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Targeting HDAC3 in the DBA/2J spontaneous mouse model of glaucoma

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Abstract

High intraocular pressure (IOP) is the most common risk factor associated with glaucoma in humans. While lowering IOP is effective at reducing the rate of retinal ganglion cell (RGC) loss, to date, no treatment exists to directly preserve these cells affected by damage to the optic nerve. Recently, histone deacetylase-3 (HDAC3) has become a potential therapeutic target because it plays an important role in the early nuclear atrophic events that precede RGC death. Conditional knockout or inhibition of HDAC3 prevents histone deacetylation, heterochromatin formation, apoptosis, and eventual RGC loss following acute optic nerve injury. Using these approaches to repress HDAC3 activity, we tested whether targeting HDAC3 protects RGCs from ganglion cell-specific BRN3A expression loss, total somatic cell loss, and optic nerve degeneration in the DBA/2J mouse model of spontaneous glaucoma. Targeted ablation of *Hdac3* activity did not protect RGCs from axonal degeneration or somatic cell death in the DBA/2J mouse model of glaucoma. However, inhibition of HDAC3 activity using RGFP966 conferred mild protection against somatic cell loss in the ganglion cell layer in aged DBA/2J mice. Further experimentation is necessary to determine whether other class I HDACs may serve as potential therapeutic targets in chronic models of glaucoma.

Keywords

glaucoma; epigenetics; RGFP966; optic nerve; retinal ganglion cell

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Declaration of competing interest

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1. Introduction

Glaucoma is the second-leading cause of blindness in the world and is associated with degeneration of the optic nerve and loss of retinal ganglion cells (RGCs) (Tham et al., 2014). To date, no therapeutic treatment has emerged to directly prevent RGC loss and preserve vision. High intraocular pressure (IOP), or ocular hypertension, is the most common risk factor associated with glaucomatous pathology at the optic nerve head (Quigley et al., 1994), and animal models to investigate the pathological effects of ocular hypertension include those in mice, rats, cats, dogs and primates (Khan et al., 2015; Kuchtey et al., 2011; Kuehn et al., 2016; Morrison et al., 2005; Morrison et al., 1998; Pederson and Gaasterland, 1984; Radius and Pederson, 1984; Sappington et al., 2010). Previously, it was shown in mouse models that retinal ganglion cells die via intrinsic apoptosis following acute or chronic optic nerve insult, and when pro-apoptotic factor BAX is knocked out, RGCs do not die (Li et al., 2000; Libby et al., 2005b; Semaan et al., 2010). In surviving *Bax*-deficient RGCs, gene silencing and nuclear atrophic events such as histone deacetylation, chromatin condensation, and deterioration of the nuclear membrane still occur, indicating that these events happen prior to committed cell death (Janssen et al., 2013).

Histone deacetylation is known to play an important role in the early events of nuclear atrophy in RGCs, and after optic nerve crush (ONC), histone deacetylation peaks at 5 days after injury (Pelzel et al., 2010). Recent studies have shown that HDACs play a critical role in gene expression change and RGC cell death in models of optic nerve injury and retinal ischemia (Alsarraf et al., 2014a; Alsarraf et al., 2014b; Biermann et al., 2011; Crosson et al., 2010; Fischer et al., 1970; Schluter et al., 2019). Class I histone deacetylases (HDACs) 1, 2, and 3 are upregulated following acute axonal injury, and the HDAC3 isoform translocates from the cytoplasm to the nucleus of RGCs undergoing apoptosis (Pelzel et al., 2010; Pelzel et al., 2012). Targeting of HDAC3 activity in the acute model of optic nerve damage was shown to protect against histone deacetylation, apoptosis, and eventual cell loss in the ganglion cell layer (GCL) (Schmitt et al., 2014; Schmitt et al., 2017). However, targeting HDAC3 activity by reducing its expression does not ameliorate the gene silencing effect post optic nerve injury, indicating a potential role for other class I HDACs in this process. In the DBA/2J mouse model of spontaneous glaucoma, broad-spectrum inhibition of HDACs partially protects against silencing of the ganglion cell specific *Fem1c* promoter and attenuates cell soma loss, but it does not protect against optic nerve degeneration (Pelzel et al., 2012). Broad-spectrum inhibition of Class I HDACs has been shown to protect RGCs in other models of retinal ganglion cell death (Biermann et al., 2011; Zhang et al., 2012), and HDAC isoform inhibitors are being heavily investigated in models of neurodegeneration (Brochier et al., 2013; Chindasub et al., 2013; Jia et al., 2012; Malvaez et al., 2013; Sun et al., 2007).

Here, we test whether *Hdac3* conditional knockout (cKO), or HDAC3-selective inhibition by systemic treatment with RGFP966, elicit therapeutic effects in the DBA/2J spontaneous mouse model of ocular hypertension.

2. Methods

2.1. DBA/2J mouse model of ocular hypertension

All mice were handled in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978), the Association for Research in Vision and Ophthalmology statement for the use of animals for research, and experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison. Mouse cohorts were age-matched and composed of similar numbers of males and females in the *Hdac3* conditional knockout study. In this study, mouse sex did not significantly affect the responses to HDAC3 ablation (data not shown). Vehicle and RGFP966 treatments were tested in male DBA/2J.BALB^{Rgcs1} mice only, since numbers of female mice were low (n=2) and significantly skewed data (data not shown). A 4 month old cohort of DBA/2J mice (3 females and 1 male) were used as a reference control for the HDAC3 inhibition experiments.

The most well-characterized spontaneous model of glaucoma is the DBA/2J mouse strain, which contains mutations in the b allele of tyrosine-related protein (*Tyrp1^b*) and transmembrane glycoprotein (*Gpnmb^{R150X}*) (Anderson et al., 2002; Howell et al., 2007b). These mutations lead to pigment dispersion, iris stromal atrophy, blockage of aqueous humor outflow, and elevated IOP with age. DBA/2J mice spontaneously develop iris disease at about 6–8 months of age, and mice between the ages of 8–13 months old have elevated IOPs that exceed 3 standard deviations over the mean IOP for young animals. Concomitant with the increase in IOP, DBA/2J mice develop an optic neuropathy and associated RGC loss in the retina that exhibits many features characteristic with glaucoma in humans (Howell et al., 2007b; Libby et al., 2005a; Schlamp et al., 2006). For *Hdac3* conditional knockout experiments in the DBA/2J mouse model, the *Hdac3^{fl/fl}* allele in mice with a C57BL/6 genetic background was backcrossed onto the DBA/2J background through congenic breeding. At the N6 generation, when the C57BL/6 genome made up <1.6% of the genetic background, mice were bred and offspring were genotyped for the *Hdac3^{fl/fl}* alleles, the *Gpnmb^{R150X}* mutation, and the *Tyrp1^b* point mutation and were entered into the experiment. In this experiment, the 4 month control group contained 8 females and 11 males.

To genotype for the *Hdac3^{fl/fl}* allele, two sets of primers were used to identify the presence of the 34bp *loxP* sites flanking exon 7: (1263T: 5'-CCA CTG GCT TCT CCT AAG TTC-3' + 2158B: 5'-CCC AGG TTA GCT TTG AAC TTC-3' wt=880bp *fl/fl*=914bp and 1133T: 5'-CTC TGG CTT CTG CTA TGT CAA TG-3' + 1597B: 5'-GGA CAC AGT CAT GAC CCG GTC-3' wt=445bp *fl/fl*=479bp). To genotype the N6 DBA/2J animals for the *Gpnmb^{R150X}* mutation, which creates a novel *PvuII* site (Howell et al., 2007b), primers *nmb7* (5'-CTA CAA CTG GAC TGC AGG GG-3') and *nmb8* (5'-AGC TCC ATT TCT TCC ATC CA-3') were used to amplify a 125bp DNA fragment. The product was then digested with *PvuII* and the mutation was confirmed by the presence of bands at 50bp and 75bp on the electrophoresis gel. Identification of the B6-derived sequence for *Tyrp1^b* was confirmed by PCR with *D4Mit178* (D2=170bp, B6=146bp) and *D4Mit327* (D2=92bp, B6=106bp) primer sets (Howell et al., 2007b).

To confirm the *Hdac3* conditional knockout, C57BL/6.*Hdac3^{fl/fl}* mice were injected with AAV2-*Pgk-Cre* virus in one eye and allowed to recover for at least one month to allow for viral transgene expression (Supplemental Figure 1). At the end of this period, mice were euthanized and the retinas harvested from enucleated eyes. Retinas were then frozen on dry ice and stored at -80°C until processing. For total RNA isolation, retinas were homogenized in TRIzol (Invitrogen, Carlsbad, CA) and processed according to the manufacturer. For qPCR, 500 ng of total RNA was mixed with 125 ng of oligo(dT)16 primer (Promega, Madison, WI) and cDNA was synthesized using M-MuLV reverse transcriptase (Promega). Real Time qPCR was conducted in a QuantStudio 7 Flex system (ThermoFisher Scientific, Waltham, MA) using the 96-well standard program. All samples were run in triplicate. Input cDNA for each sample was normalized to the level of *S16* ribosomal protein mRNA abundance and transcript levels were calculated using the absolute method (Nickells and Pelzel, 2015) based on a standard curve present in each reaction. Primers used for reactions for *Hdac3* were: Forward 5'-GTA TGA CAG GAC TGA CGA GG-3' and Reverse 5'-TTT CCT TCC CAC CAC AGA GG-3'. Primers used for *S16* were: Forward 5'-CAC TGC AAA CGG GGA AAT GG-3' and Reverse 5'-TGA GAT GGA CTG TCG GAT GG-3'.

A substrain of DBA/2J, known as the DBA/2J.BALB^{*Rgcs1*} mouse strain, was previously created by congenic breeding of the BALB/cByJ Retinal ganglion cell susceptible 1 (*Rgcs1*) locus onto the resistant DBA/2J genetic background (Dietz et al., 2008). This mouse strain displays increased susceptibility to acute optic nerve damage, and in the DBA/2J background, develops a more severe glaucomatous pathology (Dietz et al., 2008). We used this strain to test the efficacy of HDAC3-selective inhibitor RGFP966 in treating glaucomatous pathology.

2.2. IOP measurements

Measurements of IOP were taken on mice anesthetized with 2.0% isoflurane with an air flow rate of 1.5 liters/min for 8 minutes using the iCare Tonolab (Icare, Finland). IOP reads were done between 1:00 and 5:00pm to minimize differences caused by nycthemeral variations, and 5 daily mean IOP readings were taken, with each reading being the mean of 6 measurements, totaling 30 measurements. IOP measurements were done at 4 months for the healthy control animals and 6 and 10 months of age for the experimental animal groups.

2.3. AAV2/2-Pgk-Cre Injection

The AAV2/2-*Pgk-Cre* virus was purchased from the University of North Carolina (UNC) Vector core: <https://www.med.unc.edu/genetherapy/vectorcore/in-stock-aav-vectors/reporter-vectors/> (Chapel Hill, NC). One month prior to initiation of IOP readings, *Hdac3* was conditionally knocked out in the retinal ganglion cell layer of OS retinas of DBA/2J-*Hdac3^{fl/fl}* mice by injecting 1 μ l AAV2/2-*Pgk-Cre* intravitreally (equaling 10^9 genome copies) as previously described (Schmitt et al., 2014). Virally transduced controls were the OS and OD eyes of wild type DBA/2J mice injected with 1 μ l AAV2/2-*Pgk-Cre*. Mice were euthanized and retinas were harvested at 10 months of age to quantify cells expressing BRN3A, total neurons in the GCL, and the level of optic nerve degeneration.

2.4. RGFP966 treatments

Systemic injections were carried out as described by Schmitt et al (Schmitt et al., 2018). Briefly, RGFP966 was administered intraperitoneally (IP) starting at 4 months of age in the DBA/2J.BALB^{Rgcs1} substrain. A stock solution of 1mg/ml RGFP966 in 5% DMSO in 30% 2-hydroxypropyl- β -cyclodextrine, 0.1M acetate pH 5.4 (HP β CD) vehicle was made, and mice were weighed before administering the drug solution appropriately with no more than a volume of 10 μ l/g of mouse, as per IACUC guidelines. For example, a mouse weighing 23.2g would be IP injected with 46.4 μ l of stock solution (containing 0.046mg RGFP966) for a single 2mg/kg dose. RGFP966 was injected every 3 days, which was determined to provide the greatest therapeutic effect to RGCs in the acute crush model of optic nerve damage (Schmitt et al., 2017). Control animals received vehicle via IP injection. Retinas were harvested at 10 months of age in the DBA/2J.BALB^{Rgcs1} strain to measure BRN3A expression, total neuronal cell loss in the GCL, and optic nerve degeneration (see below).

2.5. Immunofluorescence

Enucleated eyes were fixed in 4% paraformaldehyde in phosphate buffered saline (150mM NaCl, 100mM NaH₂PO₄, pH 7.4) (PBS) for 1 hour before rinsing with PBS and whole mounting. Retinas were isolated from these eyes, given 4 relaxing cuts to flatten the retina, and then mounted on Superfrost Plus microscope slides (ThermoFisher Scientific, Hampton, NH) with the GCL facing up, and rinsed again in PBS. The whole mounts were then blocked in 5% bovine serum albumin (BSA) and 0.2% Triton X-100 in PBS for 3 hours at room temperature in humidified chambers before rinsing with PBS. Whole mounts were incubated in 1:50 monoclonal mouse antibody to human Brain-specific homeobox/POU domain protein 3A (BRN3A) (Cat# MAB1585, RRID: AB_94166) (EMD Millipore Inc., Billerica, MA). Optic nerves were dissected and mounted in OCT medium for cross-sectioning. Optic nerve cross-sections were incubated in 1:1,000 monoclonal mouse antibody to phosphorylated neurofilament H (pNEFL) (Covance, Princeton, NJ). Wholemounts and optic nerves were then washed three times in PBS. Tissues were then incubated in 1:1,000 goat anti-mouse FITC secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). All whole mounts and optic nerves were counter-stained for 10 minutes with 300 ng/mL 4',6-diamidino-2-phenylindole (DAPI) before washing with PBS. Lastly, whole mounts were mounted using Immumount mounting medium (ThermoFisher Scientific) and cover slipped. Fluorescent images were obtained using a Zeiss Axioplan 2 Imaging microscope with Axiovision 4.6.3.0 software (Carl Zeiss MicroImaging Inc., Thornwood, NY). Cell counts were done by collecting digital images using a 40x objective of each retinal lobe with cell nuclei immunolabeled for BRN3A or stained with DAPI, and cell numbers were determined in 24 separate 100 $\times$ 100 μ m fields for each retina. Only DAPI-stained cells with round or oval nuclei, containing prominent nucleoli, were counted because these represent either RGCs or displaced amacrine neurons. We estimate that this method samples between 5–10% of the neurons in the ganglion cell layer of the average normal mouse retina.

2.6. Bromo-deoxyUridine (BrdU) labeling

To assess proliferation of cell populations in tissues of mice treated systemically for 6 months with 2mg/kg RGFP966 or vehicle, mice were injected with 2mg BrdU (Fisher Scientific) 24 hours prior to euthanasia. After euthanasia, sections of the small intestine (inferior to the duodenum) were harvested, rinsed in ice cold PBS and incubated in 10% neutral buffered formalin for 24 hours at room temperature. Intestinal tissues were then placed in cassettes and incubated in 70% ethanol for 48 hours before embedding in paraffin and sectioning. Slides were then deparaffinized in ethanol washes, rinsed in PBS, blocked in 3% H₂O₂ in PBS for 2 hours, rinsed again in PBS, permeabilized in 0.05% trypsin in H₂O at 37°C for 10 minutes, rinsed in PBS, and labeled for BrdU using the BrdU *in-situ* detection kit from BD Pharmingen (BD Biosciences, San Jose, CA). Slides were then incubated in hematoxylin for 60 seconds and rinsed in H₂O thoroughly before cover slipping and imaging using the Zeiss Imager A2 (Carl Zeiss MicroImaging Inc.) and a digital camera attachment. Cell counts of BrdU labeled cells in treated and untreated mouse intestinal crypts were collected by first taking 10 digital images (fields) using a 20x objective. Then, using ImageJ software to do particle threshold and watershed adjustment to separate neighboring nuclei, the particle analysis plug-in was utilized to export total BrdU positive cell numbers within a 200µm length of the intestinal wall. Cell numbers from each of 10 fields per treatment group were analyzed to produce a mean value of BrdU positive cells per field for each treatment group.

2.7. Statistical Analysis

Cohorts of eyes for experiments were as follows. For the DBA/2J IOP graph; 4 month *Hdac3^{+/+}* controls n=36 eyes, 10 month *Hdac3^{+/+}* n=38 eyes, and 10 month *Hdac3^{KO}* n=44 eyes were analyzed. For the DBA/2J BRN3A positive cells/field graph; 4 month *Hdac3^{+/+}* controls: n=20 eyes, 10 month *Hdac3^{+/+}*: n=11 eyes, 10 month *Hdac3^{KO}*: n=16 eyes. For the DBA/2J DAPI positive cells/field graph; 4 month *Hdac3^{+/+}* controls: n=18 eyes, 10 month *Hdac3^{+/+}*: n= 15 eyes, 10 month *Hdac3^{KO}*: n= 28 eyes. For the DBA/2J optic nerve scoring graph; 4 month *Hdac3^{+/+}* controls: n=35 optic nerves, 10 month *Hdac3^{+/+}*: n=33 optic nerves, 10 month *Hdac3^{KO}*: n=37 optic nerves. For the DBA/2J.BALB^{Rgcs1} with RGFP966 IOP graph: 4 month controls: n=8 eyes, vehicle: n=20 eyes, and RGFP966: n=22 eyes. For the DBA/2J.BALB^{Rgcs1} with RGFP966 BRN3A positive cells/field graph, 4 month controls: n=6 eyes, 10 month vehicle treatment: n=17 eyes, 10 month RGFP966 treated: n=21 eyes. For the DBA/2J.BALB^{Rgcs1} with RGFP966 DAPI positive cells/field graph, 4 month controls: n=8 eyes, 10 month vehicle treated: n=16 eyes, 10 month RGFP966 treated: n=22 eyes. For the DBA/2J.BALB^{Rgcs1} with RGFP966 optic nerve scoring, 4 month controls: n=8 optic nerves, 10 month vehicle treated: n=19 optic nerves, 10 month RGFP966 treated: n=20 optic nerves. Optic nerves were scored for pNEFL (subunit H), a protein expressed diffusely in the axons of healthy RGCs (Sánchez-Migallón et al., 2018), by three masked observers for severity of degeneration. Optic nerves scored as having mild, moderate, and severe damage based on loss of pNEFL labeling were issued the numbers 1, 2, and 3, respectively. Consensus scores were reached by averaging scores and rounding the average to the nearest integer. This method was previously used in the Pelzel et al, 2012 study to determine optic nerve degeneration in DBA/2J mice. A weighted Kappa statistical analysis was used to determine the inter-rater agreement between observers. Observer 1 and

2 had a weighted Kappa statistic of 0.35279 (95% CI=0.18–0.53), Observer 2 and 3 had a weighted Kappa statistic of 0.62798 (95% CI=0.46–0.79), and Observer 1 and 3 had a weighted Kappa statistic of 0.33198 (95% CI=0.17–.49). We used a 3 scale scoring system of mild disease, moderate disease, and severe disease as described by Libby et al. (Libby et al., 2005a) and modified by Howell et al. (Howell et al., 2007a).

IOP data were collected from individual eyes, and box plots indicate the minimum, maximum, mean, and upper and lower quartiles of the data presented. Cell count, and optic nerve scoring data were collected from independent samples, and the data was presented as the mean± standard error. Welch's t-test is an adaptation of Student's t-test that is more reliable when the two samples have unequal variances and unequal sample size. Here, statistical analyses for cell counts were performed using Welch's two-sample t-test assuming unequal variances with statistical significance (alpha) set at p 0.05 for comparison of two groups. A χ^2 test was used to compare optic nerve scores. Bonferroni adjustments (alpha set to 0.05) were made based on fluorescence image field numbers.

3. Results

3.1 Conditional knockout of *Hdac3* in DBA/2J mouse model of spontaneous glaucoma did not protect against BRN3A expression loss, total cell loss or optic nerve degeneration

The DBA/2J model has been well characterized as a mouse model of secondary glaucoma. As the mouse ages, asynchronous RGC loss occurs due to spontaneous increase in ocular hypertension. Ocular hypertension rises in mice beginning at the age of 6 months and progresses to a peak at 10 months of age (Libby et al., 2005a). Peak retinal ganglion cell loss occurs at about 10 months of age (Jakobs et al., 2005; Schlamp et al., 2006). This mouse model has molecular and anatomical features, including RGC loss and optic nerve degeneration that are common to glaucoma. Additionally, this model is highly reproducible and elicits optic neuropathy with predictable variability (Libby et al., 2005a; Schlamp et al., 2006), making it a more interpretable model for assessing the therapeutic efficacy of targeting HDAC3 activity.

To test whether *Hdac3* cKO could protect RGCs in the DBA/2J mouse model of spontaneous glaucoma, the *Hdac3*^{fl/fl} allele was backcrossed onto the DBA/2J background for 6 generations. Because this number of generations represents a small, but significant fraction of contamination of the C57BL/6 genomic background, only wild type and *Hdac3*^{fl/fl} mice carrying the *Gpnmb* and *Tyrl* mutations, which lead to pigment dispersion and glaucomatous pathology (Howell et al., 2007b), were selected for this experiment. Here, we found that all mice developed significantly elevated IOP over the course of 10 months (Figure 1A.). Assessment of whole mounted retinas for BRN3A and DAPI positive cells (Figure 1B.-F.) by masked cell counts revealed that both groups experienced significant cell loss (****p<0.00001, Bonferroni correction: p=0.00007) and there was no statistical difference between the control and *Hdac3*-deleted mice (p>0.00007). Optic nerve scores indicated that neither wild type nor *Hdac3* cKO optic nerves were protected from degeneration (Figure 1G.-J.).

3.2. Systemic injection of 2mg/kg RGFP966 every 3 days mildly protects against total cell loss in the DBA/2J.BALB^{Rgcs1} mouse model of glaucoma

By using the treatment paradigm outlined above, DBA/2J.BALB^{Rgcs1} susceptible mice were either treated with vehicle or RGFP966 from 6 months of age until 10 months of age, when pathological loss of RGCs is observed (Dietz et al., 2008). Similar spontaneous ocular hypertension was recorded by IOP measurements at 4, 6, and 10 months of age (Figure 2A.). Total HDAC activity in the retina was measured over a time course following intraperitoneal injection of RGFP966 at 2mg/kg, and an insignificant decrease in HDAC activity was observed 1 hour after injection (Supplemental Figure 2A.). However, endogenous HDAC activity in the retina rebounds to normal levels by 72 hours post treatment. To determine whether repeated dosing of RGFP966 led to changes in proliferation of cells in the RGC layer, a BrdU incorporation assay was conducted that revealed no significant difference in proliferation in the RGC layer ($p=0.288$). To ensure that repeated systemic treatment every 72 hours (3 days) with RGFP966 over the course of 6 months did not elicit toxic side effects to the animal and to proliferating cell populations in the intestine, a BrdU incorporation assay was performed on small intestine tissue for each mouse. Cell counts indicated no significant change in proliferating cell populations (Supplemental Figure 2D.-E.). Importantly, RGFP966 did not induce a decrease in body weight (Figure 2C.), which would have been a greater indicator of overall declining health (Ray et al., 2010). Treatment with RGFP966 every 3 days for 6 months led to modest protection against total neuronal cell loss (**** $p<0.0001$ compared to vehicle treated mice) with no significant protection against BRN3A expression loss (Figure 2E.-I.). However, like the effect of *Hdac3* cKO in the spontaneous mouse model of glaucoma, HDAC3 inhibition long-term did not protect the axons from degeneration (Figure 2J.-M.).

4. Discussion

Targeting HDAC3 activity in the DBA/2J spontaneous model of glaucoma did not confer dramatic protection against RGC soma degeneration and loss. A conditional knockout of *Hdac3* had no significant effect on attenuating cell loss in the GCL. HDAC3 selective inhibition in the DBA/2J.BALB^{Rgcs1} susceptible substrain, however, led to mild but significant protection against total cell loss.. The overall protective effect of targeting HDAC3 in the DBA/2J model of glaucoma was dramatically milder than the effect of targeting HDAC3 therapeutically in an acute model of optic nerve damage (Schmitt et al., 2014; Schmitt et al., 2017). This could reflect fundamental differences in the apoptotic signaling pathways associated with ocular hypertension and acute damage and suggests that therapeutic targeting of HDAC3 might be a more effective treatment for acute optic neuropathies or optic nerve trauma.

Similarly, targeting HDAC3 had no effect in moderating optic nerve degeneration in the DBA/2J mouse model of glaucoma. These experiments suggest that HDAC3 does not mediate axon damage in response to chronic pressure insult in the DBA/2J model. These results align with the previous report that broad-spectrum HDAC inhibitor trichostatin A (TSA) also does not protect the RGCs from axonal damage in DBA/2J mice (Pelzel et al., 2012). TSA does, however, protect against loss of ganglion cell-specific *Fem1c* promoter

expression in the DBA/2J mouse model (Pelzel et al., 2012). To date, few studies have assessed the protective effect of HDAC inhibition in models of experimental glaucoma. The Class I HDAC inhibitor VPA provided at least short term protection to RGCs in ocular hypertensive rats (Alsarraf et al., 2014a). VPA also protected against cell loss in the GCL, thinning of the inner retina, and electrophysiological function loss in the glutamate/aspartate transporter knockout mouse model, which leads to RGC loss and has been described as a model of normal tension glaucoma (Kimura et al., 2015). VPA specifically leads to proteosomal degradation of HDAC2 (Krämer et al., 2003). This may explain, in part, why treatment with the HDAC3 selective inhibitor RGFP966 conferred limited protection against cell soma loss, while *Hdac3* conditional deletion did not. It is also possible that endogenous *Hdac3* expression could be affected in a compensatory manner with repeated administration of RGFP966, however this has yet to be investigated. Pharmacokinetic analysis of RGFP966 availability to the retina (Schmitt et al., 2018) showed that drug availability transiently reached the IC50 level for HDAC1 and 50% of the IC50 level for HDAC2 after IP injection. Further testing of Class I HDAC isoform inhibitors is necessary to elucidate the likely roles that HDACs 1 and 2 play in RGC death in models of chronic optic nerve injury.

The absence of a clear protective effect of HDAC3 deletion and inhibition in this glaucoma model was unexpected given the clear effect this treatment has in more acute optic nerve damage paradigms. Both optic nerve damage paradigms induce a similar molecular cascade involving activation of the Dual Leucine Zipper Kinase (DLK) – JUN-N-terminal Kinase (JNK) pathway (Welsbie et al., 2013) that eventually lead to activation of the proapoptotic BAX protein (Libby et al., 2005b). This pathway diverges, however, with JNK activation leading to both the activation of the JUN and p53 transcription factors, which in turn regulate the expression of different BAX-activating BH3-only genes (Maes et al., 2017). Of these two, HDAC inhibition most prominently affects p53 activation (Lebrun-Julien and Suter, 2015; Uo et al., 2009). It is possible that one branch of the DLK-JNK pathway is more prominent than the other depending on the mechanism of axonal damage. Alternatively, HDAC3 may have a relatively specialized function in the cell death process that is sufficient to confer protection over a short interval (such as 4–8 weeks after optic nerve crush), but is insufficient to prevent cell death over a longer interval (4 months in the DBA/2J study). This study does not rule out the possibility that an efficacious therapy could involve HDAC3 inhibition for immediate effect in conjunction with a therapeutic that has a greater long-range effect.

In conclusion, RGFP966 can retard somatic cell loss in the GCL, but unlike studies using acute optic nerve damage, this was not mimicked by conditional knock-out of *Hdac3* in RGCs. However, no axonal protection was observed in DBA/2J mice that had HDAC3 targeted therapy. Further investigation is necessary to elucidate the roles of Class I HDACs in regulating RGC apoptosis and axonal degeneration in the optic nerve in models of glaucoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Conditional knockout of *Hdac3* does not protect against RGC death or optic nerve degeneration in the DBA/2J mouse model of glaucoma.
- Selective inhibition of HDAC3 activity with RGFP966 confers mild protection against somatic cell loss in the DBA/2J mouse model of glaucoma.

Figure 1.

Conditional knockout of *Hdac3* in N6 DBA/2J mice does not confer protection against RGC cell loss. (**A.**) N6 DBA/2J-*Hdac3*^{fl/fl} (*Hdac3* cKO) and N6 DBA/2J-*Hdac3*^{+/+} mice develop spontaneous ocular hypertension with age. A control cohort of naïve N6 DBA/2J-*Hdac3*^{+/+} (Control) mice were assessed at 4 months of age for intraocular pressure (IOP), and no significant difference was observed between this group and 6 month old WT and *Hdac3* cKO treatment groups. *Hdac3*^{+/+} and *Hdac3*^{cKO} DBA/2J mice exhibited similar increases in mean IOPs at 10 months of age ($p=0.1334$), which were significantly higher than 6-month cohorts (**** $p<0.00001$). Box plots indicate the minimum, maximum, mean, and upper and lower quartiles of the data presented. 4 month controls: $n=36$ eyes, *Hdac3*^{+/+}: $n=38$ eyes,

Hdac3^{CKO}: n=44 eyes. Conditional knockout of *Hdac3* in the ganglion cell layer of N6 DBA/2J *Gpnmb*^{-/-} and *Tyrp1*^{-/-} mice does not protect against cell loss. **(B.-D.)** Fluorescence images of whole mounted **(B.)** 4 month *Hdac3*^{+/+} control, **(C.)** 10 month *Hdac3*^{+/+}, and **(D.)** 10 month *Hdac3* cKO retinas labeled with RGC marker BRN3A (green) and nuclear label DAPI (blue). Scale bar=20 μ m. **(E.)** 10 month *Hdac3*^{+/+} and *Hdac3*^{CKO} retinas had significantly fewer BRN3A positive cells per field (****p<0.00001) in comparison to 4 month controls. **(F.)** Additionally, 10 month *Hdac3*^{+/+} and *Hdac3*^{CKO} retinas had significantly fewer DAPI positive cells per field (****p<0.00001) in comparison to 4 month controls. Bar graphs indicate mean $\pm$ SEM. Bonferroni correction for significance was set to $p=7.92 \times 10^{-5}$ ($\alpha=0.05$). For BRN3A positive cells/field, 4 month controls: n= 414 fields (20 eyes), 10 month *Hdac3*^{+/+}: n= 227 fields (11 eyes), 10 month *Hdac3*^{CKO}: n=290 fields (16 eyes). For DAPI positive cells/field, 4 month *Hdac3*^{+/+} controls: n= 310 fields (18 eyes), *Hdac3*^{+/+}: n= 324 fields (15 eyes), *Hdac3*^{CKO}: n= 618 fields (28 eyes). Bonferroni correction: p=0.00007 ($\alpha=0.05$). **(G.-J.)** Conditional knockout of *Hdac3* in the ganglion cell layer of N6 DBA/2J *Gpnmb*^{-/-} and *Tyrp1*^{-/-} mice does not prevent optic nerve degeneration. Optic nerve cross-sections were stained for phosphorylated neurofilament (pNEFL) (green) and DAPI (blue), imaged using consistent exposure time across the cohort, and scored for level of degeneration by 3 masked observers. Scores were then averaged to give consensus scores. **(G.-I.)** Images that had consistent pNEFL staining (green) throughout the nerve were considered mildly degenerated or healthy **(G.)**, images with regions of reduced or no staining were considered moderately degenerated **(H.)**, and images with a predominant lack of staining were considered severely degenerated **(I.)**. **(J.)** Optic nerve scoring indicated a significant increase in degeneration in both *Hdac3*^{+/+} (n=33 optic nerves) (χ^2 value=23.32) and *Hdac3* cKO (n=37 optic nerves) (χ^2 value=23.33) optic nerves after 10 months of elevated IOP (****p<0.00001) in comparison to 4 month controls (n=35 optic nerves). (Scale bar=100 μ m)

Figure 2.

Inhibition of HDAC3 in DBA/2J.BALB^{Rgs1} mice offers mild protection against somatic cell loss, but not BRN3A expression or optic nerve degeneration. (A.) DBA/2J.BALB^{Rgs1} mice develop spontaneous elevated IOP. DBA/2J.BALB^{Rgs1} mice were age-matched and assigned to either receiving vehicle or 2mg/kg RGFP966 IP injection every 3 days from 6 to 10 months of age. IOPs were recorded in a 4-month old DBA/2J cohort, and the experimental mice before and after their treatment period from 6–10 months of age showing an age-related increase in pressure in the RGFP966 treated group (****p<0.00001). There was no significant difference in IOP between vehicle- and RGFP966-treated mice at 10 months of age (p=0.2213). Box plots indicate the minimum, maximum, mean, and upper and lower quartiles of the data presented. 4 month controls: n=8 eyes, vehicle: n=20 eyes, RGFP966: n=22 eyes. (B.-F.) Systemic injection of 2mg/kg RGFP966 every 3 days protects against total cell loss in the DBA/2J.BALB^{Rgs1} mouse model of glaucoma. DBA/

2J.BALB^{Rgcs1} mice were age-matched and assigned to either receiving vehicle or 2mg/kg RGFP966 IP injection every 3 days from 6 to 10 months of age. At 10 months of age, animals were euthanized and retinal whole mounts were analyzed for RGC loss. **(B.-D.)** Whole mounted retinas were stained for nuclear BRN3A (green) and DAPI (blue). Scale bar=20 μ m. **(E., F.)** Cell counts indicated that animals treated with either vehicle or RGFP966 had significantly fewer BRN3A and DAPI labeled cells per field in comparison to 4-month control animals (****p<0.00001). At 10 months of age, RGFP966 treated mice did not have significantly more BRN3A positive cells present in comparison to vehicle treated mice. However, RGFP966 treated mice did have significantly more DAPI labeled cells present in comparison to vehicle treated mice (****p<0.00001) Bar graphs indicate mean $\pm$ SEM. For BRN3A positive cells/field, 4 month controls: n=138 fields (6 eyes), 10 month vehicle treated: n= 397 fields (17 eyes), 10 month RGFP966 treated: n= 504 fields (21 eyes). For DAPI positive cells/field, 4 month controls: n=186 fields (8 eyes), 10 month vehicle treated: n=371 fields (16 eyes), 10 month RGFP966 treated: n= 512 fields (22 eyes) Bonferroni correction: p=0.0001 (alpha=0.05). **(G.-J.)** Systemic injection of 2mg/kg RGFP966 every 3 days does not prevent optic nerve degeneration in the DBA/2J.BALB^{Rgcs1} mouse model of glaucoma. **(G.-I.)** Masked optic nerve scoring was done, and optic nerves were scored based on phosphorylated neurofilament (P-NEFL) immunofluorescence: **(G.)** mild degeneration, **(H.)** moderate degeneration, and **(I.)** severe degeneration. **(J.)** Analysis of optic nerve scoring indicated that 4-month old control optic nerves (n=8 optic nerves) were significantly healthier when compared to vehicle (n=19 optic nerves) (****p<0.00001, χ^2 value=89.11) and RGFP966 treatment (n=20 optic nerves) (****p<0.00001, χ^2 value=78.67) optic nerves at 10 months of age. RGFP966 treated mice had significantly more degenerated optic nerves when compared to vehicle treated mice (**p<0.001, χ^2 value=13.96) (Scale bar=100 μ m).