reduced compared to vehicle-treated glaucomatous animals. Axon loss in the optic nerve was also reduced in phenytoin-treated animals, compared to controls.

CONCLUSIONS. Orally delivered phenytoin was effective in protecting neurons in an animal model of glaucoma, and merits further examination as a potential therapeutic strategy. (*Invest Ophthalmol Vis Sci.* 2005;46:4164–4169) DOI:10.1167/iov.05-0618

Voltage-gated sodium channels contribute to the development of axonal degeneration in white matter tracts, including the optic nerve, and sodium channel blocking drugs are known to have a protective effect on acutely injured white matter axons in vitro. Neuronal injury due to insult results, in part, from accumulation of $[Ca^{2+}]_i$ within injured neurons and their axons due to reverse operation of the Na^+-Ca^{2+} exchanger, which is triggered by an increase in intracellular sodium ($[Na^+]_i$) due to Na^+ influx via persistently activated voltage-gated sodium channels.^{1,2}

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Pharmacologic blockade of voltage-gated sodium channels can prevent axonal degeneration and preserve function after a variety of insults to axons. Sodium channel blockade with tetrodotoxin and tertiary and quaternary anesthetics has been shown to prevent the development of irreversible dysfunction of axons within the anoxic optic nerve.^{1,3}

In vitro studies have demonstrated that phenytoin, a drug that blocks sodium channels and inhibits persistent sodium currents,^{4,5} has a protective effect on axons within white matter subjected to anoxia.⁶ Phenytoin has recently been shown to protect against axonal degeneration of spinal cord and optic nerve axons, and improve neurologic outcome in mice with experimental allergic encephalomyelitis.^{7,8} Phenytoin has also been shown to have a neuroprotective effect, reducing the degree of retinal ganglion cell (RGC) death in a model of optic nerve crush.⁹

In the present study, we hypothesized that phenytoin would confer neuroprotection after induction of experimental glaucoma. To test this hypothesis, we examined RGC loss and density of axons within the optic nerve in animals with experimental glaucoma induced by elevated intraocular pressure (IOP) that were treated with phenytoin.

MATERIALS AND METHODS

Animal Care

Adult male Wistar rats (200–225 g) were used in this study. Experiments were carried out in accordance with National Institutes of Health and ARVO guidelines for the care and use of laboratory animals; all animal protocols were approved by the Yale University Institutional Animal Care and Use Committee. Animals were housed under a 12-hour light-dark cycle in a pathogen-free area with free access to water and food.

Phenytoin Delivery

Beginning 3 days before induction of sham surgery and glaucoma, animals ($n = 16$ per group) were fed pelleted chow (Bioserve, Frenchtown, NJ) incorporating phenytoin (5,5-diphenylhydantoin, 12 g/kg; Sigma, St. Louis, MO). Pretreatment was necessary because blood levels of orally delivered phenytoin require 3 days to reach therapeutic levels.^{7,10} A separate group of sham and glaucoma rats ($n = 16$ per group) were fed the identical base chow not containing phenytoin. Phenytoin-containing chow did not cause significant weight loss or gain. In preliminary experiments, serum phenytoin levels were measured by homogeneous enzyme immunoassay¹¹ from three additional rats on day 3, and three rats on the day of perfusion (8 weeks). Serum phenytoin levels were within the clinical therapeutic range (10–20 $\mu\text{g}/\text{mg}$).

Induction of Glaucoma

Glaucoma was induced in one eye of each rat ($n = 32$) by electrocautery of episcleral veins according to published procedures.^{12,13} Contralateral eyes served as the comparative control, and were sham operated. Rats were deeply anesthetized with a ketamine-xylazine mixture (80:5 mg/kg i.p.). The eye was held open by sutures placed through the lids, and the globe manipulated by sutures placed underneath the superior rectus muscle. The conjunctiva was separated from

the limbus using blunt dissection with spring scissors. Three of the major trunks forming limbus-derived veins were exposed at the equator of the eye by gently retracting the conjunctiva, and lifted away from the body of the eye with Dumont forceps. Cauterization of three episcleral veins per eye was performed using a high-temperature electrocautery device (Aaron Medical Industries, St. Petersburg, FL) against the tips of the forceps to avoid damage to the eye. Immediate retraction and absence of bleeding of the cauterized ends of the veins were evidence of a successful cauterization. After surgery, veterinary ophthalmic ointment (bacitracin-neomycin-polymyxin; Pharmaderm Inc., Melville, NY) was used to prevent infection.

IOP Measurement

IOP was measured before surgery, 30 minutes after surgery, and at weekly intervals after surgery by an observer blinded to the treatment status of the animals. IOP measurements were performed under light halothane anesthesia (2% by face mask), using an applanation tonometer (Tono-Pen XL; Medtronic Solan, Jacksonville, FL). To measure IOP, the tonometer probe was perpendicularly applied several times to the cornea, resulting in an output of the device averaging five consecutive applications. Probe applications were continued until three averages were obtained from the instrument, each with a coefficient of variation of <5%.¹⁴

RGC Counts

Fluorogold was used to backfill RGCs from their projections to the superior colliculus bilaterally, as described elsewhere.¹⁵ Vehicle-treated ($n = 8$) and phenytoin-treated ($n = 8$) animals were anesthetized with a ketamine-xylazine mixture (80:5 mg/kg i.p.) and mounted in a stereotaxic frame. After a scalp incision and craniotomy with a handheld drill, bilateral injections of 1 μ L 4% fluorogold (Molecular Probes Invitrogen, San Diego, CA) in phosphate-buffered saline (PBS; pH 7.4) were made bilaterally into the superior colliculi, using a micropipette attached to the needle (30 gauge) of a 5- μ L microsyringe (Hamilton; Reno, NV). Two injections (8 minutes each infusion) were made slowly on each side using coordinates obtained from a rat stereotaxic atlas: 6 mm posterior to bregma, 1 ± 0.5 mm lateral to midline and 4 mm ventrally.¹⁶ After injection, the needle was left in its final position for 5 minutes before removal to reduce spread of tracer up the needle track. Skin was sutured and animals were allowed to recover on a heating pad.

Retinas from ipsilateral (glaucoma) and contralateral eyes were collected for whole-mount and quantitative RGC densitometry 7 days after fluorogold injection. Under ketamine-xylazine anesthesia, eyes were enucleated and fixed in 4% paraformaldehyde for 60 minutes. Ipsilateral and contralateral eyes were bisected at the equator, the lens was removed, and the posterior segments were postfixed for an additional 30 minutes. Retinas were dissected from the underlying sclera and choroid, flattened by six radial cuts, and mounted vitreal-side up on glass slides (Superfrost/Plus; Fischer, Pittsburgh, PA). Slides were cover slipped with aqua polymount nonfade mounting media (Polysciences, Warrington, PA) and visualized with ultraviolet optics using a microscope (Nikon E800; Nikon, Melville, NY). Digital images were captured with a color camera (Spot RT; Spot Diagnostic Instruments, Sterling Heights, MI). An observer performed quantitative analysis using blinded protocols, whereby 15 microscopic fields were captured per retina at predetermined radial distances (1 and 4 mm) from the center of the retina for each retinal quadrant. The number of RGCs from each microscopic field was pooled as an estimate of the mean number of labeled cells for each group.

For detection of NeuN, a marker of RGCs,¹⁷ cryosections ($n = 3$ sections per animal) were mounted, and slides were incubated at room temperature in blocking solution (PBS containing 5% normal goat serum, 2% BSA, 0.1% Triton X-100, and 0.02% sodium azide) for 30 minutes; mouse anti-NeuN primary antibody (1:500; Chemicon, Temecula, CA), overnight in blocking solution; PBS, 6 times for 5 minutes each; goat anti-mouse IgG-Cy3 (1:2000; Amersham Biosciences, Pis-

cataway, NJ) in blocking solution, 2 hours; and PBS, 6 times for 5 minutes each. Control experiments were performed without primary or secondary antibodies, which yielded only background levels of signal.

Optic Nerve Axon Counts

To assess the effects of phenytoin on axonal density within the optic nerve, we performed axon counts using antibodies specific for phosphorylated (healthy myelinated axons) and nonphosphorylated (nonmyelinated and demyelinated axons) neurofilament proteins.^{7,10,18} This combination of antibodies against both phosphorylated and nonphosphorylated forms ensured labeling of the total population of axons. Nonmyelinated axons can comprise 2% to 50% of the axons in the optic nerve, depending on the location of the section.¹⁹

Optic nerves were collected from glaucomatous ipsilateral eyes of rats fed base-vehicle ($n = 8$) or phenytoin-supplemented chow ($n = 8$), as well as from the contralateral eyes of phenytoin-treated rats ($n = 8$). At 8 weeks after induction of glaucoma, rats were deeply anesthetized with ketamine-xylazine and perfused intracardially with 0.01 M PBS (pH 7.4), and then with 4% paraformaldehyde in 0.14 M Sorensen's phosphate buffer (pH 7.4). Optic nerves were carefully removed and cryoprotected overnight with 30% sucrose in PBS. Tissue from different treatment groups was processed simultaneously. Thin (12 μ m) longitudinal and transverse cryosections ($n = 5$ sections per animal) were collected immediately proximal to the retina (within 1 mm). Sections were mounted on slides and incubated at room temperature in blocking solution (PBS containing 5% normal goat serum, 2% BSA, 0.1% Triton X-100, and 0.02% sodium azide) for 30 minutes; subtype-specific primary antibodies raised in mice against phosphorylated neurofilaments (SMI-31, 1:20,000; Sternberger Monoclonals, Lutherville, MD) and nonphosphorylated neurofilaments (SMI-32, 1:20,000; Sternberger Monoclonals), overnight at 4°C on a rotating shaker; PBS, 6 times for 5 minutes each; rabbit anti-mouse IgG-Cy3 (1:2000; Amersham) in block, 2 hours; and PBS, 6 times for 5 minutes each. Control experiments were performed without primary or secondary antibodies, which yielded only background levels of signal.

An observer performed quantitative analysis using blinded protocols. Axonal densities were determined within preselected fields of 1920 μ m² within the center of the optic nerve, with location of the fields carefully conserved from group to group. Neurofilament-stained axons were manually counted from each field using application software (IPLab v3.0; Scanalytics, Fairfax, VA), as described previously.⁷

Statistical Analysis

All statistical tests were performed at the alpha level of significance of 0.05 by two-tailed analyses using parametric tests. Data were tested for significance using one-way ANOVA, followed by Bonferroni post hoc analysis. Tests of factors included pairwise comparisons with either the paired Student's *t*-test, or the two-sample Student's *t*-test. Fisher's exact test was used to compare percentages. Data management and statistical analyses were performed using application software (SigmaStat v1.0; Jandel, Point Richmond, CA), and results were graphed (SigmaPlot v7.0; Jandel) as means \pm SD.

RESULTS

Elevation of IOP

IOP values in eyes before induction of glaucoma (8.4 ± 1.6 mm Hg) were similar to those reported in the literature (Fig. 1). Thirty minutes after induction of glaucoma, a significant increase in IOP was observed in the ipsilateral eye (17.4 ± 1.3 mm Hg). Eight weeks after glaucoma induction, IOP remained significantly elevated in the ipsilateral eye (17.7 ± 2.0 mm Hg, $P < 0.05$), compared to the contralateral eye (9.6 ± 1.6 mm Hg).

In naïve animals, phenytoin treatment had no effect on IOP (7.8 ± 1.1 mm Hg, data not shown). In chronic (8 weeks)

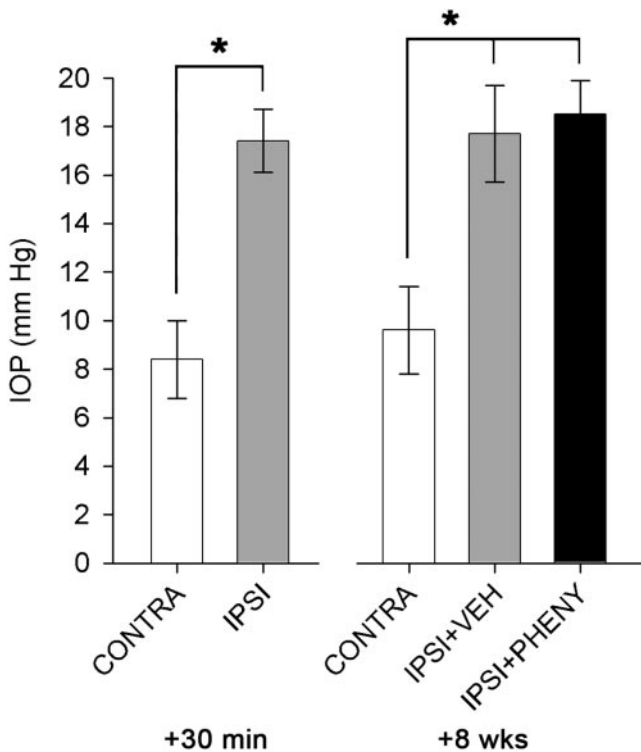


FIGURE 1. IOP measurements showing significant elevation of IOP in operated (IPSI) eyes 30 minutes after induction of glaucoma by episcleral vein cauterization, compared to the opposite (CONTRA) side ($*P < 0.05$). At 8 weeks after induction of glaucoma, IOP did not differ significantly between animals chronically treated with either vehicle (IPSI+VEH) or phenytoin (IPSI+PHENY), but remained significantly elevated relative to the CONTRA side ($*P < 0.05$).

glaucomatous animals, phenytoin had no effect on IOP for either the ipsilateral (18.5 ± 1.4 mm Hg) or contralateral (7.6 ± 0.9 mm Hg) eye (Fig. 1).

RGC Counts

RGCs were backfilled by bilateral injection of fluorogold solution into the superior colliculi (Fig. 2A). A coronal schematic section through bregma -7.6 mm shows the location of fluorogold within the superior colliculi. Very minimal fluorogold spreading was evident within adjacent brain structures. Quantitation of the mean number of backfilled RGCs is shown in Figure 2B. At 8 weeks after induction of glaucoma, the number of backfilled RGCs in the contralateral eye ($16,960 \pm 1923$ cells) was significantly higher than in the ipsilateral eye of vehicle-treated animals (8651 ± 942 cells, $P < 0.05$). In animals treated with phenytoin, the number of backfilled RGCs ($15,681 \pm 1277$ cells) was significantly higher than in animals receiving vehicle. Expressed differently, there was a 51% loss of RGCs in the ipsilateral eye in vehicle-treated animals, compared to an 8% loss of RGCs in phenytoin-treated animals.

Images of representative microscopic fields of retina are shown in Figures 2C–2H. Eight weeks after induction of glaucoma, backfilled RGC cells were present in high density in contralateral eyes in both flat-mounted and cross-sectional retina (Figs. 2C, 2F), whereas ipsilateral eyes demonstrated a reduced density of RGCs (Figs. 2D, 2G). In glaucomatous animals receiving phenytoin (Figs. 2E, 2H), density of RGCs was higher than in vehicle-treated animals in the ipsilateral eye.

Cross-sections of retina revealed NeuN immunoreactivity (Figs. 2I–2K) evident in RGCs as well as neurons of the inner

nuclear layer. The density of NeuN-positive RGCs was lower in ipsilateral eyes of glaucomatous animals treated with vehicle (Fig. 2J), compared to the contralateral eyes (Fig. 2I). Treatment with phenytoin resulted in a higher density of RGCs that were NeuN-positive (Fig. 2K). RGC densities as assessed by fluorogold and NeuN signal were equivalent.

Optic Nerve Axon Counts

At 8 weeks after induction of glaucoma, optic nerves on the contralateral side showed high density of neurofilament-positive optic nerve fibers in both longitudinal (Fig. 3A) and transverse (Fig. 3B) profiles. In the ipsilateral glaucomatous eye, both longitudinal (Fig. 3C) and transverse (Fig. 3D) profiles of the optic nerve showed loss of neurofilament signal within the nerve. When optic nerves were viewed in transverse section, axon density was seen to be most markedly reduced in the interior region of the nerve. In several ipsilateral sections of optic nerves of both vehicle- and phenytoin-treated glaucomatous animals, patchy regions of higher axon density were observed. In glaucomatous animals receiving phenytoin, axon density was relatively higher than in animals receiving vehicle in both longitudinal (Fig. 3E) and transverse (Fig. 3F) section.

Quantitation of optic nerve area (Fig. 3G) at 8 weeks revealed no significant differences between contralateral ($40,718 \pm 5413 \mu\text{m}^2$) or glaucomatous eye nerves of animals that received either vehicle ($39,415 \pm 6411 \mu\text{m}^2$) or phenytoin ($44,072 \pm 6248 \mu\text{m}^2$) treatment. However, density of axons within the optic nerve was decreased in glaucomatous eye nerves, with a significant sparing effect of phenytoin.

Quantitation of the density of axons within the optic nerve at 8 weeks (Fig. 3H) revealed that in vehicle-treated animals, glaucoma resulted in a significant reduction in the density of axons in the ipsilateral eye (1.78 ± 0.24 axons/ μm^2 , $P < 0.05$), compared to the contralateral eye (2.15 ± 0.12 axons/ μm^2 ; $P < 0.05$). Axon density was significantly higher in glaucomatous animals receiving phenytoin (2.10 ± 0.13 axons/ μm^2 , $P < 0.05$), compared to the vehicle-treated group, a 15% sparing effect.

DISCUSSION

In the present study, we examined the effects of the sodium channel blocker phenytoin on RGC loss and optic nerve axon density in experimental glaucoma. This is the first study to examine the role of phenytoin in a standardized model of glaucoma. We showed that, as measured 8 weeks after induction of glaucoma, phenytoin exerted a neuroprotective effect on RGC density, resulting in reduced loss of axons within the optic nerve.

The techniques used here focus on the histologic outcome on RGC and optic nerve axon survival after induction of glaucoma and treatment with phenytoin. The use of fluorogold as a retrograde tracer to assess RGC survival has been validated by a number of studies, including those examining RGC survival after glaucoma.^{14,20,21} In our experiments we also used an antibody selective for RGCs, NeuN. It has been shown that NeuN can be reliably used as a marker for RGCs.^{17,22} Comparing both methods, we found that after glaucoma, the degree of RGC loss and sparing with phenytoin treatment was the same as with fluorogold backfill assay techniques.

In our experiments, phenytoin had no effect on elevated IOP, which was the same as in glaucomatous animals treated with vehicle. Since the increased IOP observed in the episcleral vein occlusion model is largely a result of anatomic pathology, it is unlikely that phenytoin could affect IOP. The absence of decreased IOP in phenytoin-treated animals supports the idea that rather than reducing IOP, which would then

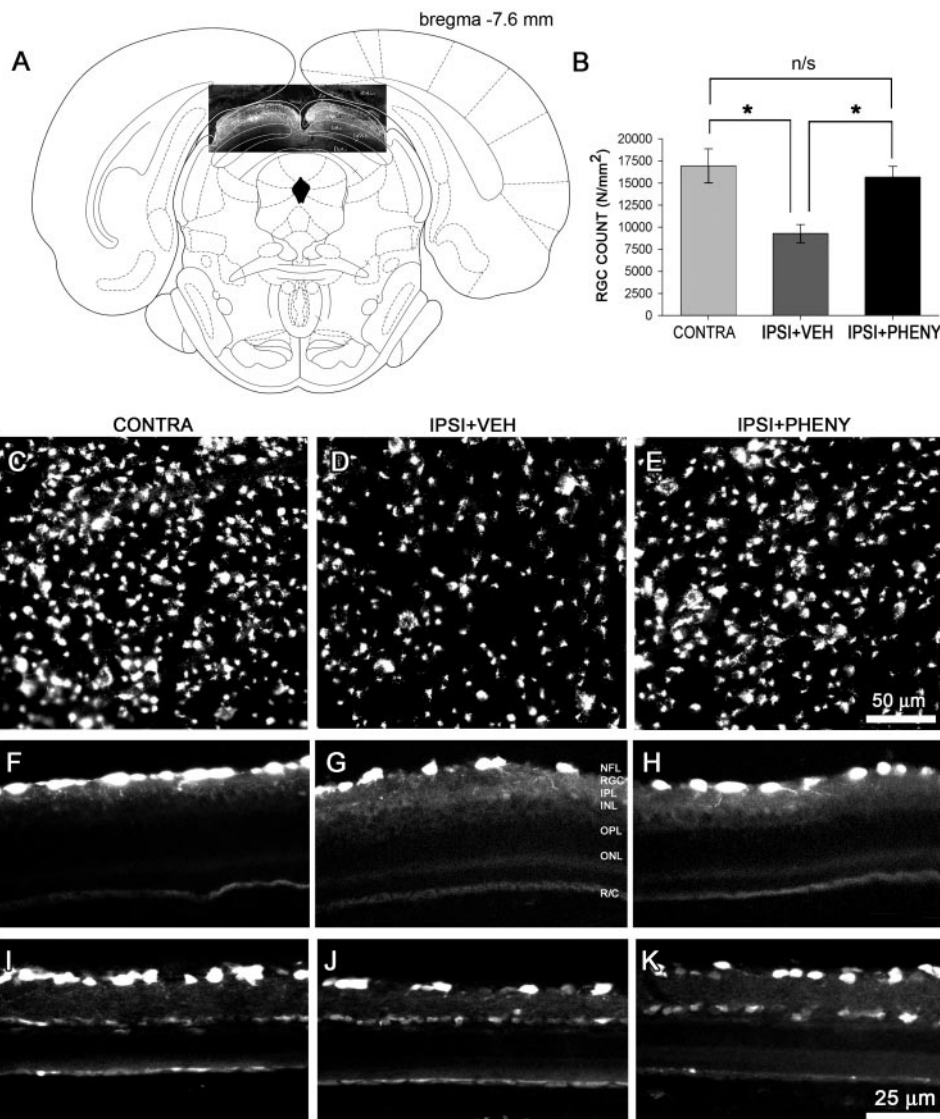


FIGURE 2. At 8 weeks after induction of glaucoma, RGCs were backfilled by injection of fluorogold solution into the superior colliculi as illustrated in this atlas section showing bregma -7.6 mm (A).¹⁶ Fluorogold signal was high within each superior colliculus, and very little tracer spread was detectable outside the injection sites. The number of backfilled RGCs in the contralateral (CONTRA) eye (C, F) was higher than in the ipsilateral eye of vehicle-treated animals (IPSI+VEH) (D, G). In phenytoin-treated animals (IPSI+PHENY) (E, H), the number of backfilled RGCs was higher than in IPSI+VEH animals. Differences in RGC density in representative eyes are obvious in retina shown flat-mounted (C–E) and in cross-section (F–H). Quantitation of RGC density (B) shows that the number of backfilled RGCs in the IPSI+VEH eye was significantly reduced compared to the CONTRA eye ($*P < 0.05$). IPSI+PHENY animals demonstrated significantly higher RGC density compared to IPSI+VEH ($*P < 0.05$). CONTRA and IPSI+PHENY values did not differ significantly. The density of NeuN-positive RGCs was lower in IPSI+VEH eyes (J), compared to CONTRA eyes (I). Treatment with phenytoin resulted in a higher density of RGCs that were NeuN-positive (K). The atlas section was reproduced with permission from Paxinos G, Watson C. *The Rat Brain in Stereotaxic Coordinates*. 2nd ed. New York: Academic Press; 1986.

in turn reduce the degree of injury to RGCs and their axons, phenytoin has a direct neuroprotective action on RGCs and optic nerve axons.

Concomitant with RGC loss, elevated IOP produces progressive changes in the optic nerve head and damage to axons within the optic nerve. In our study, nerve lesions were observed most readily in superior and central regions. Our mean findings of 17% axon loss are in general agreement with those reported by others using similar models,^{23–26} but disparities in reported loss of axons could be due to differences in detection techniques, proximity to optic nerve head, and/or surgical model.

We observed that there was 8% loss of RGCs in glaucomatous eyes of phenytoin-treated animals, compared to 51% loss with vehicle, and a dropout of axon density to 98% of the density in controls in phenytoin-treated animals, compared to 83% in vehicle-treated animals. While we do not have a direct explanation of this disparity, it could be due to a temporal factor. Since axon dropout follows death of the cell body, there could be a delay between death of the corresponding RGC and degeneration leading to elimination of its axon. It is not known how long this process takes, but death of RGCs in glaucoma is a chronic process that can be linked to the degree of increased IOP,²⁷ which in our case persisted for 8 weeks.

Axons are susceptible to degeneration partly due to detrimental effects of sodium channels in injury and/or disease states. Sodium influx via noninactivating sodium channels, and downstream events including reverse $\text{Na}^+\text{-Ca}^{2+}$ exchange, have been implicated in axonal degeneration after anoxic optic nerve injury. A significant component of axon loss after anoxic injury is due to the reverse operation of the $\text{Na}^+\text{-Ca}^{2+}$ exchanger triggered by sodium influx via persistently activated sodium channels, which causes collapse of transmembrane Na^+ gradients.^{1,2} $\text{Na}_v1.6$ voltage-gated sodium channels, which produce persistent as well as transient sodium currents,²⁸ are clustered at high densities in the axon membrane at nodes of Ranvier in white matter.²⁹ $\text{Na}_v1.6$ channels and the $\text{Na}^+\text{-Ca}^{2+}$ exchanger have been shown to colocalize along degenerating CNS axons in experimental autoimmune encephalomyelitis.³⁰

The mechanism of action of phenytoin, which is used widely in the treatment of epilepsy as a sodium channel blocker, has been known for some time.^{31–33} Phenytoin binds to and blocks active sodium channels, reducing the number of open channels along an axon or cell body. This inhibits high-frequency firing through a slowing of channel repriming, and by blockage of persistent sodium currents.^{4,5} Normal nerve function is not impaired because of the high safety factor of axons.

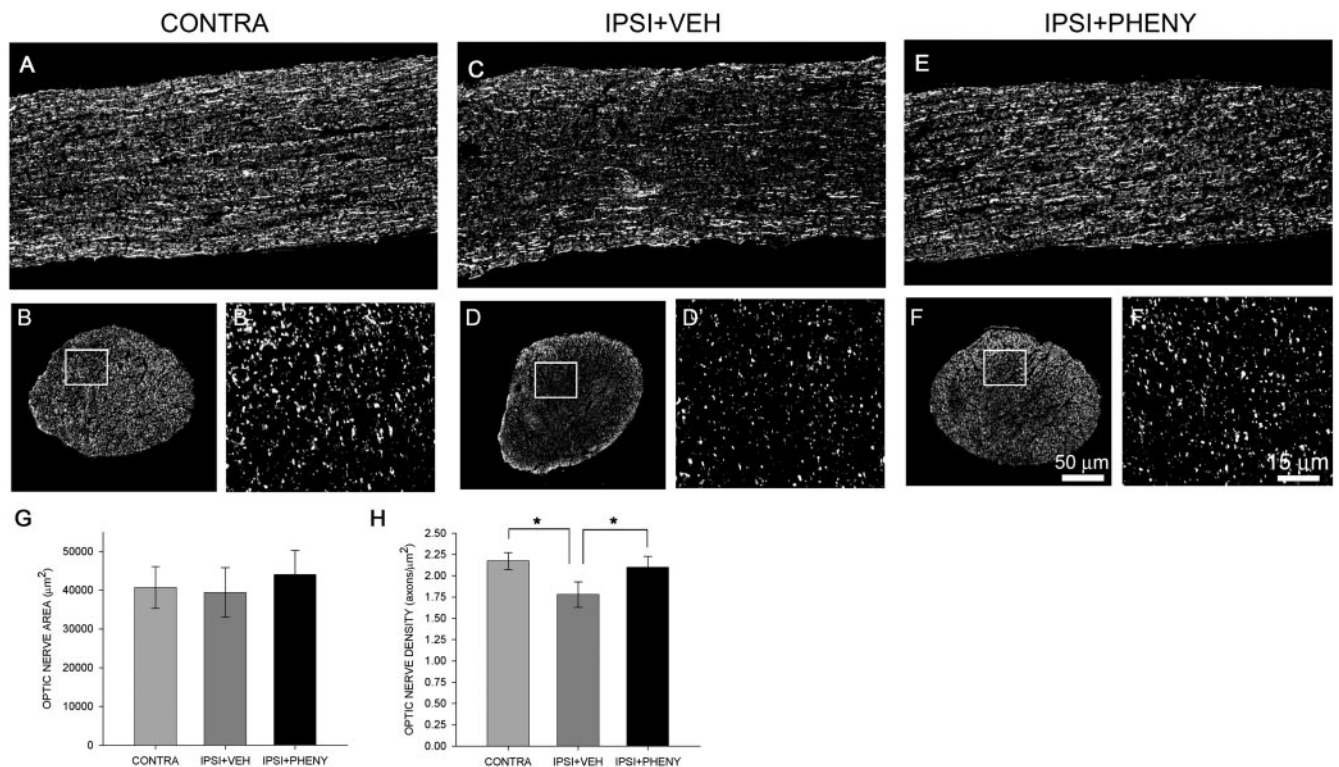


FIGURE 3. At 8 weeks after induction of glaucoma, contralateral (CONTRA) optic nerves immunostained for neurofilament protein showed uniform signal intensity and axonal density in both longitudinal (A) and transverse section at low (B) and high magnification (B'). In contrast, on the ipsilateral side, optic nerves from vehicle-treated (IPSI+VEH) animals revealed loss of neurofilament signal within the nerve, in both longitudinal (C) and transverse sections (D, D'). Patchy regions of higher axon density were observed in these nerves, with a high degree of axonal dropout in superior and temporal regions. In glaucomatous animals receiving phenytoin (IPSI+PHENY), axon density was relatively higher than in IPSI+VEH animals in both longitudinal (E) and transverse sections (F, F'). Quantitation of optic nerve area (G) at 8 weeks revealed no significant differences among CONTRA, IPSI+VEH, and IPSI+PHENY animals. Quantitation of optic nerve axonal density revealed that glaucoma resulted in a significant dropout of axons in the IPSI+VEH animals (H), compared to the CONTRA side ($*P < 0.05$). Axon density was significantly higher in IPSI+PHENY animals receiving phenytoin, compared to the IPSI+VEH group (H) ($*P < 0.05$).

Importantly, blockade of axonal sodium channels with phenytoin is known to confer protection of axons within the optic nerve in vitro subjected to anoxia.⁶ In addition, one laboratory study has examined the effects of phenytoin on RGC death in vivo; Naskar and colleagues⁹ showed that a single dose of phenytoin delivered acutely after partial crush of the optic nerve can preserve RGCs. Our study extends these results, showing that phenytoin can spare RGCs in a different injury paradigm that results in RGC loss. A few early clinical studies suggested some degree of efficacy of phenytoin in patients with glaucomatous visual field loss, with a tendency toward reversal or improvement in visual fields, and protection against further field loss despite markedly increased IOPs.³⁴ Similarly, an early report suggested that patients with ischemic optic neuropathy had significant visual improvement after phenytoin therapy.³⁵ A subsequent report did not demonstrate an improvement in vision in patients treated with phenytoin, but may have included patients in which the window of opportunity for neuroprotection had passed.³⁶

In summary, the data presented here document changes in anatomic outcomes after experimental glaucoma associated with oral phenytoin administration. We demonstrated that phenytoin confers significant neuroprotection of RGCs and optic nerve axons. Our findings lead us to suggest that treatment of glaucoma with sodium channel blockers deserves further study.

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