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Research Article

BrdU Positive Cells Induced in a Genetic Mouse Model of Glaucoma

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Abstract

Previous studies have shown that eye drop application of the selective α7 nicotinic acetylcholine receptor agonist, PNU-282987, induces neurogenesis of RGCs in adult wild-type rodents. This study was designed to test the hypothesis that PNU-282987 reverses the loss of RGCs associated with glaucoma. A DBA/2J mouse model that auto-induces a glaucoma-like condition in adulthood was used for these studies. Short-term effects using PNU-282987 and BrdU eye drop treatments were examined, as well as the effects of early treatment and the effects in a chronic early treatment group in DBA/2J mice aged 3, 6 and 10 months. With and without treatment, retinas were removed, fixed, immunostained and RGC counts were assessed. IOP measurements were obtained weekly using a Tonolab tonometer. Results showed an average typical loss of BrdU positive RGCs by 29% by 10 months of age in this DBA/2J colony corresponding with a significant increase in IOP. However, the two-week short term application of PNU-282987 and BrdU induced a significant 21% increase in RGCs for DBA/2J mice at all ages. Chronic early PNU-282987 treatment produced a similarly significant increase in RGCs, while acute early treatment had no effect on RGC numbers. IOP measurements were not affected with PNU-282987 treatment. These studies demonstrated that 2-week treatment with PNU-282987, as well as chronic long-term treatment, induced a significant increase in the number of RGCs in the DBA/2J retina, counteracting the effects of the DBA/2J genetic glaucoma-like condition. These results suggest a potential future treatment of degenerative retinal diseases with PNU-282987.

Keywords: DBA; Glaucoma; Regeneration; PNU-282987

Abbreviations

RGCs: Retinal Ganglion Cells; BrdU: 5-Bromo-2'-Deoxyuridine; IOP: Intraocular Pressure; ACh: Acetylcholine; GCL: Ganglion Cell Layer; alpha7nAChRs: Alpha7 Nicotinic Acetylcholine Receptors; RPE: Retinal Pigment Epithelium; MG: Muller Glia; MDPCs: Muller-Derived Progenitor Cells; mRNA: Message Ribonucleic Acid; RNA seq: Ribonucleic Acid Sequencing; HB-EGF/Ascl1/Lin28a: Genes Involved in Dedifferentiation; qRT-PCR: Real Time Polymerase Chain Reaction; OTX2: Orthodenticle Homeobox 2; VSX2: Visual System Homeobox 2; WMU: Western Michigan University; IACUC: Institutional Animal Care and Use Committee; PBS: Phosphate Buffered Saline; MG: Milligram; ML: Milliliter; C: Centigrade; DAPI: 4′,6-Diamidino-2-Phenylindole; Thy 1.2: Antibody also known as CD90; ANOVA: Analysis of Variance; SE: Standard Error; DBA/2J: Strain of Mouse; SVJ-129: Strain of Mouse.

Introduction

Glaucoma is a degenerative retinal disease characterized by loss of vision due to progressive death of Retinal Ganglion Cells (RGCs) and is the second leading cause of irreversible vision loss worldwide [1]. One of the primary risk factors for glaucoma is increased Intraocular Pressure (IOP), which is associated with death of RGCs. Presently, there is no cure for glaucoma and the loss of RGCs and their axons in the optic nerve is irreversible in humans [2]. All current treatments are aimed at decreasing IOP to prevent RGC death [3]. Unfortunately, these treatments can prevent further progression of the disease but cannot reverse any loss of vision. However, neuroprotection or regeneration of RGCs could potentially reverse the effects of glaucoma. Here, results are presented to provide evidence of BrdU positive RGCs after PNU-282987 eye drop treatment in adult animals using a DBA/2J genetic mouse model of glaucoma.

Recent studies from this lab have shown that neuroprotection or robust regeneration can be induced in the retinas of adult mammals when treated with a specific α7 nicotinic acetylcholine receptor agonist, PNU-282987 depending on the type of treatment [4-8]. In neuroprotective studies, PNU-282987 was shown to provide RGC neuroprotection against induced glaucoma-like conditions when it was intraocularly injected [4-6]. PNU-28987 was found to bind to alpha7 nAChRs on RGCs and mimicked the physiological neuroprotective effect of ACh typically released from starburst amacrine cells [6]. When injected intraocularly, PNU-282987 provided neuroprotection against the significant loss of RGCs that occurred in the GCL after inducing glaucoma-like conditions [6].

Adult mammalian retinal cells do not typically regenerate, but regeneration does occur naturally in many non-mammals, such as zebrafish and chick, in response to injury [9,10]. Recent studies from this lab have demonstrated that if PNU-282987 is applied as eye drops, it induces robust regeneration of new neurons in all layers of the retina without inducing an injury. Specifically, when applied as eye drops, PNU-282987 acts on alpha7nAChRs on the RPE to induce

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release of signaling molecules that cause de-differentiation of Muller Glia (MG) cells in adult mice [7-8]. Specifically, PNU-282987 has the ability to cause MG to generate Muller-Derived Progenitor Cells (MDPCs) and generate multiple types of new differentiated neurons in adult mammalian retinas [7,8]. The neurogenic response of PNU-282987 in the adult murine retina is robust and the division of the MG into progenitor cells follows a similar pattern to that seen in zebrafish regeneration [7,8]. In mRNA sequencing studies, RNAseq was performed on MG following contact with RPE cells treated with PNU-282987. Up- or down-regulated genes were compared with published literature of MG dedifferentiation that occurs in lower vertebrate regeneration or with transcript profiles during early mammalian development. These studies provided evidence that the *HB-EGF/Ascl1/Lin28a* signaling pathway was involved in MG dedifferentiation to retinal progenitor cells [11]. RNA-seq results were verified using qRT-PCR and using immunocytochemistry, the presence of retinal progenitor markers OTX2, Nestin and VSX2 in MG were identified 48 hours post treatment with PNU-282987 treated RPE supernatant [11]. However, it is unknown if PNU-282987 can regenerate or provide neuroprotection to neurons lost to retinal damage due to genetic disease.

In this study, PNU-282987 was analyzed in an *in vivo* DBA/2J mouse model. DBA/2J mice are an inbred strain of mouse characterized by a degenerative glaucoma-like condition that develops around 6-9 months of age [12]. This degenerative disease closely mimics human pigmentary glaucoma and is similarly characterized by a progressive increase in intraocular pressure that leads to retinal ganglion cell loss [2,13]. Thus DBA/2J mice are an ideal model for studying genetic glaucoma in humans. DBA/2J mice are homozygous for mutations in two separate genes. One mutation is found on the b allele of tyrosine related protein (Tyrp1b), which encodes a melanosomal protein [14,15]. The second mutant gene encodes Gpnmb, a transmembrane glycoprotein located within many cellular structures [15,16]. A distinguishing feature of the disease is loss of pigment granules within the iris. These pigment granules accumulate in the trabecular meshwork of the anterior chamber and block the drainage structures, which results in an elevated intraocular pressure [17,18]. The rise in IOP in DBA/2J mice is often reported by 6-9 months of age [19-24] and DBA/2J mice experience axonal loss and optic nerve damage shortly after the onset of elevated IOP [25,26]. Thus far, most studies involving DBA/2J mice have focused on neuroprotection, not regeneration. For example, erythropoietin and neuroglobin have both been shown to have a neuroprotective effect against the onset of the glaucoma-like disease in DBA/2J mice [27,28]. However, in this study, PNU-282987 was applied as eye drops to examine its effect in a genetic model of glaucoma. Specifically, these studies will provide evidence that early treatment with PNU-28297 can prevent or reverse the progressive loss of RGCs associated with the disease in adult DBA/2J mice.

Materials and Methods

Animals

Adult DBA/2J mice (both sexes; aged between 3 and 12 months) were used for these studies and were kept in Western Michigan University's (WMU) animal facility. The mice were obtained from Jackson laboratories. SVJ-129 wildtype mice were used as a control in one experiment. All animals were cared for in accordance with the approved guidelines of the Institutional Animal Care and Use Committee (IACUC) of WMU.

Intraocular Pressure (IOP) measurement

Intraocular pressure measurements were taken using an Icare Tonolab tonometer. Because IOP measurements tend to fluctuate throughout the day, measurements were taken at the same time each evening. IOP measurements were obtained two times each week from awake mice. Briefly, animals were removed from their home cages and held gently. A handheld Tonolab. tonometer was then applied to the cornea of experimental eyes to obtain IOP measurements before and after various eye drop treatments with PNU-282987. On each measurement day, 3 IOP measurements were obtained from each experimental animal and averaged. This was performed for between 5 and 20 animals depending on the designated end time point specified by the experimental protocols.

Experimental design

Experiments were designed to demonstrate that the α7 nAChR agonist 'PNU-282987' produced proliferation in the genetic mouse model. Three different age groups of DBA/2J mice were treated daily for 2 weeks with PNU-282987: 3 months old, 6 months old, and 10 months old. These ages were chosen to get a sampling of the different stages of the disease. 3-month-old animals have reached adulthood but have yet to develop the disease and thus have lost minimal RGCs. At 6 months old, the disease is in its early stages and at 10 months, the disease is fully developed. Five animals from each age group receive 2 weeks of treatment with PNU-282987. Their retinas were then removed and RGC survival was assessed.

Additional experiments were designed to examine the effects of early treatment with PNU-282987. The prevention of the disease with early treatment of PNU-282987 could not be shown in the previous induced surgical model of glaucoma. The use of the DBA/2J genetic model allows a unique opportunity to treat the animal in anticipation of the onset of the disease. In this experiment, DBA/2J mice received treatment before they developed their characteristic glaucoma-like condition, to determine if it would prevent the progression of the disease. There were two treatment conditions: chronic early treatment and acute early treatment. In the chronic early treatment studies, animals started receiving once weekly PNU treatment at an early age that continued until the animals reached 10 months of age. There were two time points within this group: 3 months old and 6 months old. The 3-month-old group began treatment at 3 months of age and the 6-month-old group began treatment at 6 months of age. Five mice at each time point were assessed. Each group continued receiving PNU once per week until they were 10 months old. At 10 months of age, their retinas were removed and RGC survival was assessed.

The acute early treatment animals received two weeks of PNU treatment before the onset of the disease. Following that two weeks, the animals received no further treatment and were left alone until they were 10 months old; at which point their retinas were removed and RGC survival was assessed. 3 and 6-month old mice were used for this study. Five animals at each time point received 2 weeks of PNU treatment at 3 or 6 months old and then left, with no further

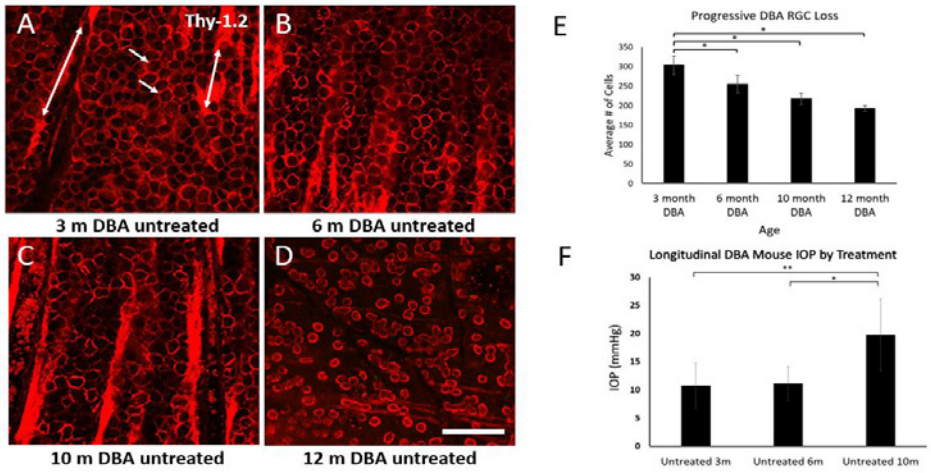

Figure 1: Progression of Retinal Ganglion Cell (RGC) loss in DBA/2J mice over time. 3, 6, 10 and 12-month-old DBA/2J mice were analyzed to quantify the increase in IOP and retinal deterioration characteristic of the DBA/2J strain.
(A): 3-month-old DBA/2J mouse retina stained with antibodies against Thy-1.2, a marker for RGCs. Arrows indicate RGCs and double-sided arrows indicate axon bundles.
(B-D): 6, 10, and 12-month-old DBA/2J mouse retinas respectively show significant degeneration of the RGC layer. **(E):** Quantification of RGCs at each time point. (F) IOP measurements in untreated 3, 6 and 10-month-old DBA/2J mice. *represents a significant difference from 3-month-old DBA/2J controls ($p<0.01$). N=5 for each condition; scale bar = 50 μm.

treatment, until they were 10 months old.

Eye drop treatment and retina preparation

Both eyes of each experimental animal were treated once daily with PBS eye drops containing 1 mg/mL BrdU and 1 mM PNU-282987. The concentration of PNU-282987 and BrdU used in eye drop experiments in rodents was previously established in dose response studies and the dosage that produced maximal effects was used [7-8,29]. Animals in the short-term treatment groups received this treatment once daily for 2 weeks while the long-term treatment animals received treatment once weekly for 3-6 months. Control animals received drops containing only PBS and 1 mg/mL BrdU. Details of the eye drop treatment are described in Linn et al. [29]. At specific times following the start of treatment, mice were euthanized. The eyes were subsequently removed, and retinas were excised, flat-mounted, and fixed in 4% paraformaldehyde overnight at 4°C [4-5].

Immunohistochemistry

Following fixation, retinas were labeled with primary antibodies including sheep anti-BrdU (7.5 µL/mL, Abcam ab1894, Cambridge, UK) and rat anti-Thy1.2 (1:200, Abcam ab 218775). Thy-1.2 is a cell surface protein used as a marker for RGCs in mice. BrdU is used as a marker for cell cycle reentry. For BrdU staining, antigen retrieval was done as in Webster et al. [8] Retinas were blocked in PBS containing 1% Triton X-100 and 1% bovine serum. Retinas were incubated in primary antibodies overnight at room temperature in PBS containing 1% bovine serum and 1% goat serum, rinsed in PBS and incubated overnight with appropriate Alexa Fluor conjugated secondary antibodies (1:300, Life Technologies) diluted in PBS without serum. Nuclei were stained with DAPI.

Cell counting and normalization

Stained retinas were then flat mounted on slides and visualized using a Nikon C2$^+$ scanning laser confocal microscope. Four images of each flat-mounted retina were taken from each of four quadrants of the retina. Each image was taken 4 mm from the optic nerve head [4-5]. To quantify the flat mounts, a fixed 200×200 μm^2 grid was applied to all images and the Thy1.2-positive RGCs within the grid for each retinal quadrant were counted. The RGC counts from the four quadrants were averaged together according to the procedure outlined in Mata et al. [5] Webster et al. [7] and Cooley-Themm et al. [6] This represented an "N" of 1 for flat-mounted retinas and "N"s of 5 were obtained for each experimental condition.

Statistical analysis

Statistical analysis was performed on all normalized data using an analysis of variance (ANOVA) as well as Tukey post-hoc analysis. $P<0.01$ was considered statistically different.

Results and Discussion

Progression of glaucoma-like RGC loss in DBA/2J mice

Experiments were designed to quantify the loss of RGCs in DBA/2J mice. (Figure 1) shows confocal microscope images obtained from untreated DBA/2J retinas of different ages. The retinas were fixed overnight in 4% paraformaldehyde and flat mounted with the retinal ganglion cell layer facing up. After thorough rinsing, the retinas were immunostained using antibodies against Thy-1.2, a marker for RGCs. RGCs were counted and averaged from 5 different animals under different age groups. (Figure 1A) illustrates an image of a DBA/2J retina from a 3-month-old mouse (Figure 1B) is from a 6-month-old DBA/2J mouse, (Figure 1C) is from a 10-month-old DBA/2J mouse, and (Figure 1D) is from a 12-month-old DBA/2J mouse. There was significant loss of RGCs in the 6, 10, and 12-month-old animals compared to the 3-month-old animals. (Figure 1E) quantifies the average number of cells lost in the different age groups. By 6 months of age, there was an average loss of 47 (SE ± 6.6) RGCs compared to 3-month-old mice, representing a 16% loss. By 10 months, there was an average loss of 88 (SE ± 5.9) RGCs (a 29% loss) and by 12 months, there was an average loss of 114 (SE ± 6.0) RGCs (a 38% loss).

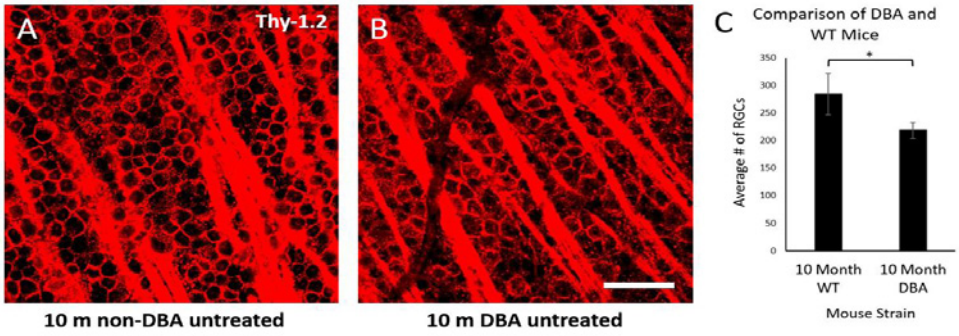

Figure 2: DBA-related RGC loss *vs.* age-related RGC loss. Comparison of RGCs in a 10-month-old DBA/2J mouse and a 10-month-old non-DBA mouse to rule out age-related degeneration as the cause of the DBA/2J RGC loss.
(A): Untreated 10-month-old non-DBA retina stained with antibodies against Thy-1.2.
(B): Untreated 10-month-old DBA retina.
(C): Quantification of RGCs between the 10-month-old DBA and 10-month-old non-DBA groups. *represents a significant difference from 10-month-old wild type ($p<0.01$). N=5 for each condition; scale bar=50 μm.

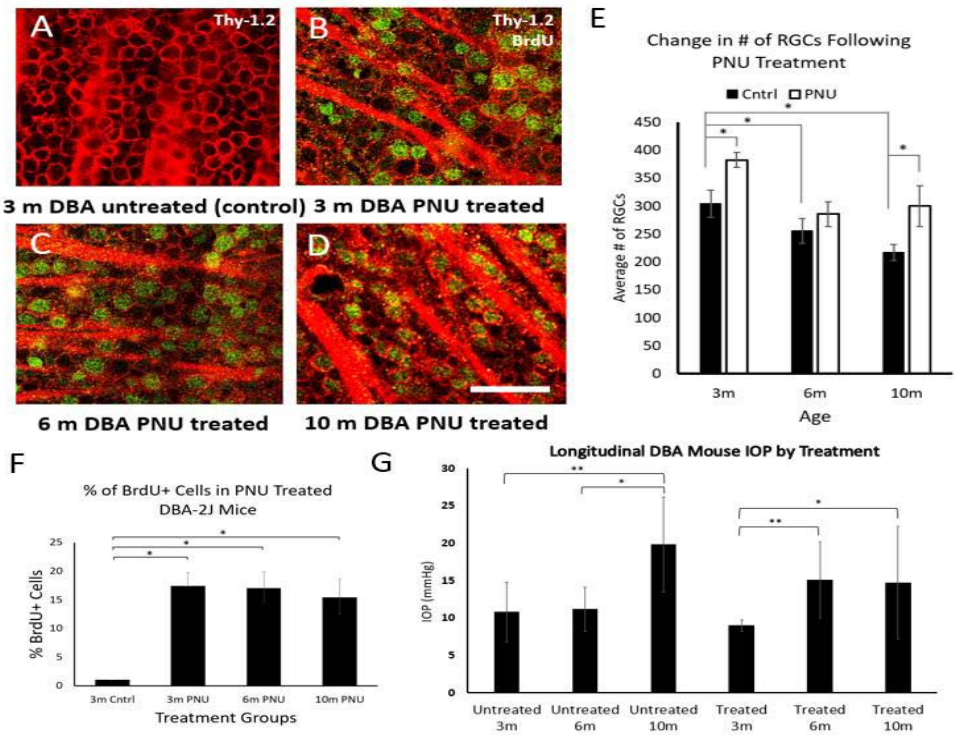

Figure 3: Two-week daily treatment of DBA/2J mice with PNU-282987. 3, 6, and 10-month-old DBA/2J mice were treated once daily with PNU-282987 for two weeks.
(A): Untreated 3-month-old DBA/2J control retina stained with antibodies against Thy-1.2.
(B-D): 3, 6 and 10-month-old DBA/2J retinas treated with PNU-282987 for 2 weeks and stained with antibodies against Thy-1.2 and BrdU. BrdU+ cells are shown in green.
(E): A quantification of RGCs in treated animals compared to control untreated animals.
(F): Quantification of BrdU$^+$ RGCs in treated 3, 6 and 10-month-old DBA/2J mice compared to a 3-month-old DBA/2J control. (G) IOP measurements in treated and untreated 3, 6 and 10-month-old DBA/2J mice. *represents a significant difference ($p<0.01$). N=5 for each condition; scale bar=50 μm.

In this model of glaucoma, loss of RGCs was preceded by a spike in IOP within the anterior chamber of the eye. Typical intraocular pressure measured from adult mice range from 10-20 mmHg [30]. We observed an average intraocular pressure of 10.69 (SE ± 4.0) mmHg at 3 months of age, 11.16 (SE ± 3.0) mmHg at 6 months, and 19.79 (SE ± 6.4) mmHg at 10 months. The increase in IOP observed in the 10-month-old animals represented a significant change compared to the IOPs from the 3 and 6-month-old animals.

DBA/2J retinal ganglion cells loss *vs.* age-related retinal ganglion cell loss

To rule out the possibility that the loss of RGCs was merely the result of natural age-related degeneration, the average number of

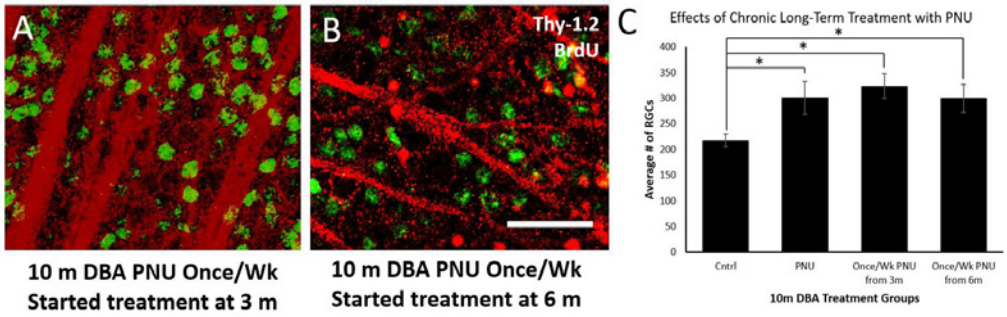

Figure 4: Chronic early treatment of DBA/2J mice with PNU-282987. 3 and 6 month old DBA/2J mice were treated with PNU once per week on a long-term basis to determine if early chronic treatment can mitigate the onset of the disease.
(A): 10-month-old DBA/2J retina treated with PNU-282987 once per week starting at 3 months of age until the animal was 10 months of age. Stained with antibodies against Thy-1.2 and BrdU.
(B): 10-month-old DBA/2J retina treated with PNU-282987 once per week starting at 6 months of age until the animal was 10 months of age. Stained with antibodies against Thy-1.2 and BrdU.
(C): Quantification of RGCs in the chronic early treatment groups compared to 10-month-old DBA/2J controls and 10-month-old DBA/2J mice who received only 2 weeks of PNU treatment. *represents a significant difference from 3-month-old controls ($p<0.01$). N=5 for each condition; scale bar=50 μm.

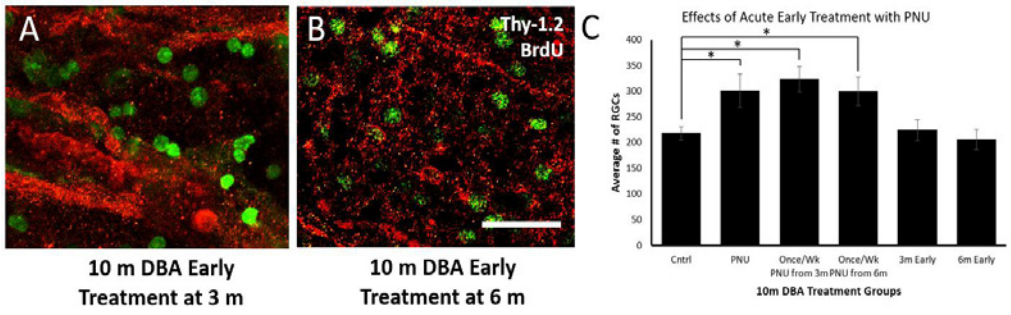

Figure 5: Acute early treatment of DBA/2J mice with PNU-282987. 3 and 6-month-old DBA/2J mice were treated early with PNU-282987 for two-weeks, then left without further treatment until 10 months of age, to determine if early acute treatment can mitigate the onset of the disease.
(A): 10-month-old DBA/2J retina treated with PNU-282987 for 2 weeks at 3 months of age. Stained with antibodies against Thy-1.2 and BrdU.
(B): 10-month-old DBA/2J retina treated with PNU-282987 for 2 weeks at 6 months of age. Stained with antibodies against Thy-1.2 and BrdU.
(C): Quantification of RGCs in 10-month-old DBA/2J animals across all treatment groups. *represents a significant difference from 3-month-old controls ($p<0.01$). N=5 for each condition; scale bar = 50 μm.

RGCs in 10-month-old DBA/2J retinas was compared to 10-month-old non-DBA retinas. The results shown in (Figure 2) indicate a significantly higher loss of cells in the 10-month DBA group compared to the 10-month non-DBA group. (Figure 2A) shows a wild type SVJ-129 retina stained with antibodies against Thy-1.2. (Figure 2B) shows an example of a 10-month-old untreated DBA retina stained with antibodies against Thy-1.2. (Figure 2C) represents a quantification of average RGC counts for DBA and non-DBA retinal images. The 10-month non-DBA mice had an average of 218 (SE ± 5.7) RGCs in the counted retinal images which was a significant difference from the 10-month DBA mice, which had an average of 291 (SE ± 13.1) RGCs, representing a significant difference of 25% between the two groups.

Treatment of DBA/2J mice with PNU-282987

To examine the effect of PNU-282987 on loss of RGCs in DBA/2J mice, 3, 6, and 10-month-old DBA/2J mice were treated with eyedrops containing 1 mM PNU-282987 and 1 mL/mg BrdU once per day for two weeks. Following treatment, the number of RGCs was assessed. (Figure 3A) shows a 3-month-old DBA/2J untreated control retina while (Figure 3B-3D) show PNU-treated DBA/2J retinas at 3, 6, and 10 months old. The retinas were stained with anti-Thy1.2, a marker for RGCs, and anti-BrdU, a marker for cell cycle reentry. The 3-month-old PNU treated DBA/2J mice showed a significant increase in the number of RGCs compared to the 3-month-old untreated DBA/2J controls. An average increase of 78 (SE ± 5.9) RGCs was measured in each confocal image, representing an increase of 26%. The 10-month-old DBA/2J mice also showed a significant increase between the treated and untreated groups. There was an average increase of 83 (SE ± 6.7) RGCs in each image, which represents an average increase of 27%. No significant difference was found between the number of RGCs counted in images obtained from treated and untreated 6-month-old DBA/2J mice. (Figure 3E) shows a quantification of the number of RGCs in the different age groups and between the control and treatment groups. (Figure 3F) illustrates quantification of the number of $BrdU^+$ cells in each treatment groups. No significant difference was found when comparing the number of BrdU+ cells between the different age groups that received the PNU-282987 eye drops, but a significant difference was apparent in retinas that did not receive PNU-282987 eyedrops. The 3-month treated DBA/2J mice showed an average of 17.4% (SE ± 2.4) $BrdU^+$ cells compared to an absence of $BrdU^+$ cells in the control. Similarly, the 6-month

and 10-month treated DBA/2J retinas showed an average of 17.0% (SE ± 2.8) and 15.4% (SE ± 3.1) $BrdU^+$ cells respectively compared to control conditions. (Figure 1G) shows intraocular pressure (IOP) in 3, 6 and 10-month-old untreated DBA/2J mice compared to 3, 6 and 10-month-old PNU treated DBA/2J mice. IOP increased from 8.96 (SE ± 0.8) mmHg at 3 months to 15.05 (SE ± 5.2) mmHg at 6 months in PNU-282987 treated animals, but values were statistically similar to those values obtained from untreated animals. PNU-282987 eye drop application had no significant effect on the normal change of IOP in these DBA/2J mice.

Effects of chronic early PNU-282987 treatment on DBA/2J RGC density

To observe the effects of repeated long-term early treatment with PNU-282987 on the onset of the glaucoma-like condition, DBA/2J mice received weekly eye drop treatments with PNU-282987 and BrdU before the full onset of the disease. One group received eyedrop treatments once per week when they were 3 months of age and continued to receive treatment until they reached 10 months of age. A second group received once-per-week treatments at 6 months of age and continued receiving treatment until 10 months of age. (Figure 5A) illustrates a retina that began treatments at 3 months of age and (Figure 5B) illustrates a retina that began treatments at 6 months of age. (Figure 5C) represents a quantification of the number of RGCs in the two groups compared to the control group and the 10-month-old 2-week PNU treatment group. We observed a significant increase in the number of RGCs in both repeated early treatment groups compared to the control. The 3 month once-per-week treatment group showed an average of 323 (SE ± 24.6) RGCs per retina compared to an average of 217 (SE ± 12.7) RGCs for 10-month-old control DBA/2J mice, representing an average decrease of 32.8%. The 6 month once-per-week treatment group showed an average of 300 (SE ± 27.5) RGCs per retina compared to an average of 217 (SE ± 12.7) RGCs for 10-month-old control DBA/2J mice, representing an average increase of 27.6%.

However, repeated long-term treatment groups elicited the same results as 10-month-old mice that only received a 2-week daily PNU treatment. The two long-term treatment groups averaged 311 RGCs, while the 10-month-old animals that received the 2-week treatment averaged 301 RGCs per retina, representing an insignificant difference of 3.1%.

Effects of acute early PNU-282987 treatment on DBA/2J RGC density

The next experiment was designed to observe the effects of acute early treatment with PNU-282987 on the onset of the glaucoma-like condition and differs from the previous experiment in the length and frequency of treatment. The previous experiment involved once-per-week treatment over the course of months, starting before the onset of the disease. This new experiment involved two weeks of daily PNU-282987/BrdU treatment at 3 months of age, before the disease develops. One group received daily eyedrop treatment with PNU-282987 and BrdU for two weeks at 3 months of age, then received no further treatment. The animals were left alone until they reached 10 months of age, when they were sacrificed and RGC survival was assessed. Another group received daily eyedrop treatment with PNU-282987 for two weeks at 6 months of age, then received no further treatment until they were euthanized at 10 months. (Figure 6) shows the effects of this acute long-term treatment with PNU-282987. (Figure 6A) shows a retina that received acute early treatment at 3 months of age and (Figure 6B) shows a retina that received acute early treatment at 6 months of age. (Figure 6C) shows the quantification of the number of RGCs compared between all control and treatment groups. Neither acute early treatment groups showed a significant difference from the untreated control retinas. The 3-month acute PNU-282987 treatment group showed an average of 224 (SE ± 20.4) RGCs and the 6-month acute PNU-282987 treatment group showed an average of 206 (SE ± 19.0) RGCs compared to the 10-month DBA/2J control, which showed an average of 218 (SE ± 12.7) RGCs.

Discussion

Based on numerous DBA/2J studies, variability in the age of onset of the glaucoma-like conditions between different colonies of DBA/2J mice has been cited [22,23,25,31]. The literature describes the onset of the disease between 6 and 10 months of age [2,25,26,32]. Thus, it was necessary to establish the rate of progression of the disease in the DBA/2J colony used in this study. DBA/2J mice of various ages were chosen and the number of RGCs were counted to determine the best timepoints to use for experimental treatments. Intraocular pressure was also measured as an additional metric for determining which time points would be best suited. Some animals in the colony did not show an increase in IOP. Therefore, only those animals that displayed an increase in IOP were used in these experiments. 10-month-old DBA/2J mice showed the most consistent increase in IOP and loss of RGCs. Therefore, the timepoints chosen for further experiments were 3, 6 and 10 months old. At 3 months, the disease has not started to develop phenotypically and shows no loss of RGCs. At 6 months, the disease has started to phenotypically develop and starts to show some loss of RGCs. And at 10 months, the disease has fully progressed, and there was a substantial loss of RGCs. This rate of RGC loss is consistent with previous studies [2,12,13]. At 12 months, the structure of the retina was almost totally degraded, and there were changes in cell morphology. Because of the severity of the damage and morphological changes, 12-month-old animals were excluded from the treatment group in these experiments. The progressive loss of RGCs across 3, 6, 10 and 12-month-old DBA/2J mice and changes in IOP measurements was demonstrated in (Figure 1).

To determine that the observed RGC loss in DBA/2J mice was the result of the progressive disease, and not just natural loss as a result of age, 10-month-old DBA/2J retinas were compared to control retinas of the same age. (Figure 2) shows the flat-mounted retina of a 10-month-old DBA/2J (Figure 2A) compared to the flat-mounted retina of a 10-month-old wild-type mouse (Figure 2B). The number of RGCs in the 10-month DBA/2J mice was significantly lower than in the comparable wild-type mice (Figure 2C), suggesting that age alone was not the driving force behind RGC loss.

Other experimental results demonstrated that PNU-282987 leads to an increase in BrdU positive RGCs in the DBA/2J genetic mouse line. Previous studies from this lab have shown the neurogenic effects of PNU-282987 in rats and wild-type mice, [7,8] but never in the DBA/2J genetic model. Other DBA/2J studies have typically focused on neuroprotection to prevent the loss of RGCs using various compounds [20,27,28,33]. However, none of these previous studies

examined the potential of regeneration, which has been shown to be triggered in these adult rodents when PNU-282987 is delivered as eye drops by acting on the RPE to release signaling molecules that causes de-differentiation of Müller glia [7,8]. (Figure 3) shows DBA/2J retinas treated with PNU-282987 and BrdU. To quantify the effect of PNU-282987, the number of total RGCs were assessed as well as the number of $BrdU^+$ RGCs. BrdU is used as an indicator of cells that have undergone the S-phase of the cell cycle. Therefore, $BrdU^+$ cells, which are shown in green, indicate newly regenerated cells in retinas that were treated with eye drops of PNU-282987 and BrdU. Previous studies have demonstrated that new RGCs induced by PNU-282987 originate from de-differentiation of Muller glia cells [7,8]. The results from this study demonstrate a significant proliferation of RGCs in all time points compared to untreated DBA/2J controls. This indicates that PNU-282987 produces consistent proliferation of RGCs across all three time points as well as a consistent percentage of $BrdU^+$ cells.

This study also assessed whether PNU-282987 had any effect on intraocular pressure when administered to DBA/2J mice. As shown in the studies summarized in (Figure 3), the change in IOP associated with different aged DBA/2J mice did not change due to PNU-282987 application. Therefore, the increase in the number of RGCs due to PNU-282987 is likely the result of regeneration rather than a prevention of loss by a decrease in IOP.

After establishing the efficacy of PNU-282987, experiments were performed to determine whether early chronic treatment could prevent the onset of the disease. In one experiment, administration of PNU-282987 began before the onset of the disease and continued on a weekly basis until the animal reached 10 months of age. As shown in (Figure 4), chronic treatment with PNU-282987 in both the 3-month and 6-month animals resulted in a significant increase of BrdU-positive RGCs. This indicates that chronic weekly treatment with PNU-282987 can significantly increase the number of RGCs in DBA/2J mice to replace the RGCs that are typically lost due to the disease. However, acute long-term treatment with PNU-282987 failed to indicate a significant increase in the number of RGCs from untreated control. A one-time, two-week acute treatment of PNU-282987 applied at 3 months or 6 months of age did not significantly affect the onset of the glaucoma-like condition in DBA/2J mice and thus, this procedure would not be a likely therapeutic approach to replace lost RGCs in future studies.

Conclusion

This study provided evidence that long-term eye drop treatment with PNU-282987 can offset the effects of the RGC loss observed in the characteristic glaucoma-like condition of DBA/2J mice. However, experiments with acute early treatment indicate that repeated administration of PNU-282987 is necessary for long-term prevention of the disease. Although neuroprotection versus regeneration was not directly addressed in this paper, previous publications analyzing this issue support the hypothesis that BrdU positive retinal cells induced by eye drop application of PNU-282987 were derived from de-differentiated MG [7,8]. Therefore, PNU-282987 may have viable therapeutic potential as a treatment for neurodegenerative diseases such as glaucoma when delivered on a long-term basis. Functional studies are also underway to verify recovery of function following treatment with PNU-282987.

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