Inhibition of Histone Deacetylases 1 and 3 Protects Injured Retinal Ganglion Cells

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Purpose. Thy-1 is a marker of retinal ganglion cell (RGC) differentiation. Optic nerve injury triggers reduction of Thy-1 promoter activation followed by retinal ganglion cell (RGC) death. This study determined whether MS-275, an inhibitor of the histone deacetylases 1 and 3, can inhibit these changes.

Methods. Mice expressing cyan fluorescent protein (CFP) under control of the Thy-1 promoter received MS-275 (subcutaneous) or vehicle three times per week starting 1 week before optic nerve crush and continuing for 6 weeks. The same retinal area was imaged using the blue-light confocal scanning laser ophthalmoscope before and after optic nerve crush every week, and fluorescent spots were counted manually. The eyes were then processed for histopathologic analysis.

Results. The mean proportions of fluorescent retinal neurons remaining in the vehicle group following optic nerve crush were 36 ± 8, 18 ± 6, 13 ± 10, 12 ± 4, 13 ± 5, and 13 ± 5% at weeks 1 through 6, respectively (n = 6). In contrast, the mean proportions of fluorescent retinal neurons remaining in the group treated with MS-275 were 59 ± 19, 39 ± 11, 34 ± 12, 33 ± 15, 32 ± 13, and 27 ± 15% at weeks 1 through 6, respectively (n = 7, $P < 0.05$ at weeks 1 through 5). Rate analysis showed that MS-275 slowed the rate of loss during the first 2 weeks by 23% ($P < 0.05$) and subsequently was similar. Histopathologic analysis revealed 27 ± 13% greater ganglion cell layer (GCL) neurons in the eyes from mice that received MS-275 treatment ($P < 0.02$).

Conclusions. These results indicate that treatment with MS-275 protects against the loss of RGC differentiation and promotes RGC survival following optic nerve injury. (*Invest Ophthalmol Vis Sci.* 2013;54:96–102) DOI:10.1167/iovs.12-10850

Growing evidence indicates that treatment with valproic acid can protect central nervous system neurons including retinal ganglion cells (RGCs) following injury.[1–3] However, this broad-spectrum inhibitor of histone deacetylases can induce a number of undesirable side effects.[4–8] The second generation histone deacetylase (HDAC) inhibitor MS-275 specifically targets HDAC-1 and HDAC-3 and is presently in clinical trials for cancer treatment.[9,10] It has fewer and milder side effects than valproic acid.[9,10] In vivo studies have shown that it can enhance differentiation of brain neuronal precursor cells.[11] It also can reduce postischemic mouse brain injury.[12] However, it is unknown whether MS-275 can protect RGCs following optic nerve injury.

Optic nerve injury induces progressive loss of specific RGC differentiation marker proteins such as Thy-1, followed by cell death.[13–16] Kinetic analysis has shown that Thy-1 mRNA and proteins are gradually lost over the first 2 weeks following optic nerve crush.[17] RGC death follows around 1 to 2 weeks after Thy-1 loss. Interventions that protect RGCs from the effects of axonal injury may diminish or delay this reduction of Thy-1 promoter activation. Recently, we have developed the capability to longitudinally measure changes in the activation of this promoter in vivo by imaging fluorescent retinal neurons of transgenic mice that express cyan fluorescent protein under control of the Thy-1 promoter.[18] We have shown that the RGC response to optic nerve crush includes an initial quick phase during which approximately half of the RGCs cease expression of CFP, followed by a prolonged phase during which loss of fluorescent cells occurs more slowly.[19]

The present study has used this method to determine whether MS-275 treatment alters the time-dependent alteration of Thy-1 promoter activation in Thy1-CFP mice following optic nerve crush. The main advantage of the optic nerve crush model is it produces simultaneous injury to optic nerve axons. This facilitates identification of time-dependent change in the rate of RGC degeneration. To determine whether these fluorescence changes were associated with protection against RGC death, the loss of ganglion cell layer (GCL) neurons was also assessed by postmortem histopathological analysis.

Methods

Animals

Adult hemizygous B6.Cg-Tg (Thy1-CFP) 23Jrs mice (both male and female) approximately 27 months old were bred at the University of California San Diego from the same stocks that provided animals for prior studies.[18,19] All experimental procedures conformed to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental Design

The experimental groups in this study were mice treated with MS-275 by subcutaneous injection and control mice treated with vehicle. The MS-275 dose, 11.3 mg/kg, was chosen because it was the lowest dose that induced maximal increase in the acetylation of histone H3 in brain

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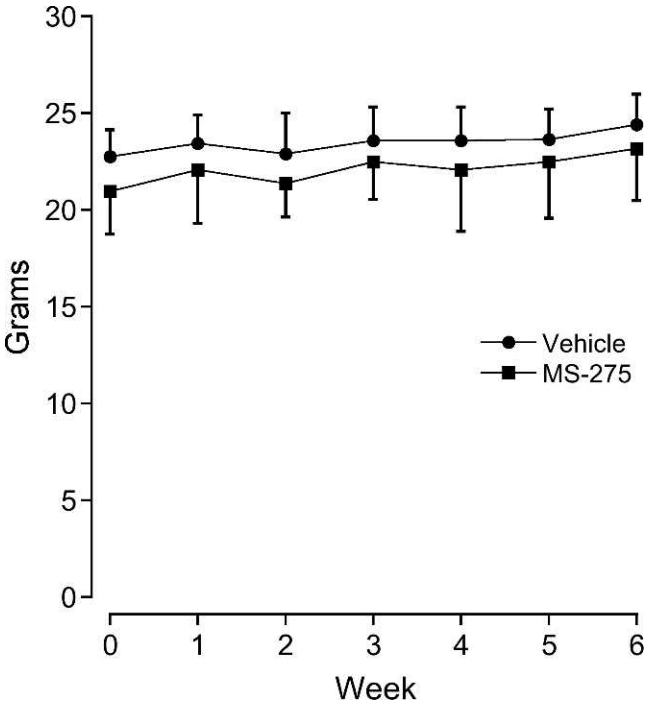

Figure 1. Body weight of mice that received vehicle control and MS-275. Error bars indicate SD. There was no significant body weight change in any of the groups ($P = 0.71$, $N = 6$ for vehicle group and $P = 0.89$, $N = 7$ for the MS-275 group).

frontal cortex.[20] MS-275 (Cayman, Michigan) was dissolved in 2% dimethyl sulfoxide in water and further diluted 1:1 with phosphate-buffered saline (PBS) just before subcutaneous injection. Control mice were injected with 2% DMSO diluted 1:1 with PBS. Control and experimental injections were given three times per week over the 7-week course of the study. Retinal images were collected as described below prior to the beginning of treatments and then weekly for 6 weeks after optic nerve crush.

Imaging

Imaging was performed as previously described.[18,19] Animals were gently restrained by hand, and no anesthesia was used. A single scan corresponding to a retina area of approximately 2 mm^2 was obtained in less than 5 seconds. The short exposure period needed for focusing and image collection was generally well tolerated by the mice. Both eyes were imaged at each imaging session. This allowed monitoring of any fluorescent cell loss for reasons unrelated to the optic nerve crush. The retinal eccentricity for optimal imaging of fluorescent GCL neurons extended from the edge of the optic nerve head outward to approximately 700 μm. Within this region, most of the fluorescent cells in a typical image field could be simultaneously imaged in focus. This facilitated obtaining images of the same retinal area over the 6 week course of the study. It was more difficult to obtain good images in more peripheral areas of the retina**.** During imaging with the blue-light confocal scanning laser ophthalmoscope (bCSLO), the mice were held steady by an assistant. Pupils were dilated with topical mydriatic agents (tropicamide and phenylephrine, 0.5% each). Imaging was noninvasive and involved no contact. No anesthesia was used. The scan rate of the modified bCSLO was 12 frames per second, with a resolution of 512 × 512 pixels at each frame. Instrument sensitivity was adjusted at each imaging session to maximize signal to noise because preliminary studies indicated that this enhanced reproducibility. Fluorescent spots in the same retinal area of each animal were counted manually.

Optic Nerve Crush

Thy-1 CFP mice were anesthetized by intraperitoneal injection of ketamine and xylazine. A drop of proparacaine hydrochloride (0.5%) was administered topically once the animal lost consciousness. Limbal conjunctival peritomy was performed in the superior region. The optic nerve was then exposed through a small window made between the superior rectus muscle and fatty tissue by gentle blunt dissection. Care was taken not to damage muscles or the blood vessels. At a site approximately 1 mm posterior to the globe, the optic nerve was clamped for 3 seconds. This site was chosen to avoid damage to the ophthalmic artery. After this procedure, antibiotic ointment was applied and eyelid was closed with 8-0 nylon suture. During the postoperative period, mice exhibited normal eating and drinking behavior. Sham procedures, in which the optic nerve was exposed without crush, were performed in contralateral eye.

Histopathologic Evaluation

Immediately after the last bCSLO image was collected, each mouse was euthanized, and eyes were immersion fixed in 4% paraformaldehyde with 20% isopropanol and 1% (w/v) zinc chloride. Oriented eyecups were embedded in paraffin and midsagittal sections through the optic nerve head were stained with hematoxylin and eosin. Counts of GCL neurons were made extending away from the GCL margin at the optic nerve head for 673 μm. The eccentricity of the retinal regions evaluated in the quantitative histological analyses corresponded to the eccentricity of the regions imaged in vivo. In the control eyes that received sham optic nerve crush, these counts typically included ~250 cells/eye. Retina cross sections allowed evaluation of whether there were treatment effects on the other layers of the retina in addition to the GCL.

Analysis

All of the bCSLO images from a particular eye were reviewed at the end of the experiment to identify a specific retinal region in which there is good quality imaging of more than 100 fluorescent cells from all of the examination sessions. For each animal, this same region was copied from the original images at different time points using Adobe Photoshop (version CS4; Adobe Systems, Inc., San Jose, CA), and fluorescent retinal neurons were manually counted in a masked fashion. The fluorescent retinal neuron counts from each crush-operated eye were expressed as a percentage of fluorescent retinal neuron counts in its contralateral sham-operated eye prior to statistical analysis. Similar normalization was made for the GCL neuron counts in the histopathologic evaluation. These corrections compensated for the normal variation in RGCs among different mice of the same strain, since the number of RGCs in fellow eyes of the same mouse typically vary by less than 3%.[21] This approach also avoided the potential influence of the normal variation in RGC density according to retinal position.[14]

Statistics

Body weight, fluorescent retinal neuron counts, and GCL neuron counts from histological sections were statistically analyzed using analysis of variance (ANOVA) and the Student t test to compare between groups at each time point.

Results

Body Weight

The body weight of animals in the vehicle group or the group treated with MS-275 did not change significantly during the course of the study (Fig. 1, ANOVA, $P = 0.71$, $N = 6$ for vehicle group and $P = 0.89$, $N = 7$ for the MS-275 group).

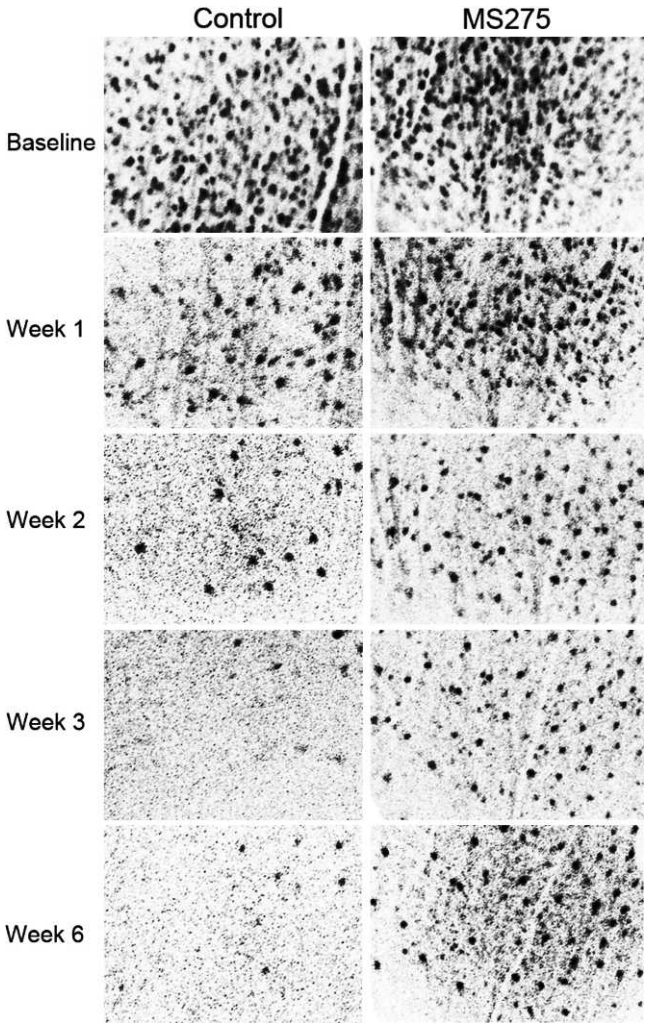

Figure 2. Inverted images of fluorescent retinal neurons in same retinal area obtained prior to optic nerve crush (baseline) and at weeks 1, 2, 3, and 6 after optic nerve crush showing protective effect of MS-275. Note that prolonged treatment with MS-275 did not generally induce fluorescence in cells that were not fluorescent at the beginning of the study.

Fluorescent Retinal Neuron Counts

After optic nerve crush, there were significant time-dependent reductions of the number of CFP-expressing fluorescent retinal neurons in both groups that were markedly less in the mice that received MS-275 than in the mice that received the vehicle control (Fig. 2). Comparison of the images from the same retinal area over time showed that nearly all of the fluorescent cells remaining later in the course of the study could be identified in the earlier images of that area based on their position relative to the other fluorescent neurons. Counts of fluorescent cells in the defined retinal areas at one week after the crush showed a 64% average decline in the vehicle treated animals while the average decline was only 41% in the animals that received MS-275. The magnitudes of the declines were less in the second week. By the third week following the optic nerve crush, the counts of fluorescent cells in the defined retinal areas of both the vehicle and MS-275 treated animals had reached plateaus (Fig. 3). However, the mean number of fluorescent neurons remaining was 31 to 34% of baseline in the animals that received MS-275 while it was only 12% to 14% of baseline in the animals that received vehicle. Overall, the

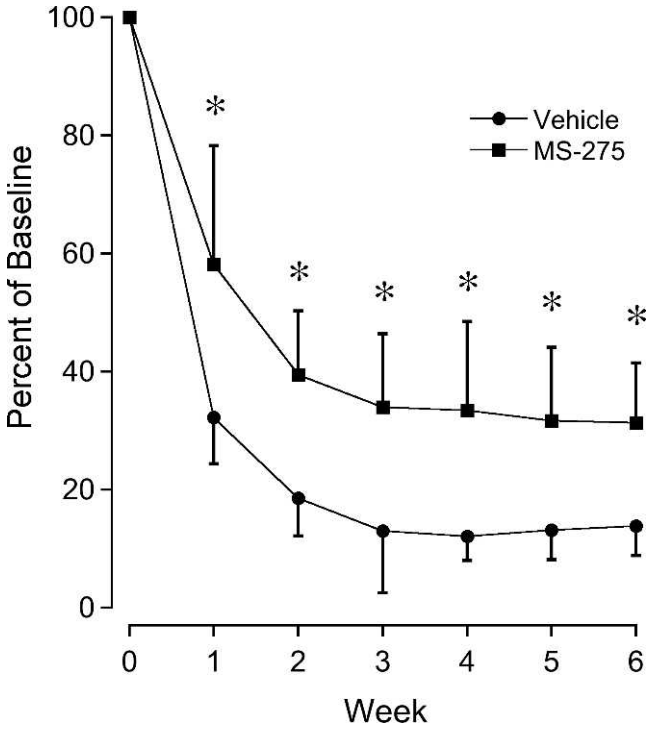

Figure 3. Counts of retinal neurons that continue to express CFP after optic nerve crush expressed as a percentage of baseline count. $N = 6$ in the vehicle control group and 7 in the MS-275 group. Bars indicate SD. *Asterisk* indicates $P < 0.05$, t test.

number of fluorescent retinal neurons was significantly greater at weeks 1 through 6 in the animals that received MS-275 than in the animals that received vehicle ($P < 0.05$).

Rate Analysis

The rate of loss of fluorescent retinal neurons were calculated on a weekly basis for each eye over the course of the study. These results showed that in the MS-275 group, the rate of loss was greatest in the first week after optic nerve crush in 4 of 7 eyes, similar for weeks 1 and 2 in 1 of 7 eyes, and greatest in the second week in 2 of 7 eyes. In view of this variability, the rate of loss for each eye was recalculated for two-week periods over the course of the study. As shown in Figure 4, the mean rate of loss in the first two weeks after optic nerve crush was 26% slower in the MS-275 group than in the vehicle group ($P < 0.05$, Bonferroni t-test). In contrast, the mean rate of loss was both less than 5%/week and differed insignificantly in the MS-275 and vehicle groups during the second two weeks and third two weeks following optic nerve crush.

Potential Induction of Fluorescence

To evaluate the possibility that MS-275 might induce fluorescence in GCL neurons that were not fluorescent at the beginning of the study, the position of fluorescent retinal neurons in specific retinal areas within the sham-operated eyes at the end of the study were compared to the position of fluorescent retinal neurons in these same specific retinal areas that were imaged before the initiation of MS-275 treatments. The same comparison was performed for the vehicle group. As illustrated in Figure 5, the cells observed at the end of the study in specific retinal areas had the same positions as cells that had been observed at the beginning of the study; i.e., no new fluorescent cells appeared during the course of the study. Supporting this observation,

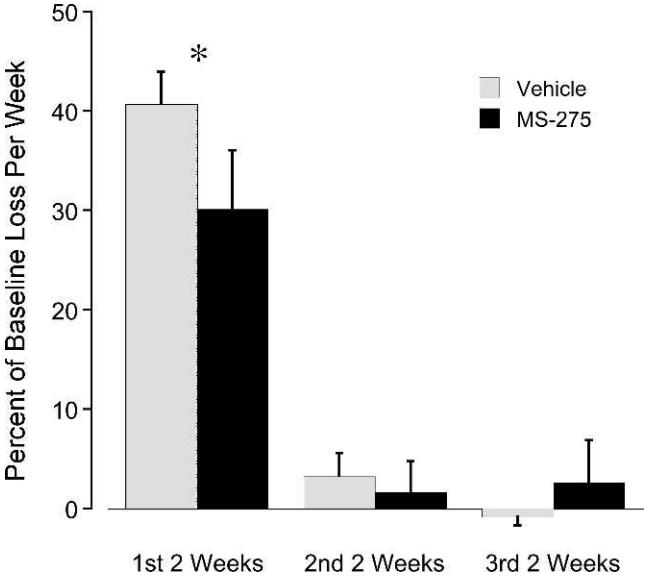

Figure 4. The mean rate of loss of fluorescent retinal neurons in the first two weeks, the second two weeks, and the third two weeks after optic nerve crush ($N = 6$ in the vehicle control group and 7 in the MS-275 group). Bonferroni *t*-test analysis of all pair-wise comparisons indicated that the mean rate of loss was significantly slowed by MS-275 treatment during the first two weeks (*asterisk*). It also indicated that the rate of loss during the first two weeks was significantly faster in each treatment group than the rate of loss in the second two weeks or third two weeks for both treatment groups (not indicated).

counts of the fluorescent retinal neurons in the specific retinal areas of the MS-275 treated animal sham eyes at the end of the study differed insignificantly from cell counts of the same areas of baseline images obtained at the beginning of the study ($P = 0.22$, $N = 7$). This is further supported by the lack of change in the number of fluorescent retinal neurons in the vehicle control sham eyes ($P = 0.55$, $N = 6$).

Histopathology and Quantitative Analysis

Histopathologic analysis of eyes collected at the end of the study revealed normal structure in the sham-operated eyes of mice that received either vehicle or MS-275 treatments (Fig. 6). The density of GCL neurons was always less in the eyes that received optic nerve crush than in the contralateral sham-operated eyes. Moreover, the density of GCL neurons was less in these eyes of mice that received vehicle than those that received MS-275. This pattern was present in central retina, mid-peripheral retina, and in the peripheral retina. The other retinal cell layers in these eyes had normal appearance. Quantitative analysis showed there were 27% more GCL neurons remaining in the crush-operated eyes of mice that receive MS-275 than in the crush-operated eyes of mice that received vehicle ($P < 0.05$, Table 1). The GCL neuron counts in sham-operated eyes of mice that received MS-275 did not differ from corresponding counts in sham-operated eyes of mice that received vehicle ($P > 0.05$).

Discussion

These results indicate MS-275 protects against loss of retinal Thy-1 promoter activation that occurs following optic nerve crush, as well as protects against reduction in the number of GCL neurons. Specifically, the proportion of initially fluorescent retinal neurons in each group that remained fluorescent

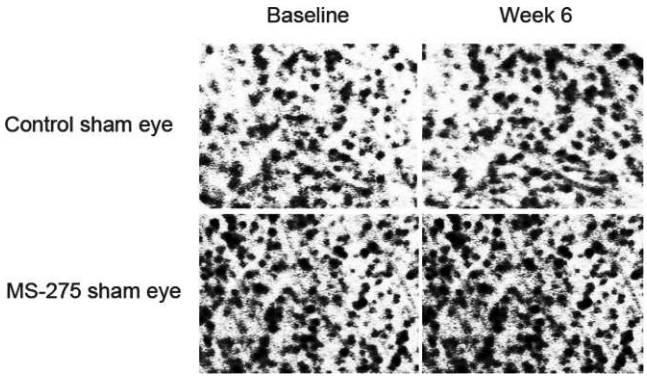

Figure 5. Comparison of fluorescent retinal neurons in sham operated eyes of mice treated with vehicle control or MS-275 and imaged by bCSLO prior to initiating the study (baseline) and at the end of the study (week 6). Note that for both the control vehicle treated mice and MS-275-treated mice, the cells observed at the end of the study were the same as was observed at the beginning of the study.

after optic nerve crush was up to 175% greater in the animals that received MS-275 than in corresponding control animals. The rate of decline in the number of fluorescent retinal neurons was significantly slowed by the MS-275 treatment during the first two weeks after optic nerve crush. Both groups reached a plateau by the third week with counts of the fluorescent retinal neurons remaining over 2-fold greater in the animals that received MS-275 than in the animals that received vehicle. In addition to RGC differentiation, histopathologic analysis of the eyes at the end of the study that showed the number of remaining GCL neurons in the mice that received MS-275 was significantly greater than in the mice that received vehicle. Together, these results indicate that MS-275 protects both RGC differentiation and RGC survival following optic nerve crush.

The current method for analysis of the bCSLO images is modification of the method of Leung et al.[18,19] that scored brightly fluorescent retinal neurons and excluded dimly fluorescent neurons. More than 96% of the brightly fluorescent retinal neurons in Thy1-CFP23Jrs mice were RGCs since they could be labeled by injection of a retrograde tracer into the superior colliculus.[19] A subsequent study by Raymond et al. showed that the dimly fluorescent retinal neurons in this strain included cholinergic amacrine cells.[22] Quantitative analysis showed 80% of all fluorescent neurons were RGCs and 20% were cholinergic amacrine cells. Because the fluorescent retinal neurons that respond to optic nerve crush first become dimmer before CFP expression ceases, it is likely that dimly fluorescent cholinergic amacrine cells are included in the fluorescent retinal neuron counts. As cholinergic amacrine cell death following optic nerve injury is minimal,[23] it is probable the observed decline in retinal neurons expressing CFP primarily reflects down regulation of Thy-1 gene expression in RGCs.

The possibility that the increased numbers of fluorescent retinal neurons in the Thy1CFP23Jrs mice might reflect induction of CFP expression in some of the originally nonfluorescent amacrine cells was investigated and found to be either minimal or nonexistent. Specifically, careful comparison of the images from the eyes that received optic nerve crush (Fig. 2) and in the sham eyes (Fig. 5) showed that MS-275 treatment did not induce fluorescence in cells that were not fluorescent prior to the beginning of the study. Thus, MS-275 preserved fluorescence in cells that were initially fluorescent without inducing initially nonfluorescent cells to become fluorescent.

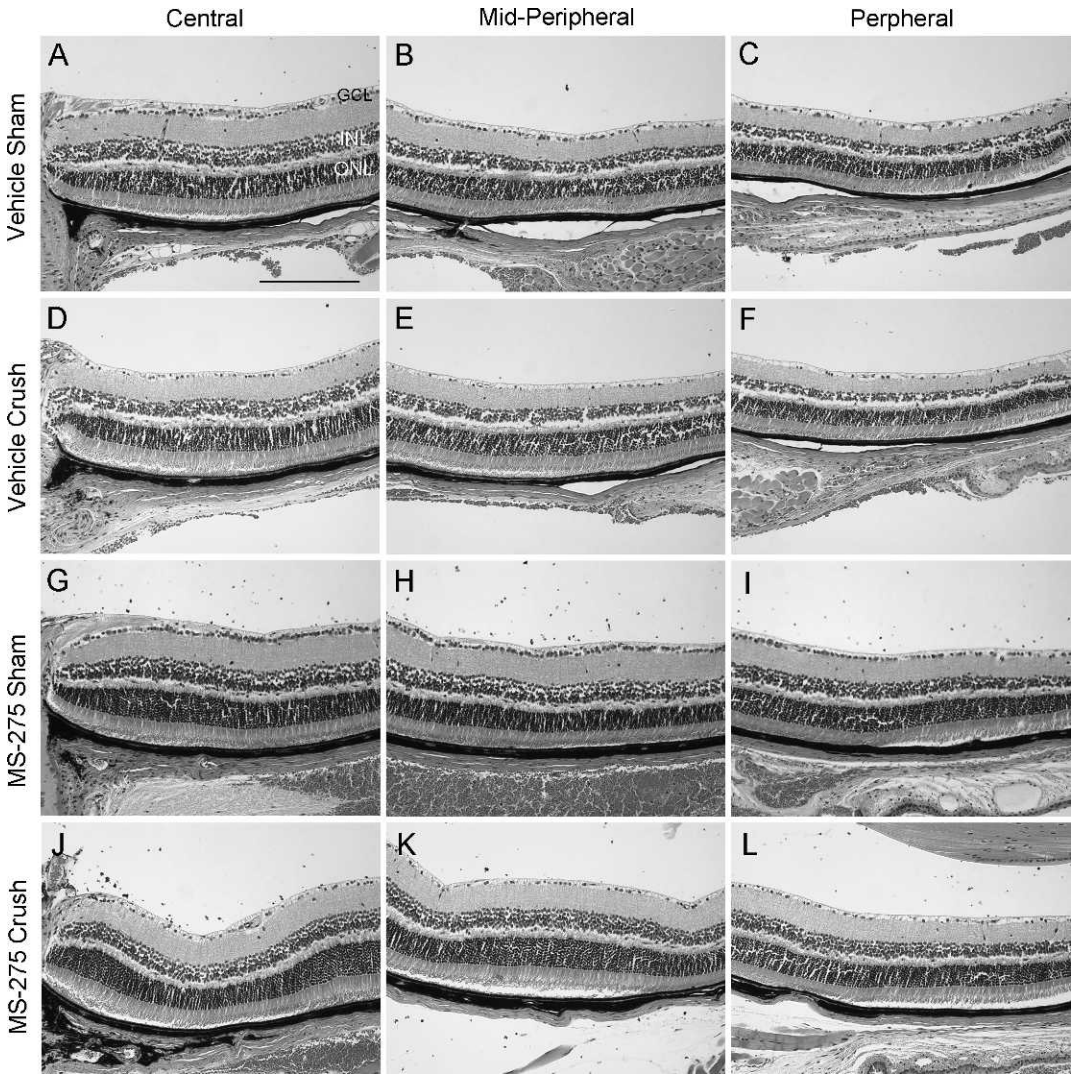

Figure 6. Conventional histological sections of the central retina (*left column*), mid-peripheral retina (*middle column*), and peripheral retina (*right column*), from mice that received vehicle (*top two rows*) or MS-275 (*bottom two rows*) illustrating MS-275 treatment protection of GCL neurons. Among the sham operated eyes, retinas from vehicle-treated animals (**A–C**) or MS-275-treated animals (**G–I**) had normal appearance. Among the eyes that received optic nerve crush, retinas from MS-275-treated animals (**J–L**) had greater numbers of GCL neurons than vehicle-treated animals (**D–F**). Note that the other retinal layers from each of these experimental conditions had normal appearance. Figure labels: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. *Scale bar* = 20 μm.

The absence of any significant change in body weight during the course of the study suggests that MS-275 had minimal impact on the general metabolism of the study mice. Hence, the increased numbers of fluorescent retinal neurons imaged in vivo and the greater numbers of GCL neurons observe in the histopathological analysis in the mice that received MS-275 likely reflects a neuroprotective effect. In view of the potentially wide spread effects of HDAC activity alterations on global gene expression[6]; however, further study will be needed to confirm this point.

Table 1. Enhanced GCL Survival with MS-275 Treatment

Treatment	GCL Density Crush Eyes,* Mean ± SD	GCL Density Sham Eyes, Mean ± SD
Vehicle	44.8 ± 4.8	70.5 ± 8.3
MS-275	56.9 ± 5.9	81.0 ± 4.9
Ratio	1.27	1.15
t-test ($P < 0.05$)	+	−

*GCL neurons/mm, mean ± SD, $N = 4$ eyes/group.

Turning to the experimental results, the number of fluorescent retinal neurons observed in the crush-operated eyes of MS-275-treated mice was substantially greater than the mice that received vehicle. At 2 weeks after crush, when the number of fluorescent retinal neurons in vehicle-treated mice first declined to less than 20% of baseline counts, the remaining fluorescent retinal neurons in the crush-operated eyes of mice that received MS-275 was 40% of baseline counts. Thus, after subtracting the 20% proportion of amacrine cells present at baseline, there remained at least 20% of the original fluorescent retinal neurons that were RGCs in which CFP expression was preserved by MS-275 treatment. At three weeks and later, counts of fluorescent retinal neurons in animals that received MS-275 reached a plateau around 33% during weeks 3 through 5 with a decline to 27% at week 6. These values were more than twice the corresponding values for fluorescent retinal neurons present at each time point in the vehicle treated mice. Thus, MS-275 provided substantial protection

Table 2. Estimate of Increase in RGC Survival with MS-275 Treatment

Treatment	GCL Density Crush Eyes,* Mean ± SD	GCL Density Sham Eyes, Mean ± SD	Estimated Amacrine Cell Density†	Estimated RGC Survival‡	Adjusted RGC Survival§
Vehicle	44.8 ± 4.8	70.5 ± 8.3	41.6	3.2	3.2
MS-275	56.9 ± 5.9	81.0 ± 4.9	47.8	9.1	7.9
Ratio (MS-275/vehicle)	1.27	1.15	1.15	2.84	2.47

*GCL neurons/mm, mean ± SD, $N = 4$ eyes/group.
†GCL density in sham eyes × 59% amacrine cells in GCL,[26] amacrine cells/mm.
‡GCL density in crush eyes – estimated amacrine cell density, RGCs/mm.
§Estimated RGC survival/(MS-275-to-vehicle ratio of GCL density in sham eyes), RGCs/mm.

against loss of Thy-1 promoter activation that persisted to the end of the study.

Within vehicle-treated mice, the loss rate of fluorescent retinal neurons was over 40%/week during the first 2 weeks, less than 5%/week during the second two weeks, and negligible during the third 2 weeks. Treatment with MS-275 significantly slowed the rate of RGC loss during the first two weeks while differences in the rate of loss differed insignificantly between the two groups at later time points. First, this indicates that MS-275 slowed the rate of de-differentiation that occurred after optic nerve injury. Second, and perhaps of greater importance, the stable preservation of 2-fold greater numbers of fluorescent retinal neurons in the animals that received MS-275 suggests there is a subset of RGCs that are stably maintained following optic nerve injury that otherwise would de-differentiate and perhaps later die.

Several considerations allow estimation of the effect of MS-275 treatment on RGC survival in the present study. First, the background strain for the Thy1-CFP23Jrs mice used in this study is the C57BL/6 mouse.[24] A previous study showed that 59 ± 4% of GCL neurons in C57BL/6 mice are displaced amacrine cells.[25] Also, though the number of RGCs, though the number of RGCs can vary widely among individual mice, the RGC difference between left and right eyes within the same mouse is typically less than 3%.[21] Thus, as shown in Table 2, it is reasonable to estimate that the average density of amacrine cells adjacent to the optic nerve at the beginning of the study by calculating 59% of the GCL counts in the sham eyes. This yielded densities of 41.6 amacrine cells/mm in the vehicle group and 47.8 amacrine cells/mm in the eyes of the MS-275 treated mice. Because prior studies indicate that amacrine cell survival is not altered by optic nerve injury,[23,26] the average survival of amacrine cells in the crush eyes of the vehicle and MS-275 mice were likely similar to the average amacrine cell survival in the sham eyes of these experimental groups. Thus, subtracting the estimated amacrine cell density for each group from the observed crush eye GCL densities for each group yields RGC density estimates of 3.2 RGCs/mm in the vehicle-treated group and 9.1 RGCs/mm in the group that received MS-275. The 15% difference in the mean sham eye GCL counts in the vehicle and MS-275 groups, which likely reflects normal variation, can be compensated for as shown in footnote § for Table 2. This adjustment yielded a final estimate of 7.9 RGCs/mm in the eyes of mice that received MS-275. As shown in the last column of Table 2, these calculations indicate RGC survival was 147% greater in the crush eyes of mice that received MS-275 than in the crush eyes of mice that received vehicle. Hence, in addition to protection of RGC differentiation, MS-275 treatment also protected RGC survival.

In conclusion, these results indicate that the HDAC-1/HDAC-3 inhibitor MS-275 protects against the loss of RGC differentiation and promotes RGC survival following optic nerve injury. These results justify further studies to determine whether treatment with MS-275 or other histone deacetylase inhibitors may protect vision in pressure-related experimental models of glaucoma and in patients with optic nerve injuries such as ischemic optic neuropathy, optic nerve compression injuries, or glaucoma.

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