

Silencing of *Fem1c*^{R3} Gene Expression in the DBA/2J Mouse Precedes Retinal Ganglion Cell Death and Is Associated with Histone Deacetylase Activity

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PURPOSE. Downregulation of normal gene expression in dying retinal ganglion cells has been documented in both acute and chronic models of optic nerve disease. The authors examined the mechanism and timing of this phenomenon in DBA/2J mice, using genetically modified substrains of this inbred line.

METHODS. DBA/2J mice, doubly congenic for the *Bax* mutant allele and the ganglion cell reporter gene *Fem1c*^{Rosa3} (R3), were evaluated to elucidate the timing of loss of normal gene expression during the apoptotic process. The localization of histone deacetylase 3 (HDAC3) and nuclear histone H4 acetylation were examined by immunofluorescence in dying cells. The role of HDACs in gene silencing during glaucoma was interrogated using the global HDAC inhibitor trichostatin A (TSA).

RESULTS. Silencing of the R3 allele occurred in *Bax*^{-/-} ganglion cells, indicating that this process preceded the committed step of the intrinsic apoptotic pathway. Weekly TSA treatment, between the ages of 6 and 10 months, was able to attenuate the loss of R3 expression in the retina, but had no effect on optic nerve degeneration. Dying cells in aging DBA/2J mice exhibited nuclear localization of HDAC3 and a decrease in the level of H4 acetylation.

CONCLUSIONS. Retinal ganglion cells exhibit a loss of normal gene expression as an early (pre-BAX involvement) part of their apoptotic program during glaucomatous degeneration. This process can be ameliorated, but not completely blocked, using HDAC inhibitors. Epigenetic changes to active chromatin, such as deacetylation, may be mediated by HDAC3 in dying neurons. (*Invest Ophthalmol Vis Sci.* 2012;53:1428–1435) DOI:10.1167/iovs.11-8872

Axonal degeneration and retinal ganglion cell body atrophy and death are the fundamental characteristics of glaucomatous optic neuropathies.¹ The identification of intrinsic apoptosis in glaucomatous ganglion cell somas² has led to the hypothesis that disruption of this program may be a viable

target to prevent ganglion cell neurodegeneration and, combined with conventional pressure-lowering therapy, may yield more effective preservation of vision. Substantial focus, therefore, has been given to obtaining a better understanding of the temporal sequence of events being executed in apoptotic ganglion cells, particularly to the early events that may critically activate subsequent and irreversible steps in the apoptotic cascade.^{1,3}

One of the early events associated with ganglion cell death is the silencing of normal gene expression. This phenomenon has been documented in both acute and chronic (experimental ocular hypertension) models of optic nerve disease.^{4–8} These studies have typically reported an exponential decay in the mRNA abundance of genes that are normally expressed in ganglion cells. In all studies to date, the loss of mRNAs occurs well in advance of a measurable loss of ganglion cell bodies. Our initial characterization of this phenomenon was a quantitative analysis of the transcript abundance for the *Thy1* gene. *Thy1* mRNA becomes depleted before cell loss in mouse models of optic nerve crush, and intravitreal injection of the glutamate analog, *N*-methyl-D-aspartate. Additionally *Thy1* transcripts were depleted in advance of cell loss in a rat model of experimental glaucoma.^{4,5} Loss of this mRNA was also observed in *Bax*-deficient retinal ganglion cells after optic nerve crush, even though these cells were completely arrested in the apoptotic program. Similar kinetics of decay of other ganglion cell-specific genes, including *Brn3b*,⁹ *Nrn1*, and *Sncg*^{6,7} have now been reported.

Collectively, these data indicate that downregulation, or the silencing of normal gene expression, is an early event in the apoptotic program, occurring before the point at which BAX is activated. The global nature of the effect suggests a coordinated mechanism that can simultaneously affect multiple actively expressed genes. One mechanism that could account for this effect is rapid modification of histones in the promoters of genes in an “open” conformation, particularly by histone deacetylation, which has been described as a principal epigenetic change that can rapidly silence active genes.¹⁰ Using the mouse optic nerve crush model to activate ganglion cell apoptosis, we observed that histones in the promoters of actively expressed genes were rapidly deacetylated, which was associated with the translocation of histone deacetylase 3 (HDAC3) from the cytoplasm to the nuclei of dying cells. Moreover, the downregulation of the *Rosa3* ganglion cell reporter gene (*Fem1c*^{R3})¹¹ could be attenuated in mice pretreated with the broad-spectrum HDAC inhibitor trichostatin A (TSA).⁸

Here, we report that DBA/2J mice, carrying the *Fem1c*^{R3} reporter gene, exhibit loss of expression of this gene before glaucomatous ganglion cell loss, similar to reports made for other genes in this mouse strain.⁷ This downregulation preceded the function of the proapoptotic BAX protein. Evaluation of dying cells in DBA/2J mice indicated both the nuclear

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accumulation of HDAC3 and deacetylation of histone H4, suggesting an epigenetic mechanism affecting gene transcription similar to that reported after acute optic nerve damage. Continuous treatment of mice with TSA was able to ameliorate the silencing of *Fem1c*^{R3}, and therefore somewhat preserve retinal integrity. This effect was not translated to optic nerve axons, however, which still exhibited a similar level of damage compared with control treatment groups.

MATERIALS AND METHODS

Experimental Animals

All animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research using protocols approved by the Animal Care and Use Committee of the University of Wisconsin. Mice were housed in microisolator cages and kept on a 12-hour light/dark cycle. They were maintained on a 4% fat diet (8604 M/R; Harland Teklad, Madison, WI). DBA/2J^{R3/R3} mice were used as a chronic disease model of retinal ganglion cell loss. These mice develop a spontaneously occurring secondary glaucoma as they age. These mice also carry the β Geo reporter trap gene in the first intron of the *Fem1c* gene (*Fem1c*^{R3}). Expression of this allele produces a fusion protein (β Geo), which has β -galactosidase activity and can process X-Gal as a substrate. Expression from this gene decreases as these mice age and the disease progresses.¹² In addition, a second substrain of DBA/2J mice, containing a mutant *Bax* allele (DBA/2J^{Bax^{-/-}R3/R3}), was used to examine the timing of gene silencing in retinal ganglion cells unable to execute a full apoptotic program.

Intraocular pressures (IOPs) were measured by applanation tonometry (Tono-Lab; Colonial Medical Supply, Franconia, NH), on mice that were anesthetized with ketamine (6 mg/mL) and xylazine (0.4 mg/mL). Measurements were taken 5 to 10 minutes after anesthesia, when normalized pressures under this anesthetic are most similar to IOPs in awake mice.¹³ Buphthalmia was scored as a presence or absence of eye bulging, also while the mice were anesthetized. Transillumination defects were scored on a 4-point scale, with 1 representing no defect and 4 representing complete loss of pigment from the iris.

Histochemical Staining for β Geo Activity

Ganglion cells expressing β Geo were identified histochemically in DBA/2J mice 10 to 22 months of age by staining retinal preparations with X-Gal, followed by whole mounting as previously described.¹¹ Briefly, the mice were euthanized and the eyes were collected and fixed in 4% paraformaldehyde in PBS at room temperature for 30 minutes. The eye was then rinsed in PBS, and the anterior portion of the eye was removed and discarded. The resulting eyecup was washed in PBS containing 2 mM MgCl₂ and 2 μ M CaCl₂ and stained by incubation in 1 mg/mL X-gal solution at 37°C for 18 hours. After staining, the retina was dissected from the eyecup and whole mounted on glass slides. The mounted retinas were then Nissl-stained with cresyl violet to enable counting of all cells in the ganglion cell layer.¹⁴ The slides were examined and photographed using a light microscope (Olympus BX40; Olympus America Inc., Center Valley, PA) and a digital camera attachment.

Double-stained cells were counted by overlying ($\times 40$) digital images with a 100 \times 100- μ m grid. Total cell number was determined by counting Nissl-stained cells exhibiting large round nuclei with prominent nucleoli. This metric essentially estimates all the neurons in this layer, including both ganglion and amacrine cells. After counting of all cells, the percentage of cells positive for X-Gal deposits was recorded. Regions with intense X-Gal staining were scored as 100% since it was not possible to sort positive from negative staining cells. Numbers of eyes examined were 18 each for *Bax*^{+/+} and *Bax*^{+/-} mice, and 14 for *Bax*^{-/-} mice. A minimum of 10 fields were scored for each eye.

Immunofluorescence

Immunofluorescence was performed on retinal cryosections, which were prepared in the following manner. Whole eyes were fixed in PBS

(150 mM NaCl, 100 mM NaH₂PO₄, pH 7.4) containing 4% paraformaldehyde for 1 hour at room temperature. The anterior chamber, including the lens, was removed, and the eyecup was then stored in 0.4% paraformaldehyde in PBS at 4°C. Before embedding the tissue, the eyecup was placed in 30% sucrose overnight at 4°C. The tissue was embedded in optimum cutting temperature specimen matrix for cryostat sectioning (Tissue-Tek OCT compound; Fisher Scientific, Pittsburgh, PA), flash frozen using dry ice, and sectioned in 5- μ m slices in the midregion of the retina containing the optic nerve. Antibody labeling and washes were done as described previously.^{8,15} Primary rabbit polyclonal antibodies were used for HDAC3 (Santa Cruz Biotechnology, Santa Cruz, CA) and acetylated histone H4 (Millipore Inc., Billerica, MA). The primary antibody for γ H2AX was a mouse monoclonal (Millipore). All primary antibodies were used at a 1:100 dilution. Secondary antibodies used were goat anti-rabbit with FITC label (1:100) and goat anti-mouse with a Texas Red label (1:100) (both from Jackson ImmunoResearch Laboratories, West Grove, PA). The images were obtained using an imaging microscope (Zeiss Axioplan 2; Carl Zeiss Microimaging Inc., Thornwood, NY) and processed by extended focusing with application software (AxioVision 4.6.3.0 software; Carl Zeiss Microimaging).

Cells undergoing apoptosis in aged DBA/2J retinas were identified by staining them for a phosphorylated histone H2 variant (γ H2AX). This variant becomes concentrated in nuclei of dying cells, coinciding with DNA damage that precedes mitochondrial involvement in apoptosis.¹⁶ After optic nerve crush, we previously reported that γ H2AX exhibited three distinct stages of localization, including minimal expression except for a small concentration of protein near the nucleolus (stage I), the formation of a perinuclear ring of protein early during the apoptotic process (stage II), and, finally, concentration of γ H2AX within the nucleus (stage III).⁸ The localization patterns of this histone variant also exhibited distinct patterns of colocalization with both HDAC3 and acetylated histone H4. HDAC3 distribution shifted from cytoplasmic to nuclear in stage II γ H2AX-labeled cells, and eventually colocalized with this histone variant in the nuclei of stage III cells. Conversely, stage II cells nearly always exhibited prominent acetylation of H4, whereas stage III cells featured dramatic loss of acetylation of H4. A flow chart of the changes in distribution of HDAC3 and acetyl H4 with γ H2AX labeling has been published elsewhere as a supplemental figure.⁸

For immunofluorescent labeling experiments, a total of 35 eyes, from mice between 10 and 12 months of age, were analyzed (minimum of two sections each eye). Assuming uniform loss of 60,000 ganglion cells per eye, over a period of 4 months (9 to 12 months of age), we could predict a loss of approximately 500 cells per day. The frequency of finding cells in stage III labeling was low (<1%) using longitudinal sections in this study, consistent with the predicted rate of cell loss.

In Vivo HDAC Inhibitor Studies

Systemic TSA treatments were used to inhibit HDAC activity in the retina of DBA/2J^{R3/R3} mice. TSA was delivered via intraperitoneal (IP) injections given as 1 mg/kg TSA in dimethyl sulfoxide (DMSO). Vehicle injections consisted of an equal volume of DMSO. Injections were given weekly beginning at 6 months until 10 months, when the animals were euthanized and the retinas were collected. Three cohorts of mice were followed for this experiment, including animals receiving TSA (25 mice, 50 eyes), DMSO (24 mice, 48 eyes), or no treatment (23 mice, 46 eyes). Since disease develops independently in each eye of DBA/2J mice,¹² each eye was considered an independent sample for analysis.

The level of *Fem1c*^{R3} expression was quantified as a function of β Geo enzyme activity assessed by β -galactosidase solution assay (Promega, Madison, WI). Briefly, dissected retinas were individually homogenized in 400 μ L 1 \times reporter lysis buffer. After a 15-minute incubation at room temperature, the samples were vortexed for 15 seconds and centrifuged to pellet cellular debris. A portion of the resulting supernatant (50 μ L/well), along with the prepared standard

curve (10–50 μL of a 1:10⁴ dilution of the β -galactosidase provided in the kit), was plated in duplicate for the assay. After a 1-hour incubation at 37°C, the plates were read with a microplate reader (EL_x800; Bio-Tek Instruments Inc., Winooski, VT). Background levels of β -galactosidase activity were determined from age-matched DBA/2J mice not carrying the R3 allele. Total protein in each sample was determined using a bicinchoninic acid protein assay (BCA; Thermo Fisher Scientific, Rockford, IL). The total enzyme activity measured in the samples was normalized to the amount of protein in each sample, minus the background level.

Evaluation of Optic Nerve Degeneration

Optic nerve degeneration in 10-month-old mice was scored using a modified mild, moderate, or severe grading scale previously described.^{17,18} Optic nerves from each eye used for β GEO activity assays were harvested, fixed in 4% paraformaldehyde, and embedded in glycolmethacrylate (JB-4 Plus; Polysciences, Inc., Warrington, PA). Sections (1 μm) were cut from the postlaminal region, where the axons are myelinated, and stained with a chicken polyclonal antibody against β III tubulin (Chemicon International, Temecula, CA) and a rabbit anti-chicken IgG conjugated to rhodamine as the secondary antibody (Chemicon International). Optic nerve sections were scored by three masked observers. Discrepancies in scores were rectified by consensus among the three observers. Nerves with $\geq 95\%$ of their area positive for β III tubulin were considered to be mild, nerves with 95%–50% staining were considered moderate, and nerves with $< 50\%$ staining were considered as severe.

Statistical Analyses

Statistical evaluation of means between two data sets was performed by Student's *t*-test. Comparisons of multiple data sets were conducted by one-way ANOVA. Statistical evaluation of optic nerve damage was performed by χ^2 test. Significant difference among groups was set at $P \leq 0.05$.

RESULTS

Gene Silencing Occurs in Protected Retinal Ganglion Cells in a Glaucoma Model

DBA/2J^{R3/R3} mice, with different genotypes for a functional *Bax* allele, were aged between 10 and 22 months and euthanized. Previously, we had observed that neither the *Fem1c*^{R3} nor the mutant *Bax* allele prevented the development of elevated IOP and glaucomatous optic nerve disease in DBA/2J mice.^{12,17} By combining these alleles on the DBA/2J genetic background, we were able to more precisely investigate the timing of ganglion cell gene silencing in glaucoma. Isolated retinas were double-labeled for total cells present in the ganglion cell layer and the cells positive for the β GEO reporter protein. Morphometric analysis of randomly selected areas of 100 \times 100 μm revealed roughly three distinct phenotypes in these mouse retinas when stratified by the percentage of β GEO expression and cell density (Fig. 1, Table 1). In wild-type mice, regions with phenotype 1 (100–80% of the cells expressing β GEO) exhibited the highest density of neurons in each region. This phenotype was also typically observed in young mice before the development of disease (Schlamp et al.¹² and data not shown). Phenotype 2 (80–20% of the cells expressing β GEO) exhibited cell densities similar to those of the phenotype 1 group (78 \pm 2.3 cells/region vs. 84 \pm 1.6 cells/region). Phenotype 3 ($< 20\%$ cells expressing β GEO) exhibited the lowest density of neurons in each selected region (52 \pm 0.7 cells per region, $P \ll 0.001$; Fig. 1, Table 1). Using the level of β GEO expression as the principal criterion for each phenotype, we then examined regions in *Bax*-deficient mice. *Bax*^{+/-} mice exhibited cell densities to those of wild-type

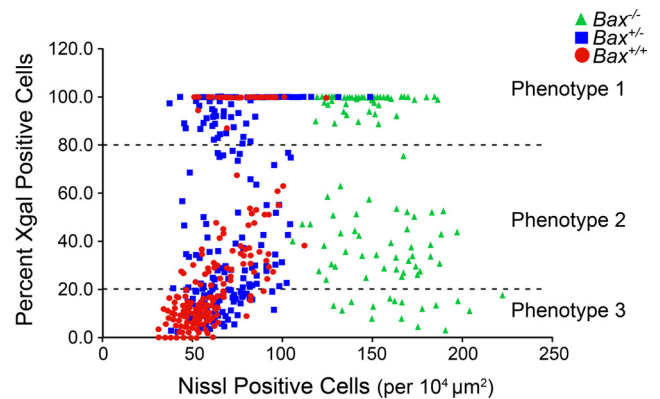


FIGURE 1. Scatterplot of total neuron counts relative to percentage of β GEO expression in aged DBA/2J^{R3} mice with different *Bax* genotypes. Randomly selected 100 \times 100- μm fields were evaluated in double-labeled retinal whole mounts of mice 10 months of age. Each field was also designated as one of three phenotypes based on the apparent percentage of cells expressing β GEO based on X-gal staining. Wild-type (red circles) and *Bax*^{+/-} (blue squares) mice exhibited similar cell densities for phenotype regions 1 and 2, but a lower density of cells in phenotype 3 regions. *Bax*^{-/-} mice (green triangles) exhibited approximately twice the density of cells, which did not decrease in regions with phenotype 3. See Table 1 for quantitative values and statistics.

animals for phenotypes 1 and 2, whereas density in phenotype 3 regions was also significantly decreased (67 \pm 1.4 cells/region, $P \ll 0.001$; Fig. 1, Table 1). Mice completely null for a functional *Bax* gene contained nearly twice the density of neurons than that of *Bax*^{+/-} and *Bax*^{+/+} mice (Table 1), consistent with previous reports that the absence of *Bax* prevents normal programmed cell death of this layer during development.¹⁹ Even though *Bax*-deficient ganglion cells are resistant to apoptosis in glaucoma,² there are still regions with nearly complete loss of β GEO activity, indicative of silencing of the *Fem1c*^{R3} reporter gene, but no significant cell loss ($P = 0.108$; Fig. 1, Table 1). Additionally, in some DBA/2J^{Bax-/-,R3/R3} mice, loss of β GEO activity occurred in distinct wedge-shaped regions extending from the optic nerve head (Fig. 2), typical of the pattern of cell loss observed in wild-type mice with glaucomatous retinal degeneration.

Nuclear HDAC3 Localization and Histone H4 Deacetylation Are Associated with Apoptotic Cell Death in Aging DBA/2J Mice

Previously, we reported that apoptotic retinal ganglion cell death after optic nerve crush was associated with the nuclear accumulation of HDAC3 and the deacetylation of histone H4.⁸ To investigate if similar events occur in dying cells in this glaucoma model, frozen sections from DBA/2J mice at different ages were stained to examine changes in both HDAC3 localization and nuclear acetylation. Staining for γ H2AX was used to identify cells in early or later stages of apoptosis. Double-label immunostaining for γ H2AX and HDAC3 on sections of DBA/2J mouse retinas revealed that dying cells with either stage II or stage III γ H2AX localization exhibited increasing concentration of nuclear HDAC3 localization. Relatively normal appearing cells (stage I γ H2AX localization) exhibited minimal labeling HDAC3 (Fig. 3).

Similarly, colocalization of acetylated histone H4 (ACh4) and γ H2AX showed that normal-appearing (stage I) cells had robust ACh4 labeling (Fig. 4). Stage II cells had variable staining for ACh4, whereas stage III cells typically had little to no staining for ACh4. DAPI counterstaining of cells also revealed that stage III cells with minimal ACh4 staining also exhibited

TABLE 1. β GEO Activity Is Depleted Prior to Cell Loss in Aged DBA/2J^{R3} Mice

Phenotype	<i>Bax</i> ^{+/+}		<i>Bax</i> ^{+/-}		<i>Bax</i> ^{-/-}	
	Percentage of Cells β GEO Positive	Nissl-Stained Cells*	Percentage of Cells β GEO Positive	Nissl-Stained Cells	Percentage of Cells β GEO Positive	Nissl-Stained Cells
1	99.58 \pm 0.31	78 \pm 2.3	97.15 \pm 0.51	81 \pm 2.0	98.63 \pm 0.33	145 \pm 1.7
2	36.94 \pm 2.26	84 \pm 1.6	52.04 \pm 2.69	75 \pm 3.2	39.28 \pm 1.75	155 \pm 3.8
3	12.27 \pm 0.77	52 \pm 0.7	16.79 \pm 0.75	67 \pm 1.4	11.74 \pm 1.39	158 \pm 5.9

DBA/2J^{R3} mice \geq 10 months of age were examined by double labeling for ganglion cell gene activity and total cell number. Cell counts were obtained in sample areas of 100 \times 100 μ m randomly placed on whole-mounted mouse retinas (10–15 fields per retina) double labeled for β GEO enzyme activity (ganglion cells) and Nissl substance (all neurons). Each sample was assigned a phenotype principally based on the percentage of cells expressing β GEO and secondarily the overall density of total neurons present. These phenotypes were first designated for wild-type mice. The criteria for β GEO expression were then applied to both the *Bax*^{+/-} and *Bax*^{-/-} mice. Phenotype 1 contained 80–100% expressing cells, phenotype 2 80–20% expressing cells, whereas phenotype 3 contained fewer than 20% expressing cells. All data are shown as mean \pm SE. Retinas from both wild-type ($n = 18$ eyes) and *Bax*^{+/-} ($n = 18$ eyes) mice show regions of normal cell concentration ($P = 0.07$), but significantly reduced β GEO activity when assigned to phenotype 2 ($P \ll 0.001$, compared with phenotype 1). The nearly complete loss of β GEO activity (phenotype 3), however, is associated with a significant decrease in cell density relative to phenotypes 1 and 2 ($P \ll 0.001$). If cell death is prevented in *Bax*^{-/-} mice ($n = 14$ eyes), the loss of β GEO activity is not associated with cell loss in any phenotype group ($P = 0.108$), indicative of silencing of the *Fem1c*^{R3} gene early in the apoptotic pathway.

* Cells counted per 10,000 μ m².

abnormal nuclear structures indicative of chromatin condensation.

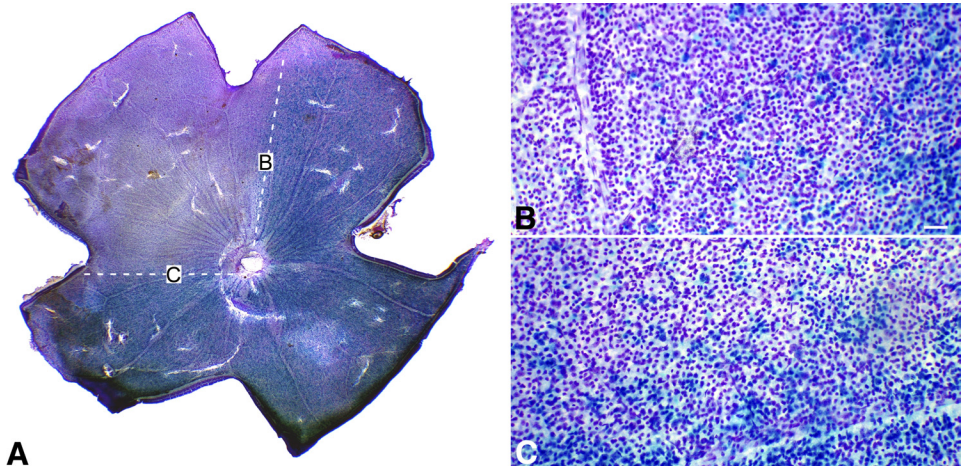
Inhibition of HDAC Activity Attenuates *Fem1c*^{R3} Silencing in Aged DBA/2J Mice

To establish that HDAC activity was linked to the downregulation of gene expression in glaucomatous mice, we treated DBA/2J^{R3/R3} mice once per week with the broad-spectrum HDAC inhibitor TSA. To mimic a potential treatment paradigm for human glaucoma, we initiated TSA treatment in mice that were 6 months old, and continued this once every week until mice reached 10 months of age. This period coincided with the reported longitudinal progression of disease in this model, when mice first begin to show elevation of IOP, to a point where the majority of mice exhibit some level of retina degenerative effects.^{12,17} At 10 months of age, all eyes in the study were also scored for IOP, iris pigment dispersion and transillumination defects, and buphthalmia (Table 2). Eyes from mice treated with TSA showed, on average, statistically equal elevations of IOP and anterior chamber defects as those of both the DMSO and nontreated mice ($P = 0.307$), whereas these metrics were significantly increased in all treatment groups relative to 6-month-old mice ($P \ll 0.001$). TSA-treated mice also exhibited relatively high levels of buphthalmia. Collectively, these results indicated that TSA did not interfere with the

development of the characteristic features of anterior chamber pathology and elevation of IOP typical for this strain.

The effect of TSA treatment on the silencing of the *Fem1c*^{R3} reporter gene, as a function of β GEO enzyme activity, was measured from retinal homogenates of eyes harvested at 10 months of age. TSA-treated mice ($n = 50$ eyes) retained significantly more β GEO enzyme activity/ μ g of total protein in retinal homogenates than did either nontreated ($n = 48$) or DMSO-treated ($n = 46$) animals ($P = 0.032$ and $P = 0.049$, respectively; Fig. 5). TSA treatment, however, appeared to only partially prevent the decrease in β GEO activity, which was approximately 80% of the mean level detected in 6-month-old animals. This difference was not statistically significant ($P = 0.314$). No treatment, or DMSO injections alone, resulted in an approximate 50% decrease in β GEO activity relative to younger mice ($P = 0.021$ and 0.044 , respectively). Some mice were also aged to 12 months, with weekly injections of TSA. In these mice ($n = 6$ eyes), β GEO activity was nearly at undetectable levels (6% of levels found in young mice), suggesting that TSA was only able to delay the progressive damage to ganglion cells, leading to gene silencing. The small sample size for the 12-month-old animals combined with the high degree of variability for disease progression in these mice warrant further investigation of the effects of HDAC inhibitors on long-term glaucoma.

FIGURE 2. Regional loss of β GEO activity occurs in 10-month-old *Bax*^{-/-} DBA/2J^{R3/R3} mice. Cells with β GEO activity appear blue, whereas Nissl-stain appears violet. (A) Whole-mounted retina, showing a wedge-shaped region devoid of β GEO activity (defined by white hashed lines). Regions (B) and (C) in borders of the wedge are shown in higher magnification. Although cells have stopped expressing β GEO, they are still present in the wedge region. Scale bar for (B) and (C): 50 μ m.



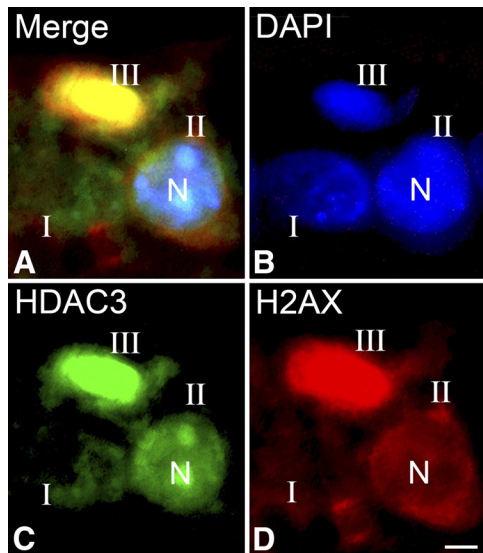


FIGURE 3. HDAC3 concentrates in nuclei of apoptotic cells. Immunofluorescent double labeling of cells in the ganglion cell layer of a DBA/2J mouse. Frozen sections were double labeled with antibodies against the histone variant γ H2AX to identify dying cells, HDAC3, and counterstained for DNA using DAPI. (A) Merged image of all three labels in a representative section. (B–D) Individual channels for DAPI, HDAC3, and γ H2AX. Based on the γ H2AX labeling, the nuclei in the section were identified as stages I, II, or III. Stage II and stage III cells exhibit nuclear localization of HDAC3. Stage II and stage III cells also appear to contain fragmented or highly condensed chromatin, respectively. N, representative nucleus. Scale bar: 5 μ m.

Several groups have now determined that axonal damage and degeneration precede the activation of apoptosis in ganglion cells in these mice.^{12,20–23} To determine whether TSA treatment was also able to attenuate optic nerve degeneration, we examined every nerve from each 10-month-old mouse for β III tubulin immunoreactivity and scored them for mild, moderate, and severe disease (Figs. 6A–C). Nerves in TSA-treated eyes ($n = 43$) exhibited the same frequency of severe and moderate disease as DMSO-treated ($n = 41$) and untreated ($n = 41$) mice (χ^2 test, $P = 0.207$ vs. DMSO, and $P = 0.051$ vs. no injection) (Fig. 6D).

DISCUSSION

Loss of β GEO Expression During Glaucomatous Degeneration

Three phenotypes, based on the level of β GEO expression and cell density, were defined in randomly selected 100×100 - μ m regions of retinal whole mounts of aged DBA/2J^{R3/R3} mice. The most informative phenotype was in regions of retinas that exhibited a decrease in β GEO activity without a concomitant decrease in cell density (phenotype 2). This phenotype was characteristic only of elderly mice, at ages where we, and others, have observed retinal damage as a result of glaucomatous damage.^{12,17,20,24,25} Regions with phenotype 2 cells are consistent with ganglion cells that have become damaged and are suspected of downregulating the expression of normal gene expression before the completion of the apoptotic program observed previously in this model.⁷ In mice, wild-type for a functional *Bax* gene, some regions with substantially decreased β GEO activity (phenotype 3) also had significantly fewer cells, which we interpret as regions that have lost retinal ganglion cells due to completion of the apoptotic program.

To confirm that downregulation of the *Fem1c*^{R3} gene occurred early in the process of ganglion cell apoptosis, we also observed loss of expression in cells deficient for the *Bax* gene. In both *Bax*^{+/-} and *Bax*^{-/-} mice, regions with phenotype 3 still exhibited a high density of cells remaining, although some cell loss was detected in *Bax*^{+/-} animals. In these mice the onset of cell death may be delayed or retarded by the reduction in BAX protein levels, similar to findings in heterozygous mice after acute optic nerve crush.²⁶ Further evidence that the silencing of *Fem1c*^{R3} was a characteristic event of ganglion cell damage was noted in the formation of entire regions of affected cells forming a wedge-shaped pattern radiating from the optic nerve head. This same pattern has been observed for cell loss in both the DBA/2J mouse model^{20,21} and rat models of experimental glaucoma,²⁷ indicating that gene silencing is an early stage of ganglion cell pathology leading to soma loss in this disease.

Gene Silencing and HDAC3 Activity

In the mouse optic nerve crush model of ganglion cell death, the downregulation of gene expression is associated with the nuclear translocation of HDAC3 and deacetylation of histone H4. Inhibition of HDAC activity is able to partially attenuate this silencing phenomenon and delay cell death. Importantly, in these more acute studies, temporal observation of cells indicated that HDAC3 translocated early in the apoptotic process, and was followed by global histone deacetylation in the nuclei of damaged cells.⁸ Determining the same role for HDAC3 in ganglion cells affected by glaucoma is technically more challenging due to the chronic nature of disease progression. Using γ H2AX expression as a marker of dying cells, however, allowed us to evaluate HDAC3 translocation and histone deacetylation at the resolution of different stages of the apoptotic process in individual cells. Similar to dying cells in the acute model, HDAC3 localized to the nuclei of cells with stage II γ H2AX staining, whereas deacetylation was more com-

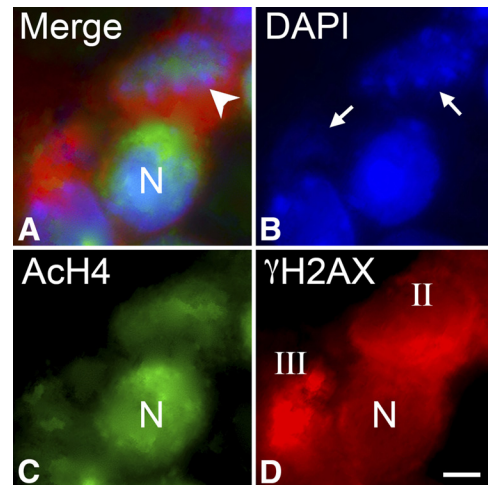


FIGURE 4. Apoptotic cells exhibit reduced staining for acetylated histone H4. Immunofluorescent double labeling of cells in the ganglion cell layer of a DBA/2J mouse. Frozen sections were double labeled with antibodies against the histone variant γ H2AX to identify dying cells, acetylated histone H4 (ACh4), and counterstained for DNA using DAPI. (A) Merged image of all three labels in a section. (B–D) Individual channels for DAPI, ACh4, and γ H2AX, respectively. Representative stages I, II, or III nuclei (N), based on the γ H2AX labeling, are indicated. Stage I cells exhibit strong labeling for ACh4, whereas the single stage III cell in the image has virtually no ACh4 present. Stage II cells show some ACh4 staining, but it is typically intermediate between stage I and stage III labeling. Scale bar: 5 μ m.

TABLE 2. TSA Treatment of DBA/2J^{R3} Mice Does Not Affect Development of Ocular Hypertension

Treatment	n (Eyes)	Mean IOP (mm Hg ±SD)*	IOP Range (mm Hg)	Mean AC Score (± SD)†	Buphthalmia‡
DMSO	46	20.56 ± 7.82	9–40	1.2 ± 0.7	26.1%
TSA	50	19.88 ± 5.96	7–35	1.6 ± 0.9	42.0%
No injection	48	20.13 ± 6.43	11–41	1.6 ± 0.7	22.9%

* Mean intraocular pressure (IOP) in 6-month-old mice is 12.5 ± 3.0 mm Hg (range, 8–20, $n = 18$). Note that IOPs were taken under ketamine and xylazine anesthesia, which may yield artificially low values, particularly in ocular hypertensive mice.¹⁵ There was no statistical difference between IOP levels of all treated groups (ANOVA, $P = 0.307$), but all treated groups were significantly different from 6-month-old mice ($P < 0.001$). These values are also comparable to the mean IOPs reported for age-matched DBA/2J animals that do not carry the R3 allele.¹⁷

† Anterior chamber (AC) score was based on a qualitative evaluation of iris pigment dispersion and transillumination defects by two observers. Scores were made on a 1 to 4 scale, where 1 is no damage and 4 is severely damaged, and the average taken. All treated groups exhibited significantly more AC defects than 6-month-old mice ($P < 0.003$), but there was no significant difference among treated groups ($P > 0.32$).

‡ Buphthalmia, which is characterized by bulging of the eye from the orbit as a consequence of elevated IOP, scored as + or – based on agreement between two observers. The value given is the percentage of eyes in the cohort that were scored as “+.”

mon in stage III labeled cells. The timing of these changes in dying cells supports the role for HDAC3 in establishing early epigenetic modifications in apoptotic nuclei, first suggested in

the acute injury model. Furthermore, TSA treatment was also able to attenuate the loss of *Fem1c^{R3}* gene expression in this glaucoma model, confirming a link between HDAC activity and the silencing of normal ganglion cell expression.

At this point, there is no direct evidence linking HDAC3 activity as a central molecule in the apoptotic pathway executed by retinal ganglion cells. Recently, however, Bardai and

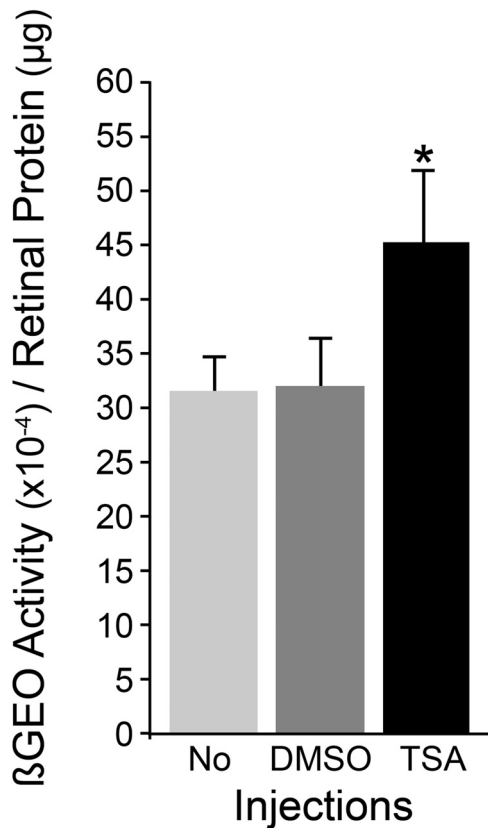


FIGURE 5. TSA treatment attenuates the downregulation of *Fem1c^{R3}* expression. Mice treated weekly with vehicle (DMSO), or TSA, between 6 and 10 months of age, were compared with age-matched mice that had no treatment (No). The level of *Fem1c^{R3}* gene expression was assessed as a function of the amount of betaGEO fusion protein activity relative to total protein in retinal homogenates. TSA provided a significant level (asterisk) of attenuation of the loss of betaGEO enzyme activity observed in both noninjected and DMSO-injected mice ($P = 0.032$ and $P = 0.049$, respectively). The preservation of betaGEO activity afforded by TSA is only partial, however, and represents approximately 80% of the level observed in young DBA/2J mice without disease.

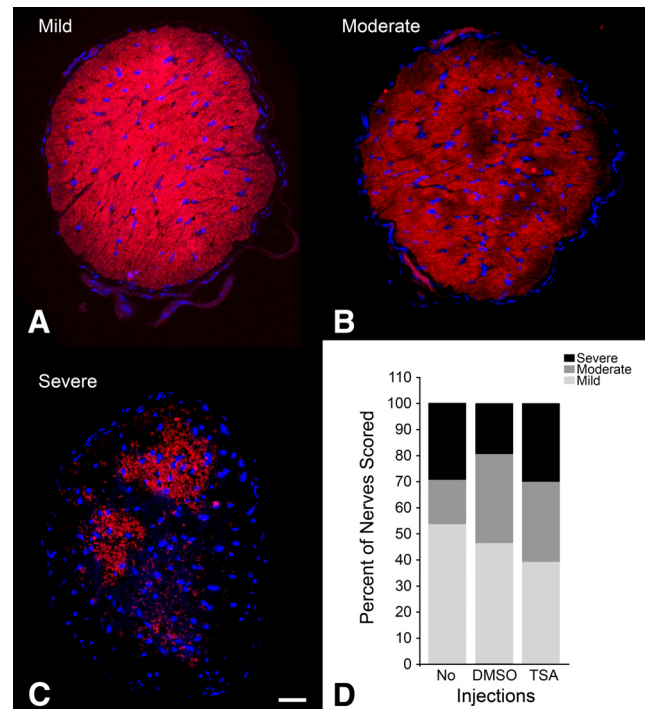


FIGURE 6. Optic nerve scores for treated 10-month-old mice. Optic nerves for each eye harvested for betaGEO activity (Fig. 5) were also examined for optic nerve degeneration. Sections were immunostained for betaIII tubulin and counterstained with DAPI. Digital images were scored by three masked observers for (A) mild, (B) moderate, and (C) severe damage, based on the extent of positive staining for betaIII tubulin. The frequency of each category, for each treatment group, was then graphed and evaluated (D). TSA treatment provided no statistical difference in the frequencies of mild, moderate, or severe damage to the optic nerves of mice, compared with either noninjected mice (No), or vehicle-injected mice (DMSO) (χ^2 test, $P = 0.051$ and $P = 0.207$, respectively). Scale bar for (A–C): 100 μm.

D'Mello²⁸ reported that forced expression of exogenous HDAC3 in a variety of neurons was toxic, whereas similar overexpression in nonneuronal cells had no effect. Silencing of *Hdac3* expression using short hairpin RNAs increased resistance of neurons to damaging stimuli. Additionally, selective HDAC3 inhibitors have been found to be therapeutic in a mouse model of Friedreich's ataxia,^{29,30} principally by reversing the silencing of frataxin expression.

A major contribution of HDAC3 in the process of cell death may also be in the formation of heterochromatin and nuclear condensation. Histone deacetylation is a fundamental characteristic of heterochromatin,¹⁰ which in turn is associated with transcriptionally silent gene activity.^{31,32} One of the earliest steps in the apoptotic program of retinal ganglion cells is the atrophy and shrinkage of the cell nucleus (Janssen KT, et al., manuscript submitted, 2012). This process occurs within 2 hours after acute optic nerve damage and is associated with the deacetylation of histone H4 and the formation of heterochromatin. Additionally, this process occurs in the absence of BAX activation. Ultimately, the condensation of chromatin is considered a hallmark of the apoptotic process.³³ Although some have suggested that the formation of heterochromatin is dependent on caspase activation,^{34,35} this may be more likely a secondary process associated with DNA fragmentation that is a consequence of caspase activity.³⁶ It is possible heterochromatin formation is a multistep process, requiring both early deacetylation of histones, followed by caspase-mediated activation of apoptotic endonucleases. Certainly, it is plausible that cellular mechanisms designed to facilitate soma and nuclear condensation and shrinkage may also secondarily affect active gene transcription through the process of histone modification.

Inhibition of HDAC Activity and Attenuation of Neuronal Apoptosis

There is growing evidence that HDAC inhibitors, in general, have a protective effect on neurons in several animal models of chronic neurodegeneration.^{37,38} Several studies, in fact, have documented neuroprotection of retinal ganglion cells treated with the HDAC inhibitors valproic acid, sodium butyrate,^{39,40} and TSA.^{8,41} Importantly, these previous studies examined the effects of HDAC inhibitors in more acute models of ganglion cell death or on isolated ganglion cells in culture. Using a continuous paradigm of TSA treatment, our experiments document a similar protective effect, specifically on the ganglion cell gene expression, in a chronic model of secondary glaucoma. Although we did not explicitly examine ganglion cell somas after treatment, we interpret the relative preservation of *Fem1c*^{R3} gene expression as an indicator that ganglion cell somas were not only present, but somewhat less affected in disease. This effect was apparently not translated to the axons in the optic nerve, however, which still exhibited a similar level of degenerative effects compared with DMSO-injected and noninjected control groups. This observation suggests that the principal effects of an HDAC inhibitor on neuronal survival are restricted to the soma, possibly by directly influencing progression of the apoptotic program. An analogous finding was made in *Bax*-deficient DBA/2J mice with glaucoma,² and is consistent with the concept that some damage is the downstream effect of initial axonal damage.

Although HDAC inhibitors are now being recognized as a potential therapeutic to prevent ganglion cell death, the mechanism of their protective function is still being elucidated. Our studies suggest that one mechanism may be ameliorating the process of gene silencing in affected retinal ganglion cells. This could provide cells with a more stabilized molecular environment, allowing them a better chance to recover from damaging

insults. Alternatively, HDAC inhibitors may also influence the expression of other genes that help to prevent apoptosis. For example, valproic acid and TSA have been reported to stimulate the upregulation of antiapoptotic members of the *Bcl2* gene family.^{42,43} Preliminary data from our laboratory suggest that TSA may similarly enhance *BclX* expression in retinal ganglion cells (Pelzel HR, et al., manuscript in preparation). It is likely that the combination of these two effects provides an important advantage to neurons under stress conditions.

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