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Evidence of BrdU Positive Retinal Neurons after Application of an Alpha7 Nicotinic Acetylcholine Receptor Agonist

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Abstract

Irreversible vision loss due to disease or age is responsible for a reduced quality of life. The experiments in this study test the hypothesis that the $\alpha 7$ nicotinic acetylcholine receptor agonist, PNU-282987, leads to the generation of retinal neurons in an adult mammalian retina in the absence of retinal injury or exogenous growth factors. Using antibodies against BrdU, retinal ganglion cells, progenitor cells and Müller glia, the results of this study demonstrate that multiple types of retinal cells and neurons are generated after eye drop application of PNU-282987 in adult Long Evans rats in a dose-dependent manner. The results of this study provide evidence that progenitor cells, derived from Müller glia after treatment with PNU-282987, differentiate and migrate to the photoreceptor and retinal ganglion cell layers. If retinas were treated with the $\alpha 7$ nAChR antagonist, methyllycaconitine, before agonist treatment, BrdU positive cells were significantly reduced. As adult mammalian neurons do not typically regenerate or proliferate, these results have implications for reversing vision loss due to neurodegenerative disease or the aging process to improve the quality of life for millions of patients.

Keywords

$\alpha 7$ nAChRs; Müller glia; retinal ganglion cells; BrdU; retina; Long Evans rats

Introduction

Irreversible vision loss is one of the top ten disabilities worldwide. It is responsible for a reduced quality of life and a substantial burden on national healthcare systems (Saaddine et al., 2003). Glaucoma is a neurodegenerative disease of the retina that can lead to irreversible blindness due to loss of retinal ganglion cells (RGCs) (Rossetti et al., 2015; Foster et al., 1992; Guo et al., 2005). Although the cause of glaucoma is unknown, the primary risk factor associated with the disease is an increase of intraocular pressure (IOP) (Chauhan et al., 2002; Levkovitch-Verbin et al., 2002; Damji et al., 2003). All current treatments are focused

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on reducing IOP (Bucolo et al., 2015). However, these treatments alone are often insufficient to halt the progression of blindness associated with glaucoma, as RGCs can continue to die even after successful IOP reduction (Beidoe and Mousa, 2012). Regeneration or proliferation of RGCs in adult mammalian systems could potentially reverse vision loss caused by glaucoma.

Previous studies from this lab have demonstrated that intravitreal injections or eye drop application of the $\alpha 7$ nAChR agonist, PNU-282987, in an *in vivo* rat model prevented the loss of RGCs normally associated with a procedure to induce glaucoma-like conditions (Iwamoto et al., 2014; Mata et al., 2015). However, these same studies also demonstrated that application of the $\alpha 7$ nAChR agonist significantly increased the number of RGCs in the adult retina compared to internal controls, suggesting proliferation of new RGCs. As adult mammalian neurons are not known to possess regenerative capabilities, the objective of this study was to characterize the neurogenic effect of the $\alpha 7$ nAChR agonist, PNU-282987, on adult mammalian retinal neurons.

Binding studies in rat chimera cells using PNU-282987 have demonstrated that PNU-282987 is a potent and specific agonist for $\alpha 7$ nAChRs (Bodnar et al., 2005; Walker et al., 2006). These studies demonstrated that methyllycaconitine (MLA), a specific $\alpha 7$ nAChR antagonist, competitively bound to $\alpha 7$ nAChRs when both PNU-282987 and MLA were present. In electrophysiology studies using rat hippocampal neurons, PNU-282987 evoked a rapidly desensitizing inward whole-cell current associated with the opening of the $\alpha 7$ nAChR channel. This current was eliminated if MLA was introduced before PNU-282987 (Bodnar et al., 2005). Previous pharmacological studies from this lab have blocked $\alpha 7$ nAChRs with MLA in the retina of an *in vivo* rat glaucoma model to prevent PNU-282987's effect on RGC loss (Mata et al., 2015; Birkholz et al., 2016). Additionally, immunocytochemical studies using antibodies specific to different AChR subunits were performed *in vitro* to verify that PNU-282987's effects on mature mammalian RGCs (porcine) were mediated through $\alpha 7$ nAChRs and to rule out activity on other nAChRs or serotonin receptors (Thompson et al., 2006).

In this study, the neurogenic effect of PNU-282987 on uninjured adult rat retinas was analyzed using 5-bromo-2'-deoxyuridine (BrdU). BrdU labels both mitotically active cells and cells undergoing unscheduled DNA synthesis (e.g. DNA repair). Previous studies have demonstrated the ability to regenerate rod, bipolar and amacrine cells to repair injured retinal tissue in mammals to a limited degree (Li and Zhou, 2015). However, here we demonstrate the neurogenic effect of an $\alpha 7$ nAChR agonist without the necessity of prior damage or insult to the retina. Results are also presented to support the hypothesis that new retinal cells originate from Müller glia. These are the first studies to investigate generation of new RGCs in adult mammalian retinas using an $\alpha 7$ nAChR agonist.

Experimental Procedures

Animals

Adult Long Evans rats (males and females 3 months of age), purchased from Charles River and bred on site, were used for all studies. A total of 145 mature rats were used in these

studies. The number of rats used for each experiment is listed in the figure legends. Rats were kept at Western Michigan University's (WMU) animal facility and were cared for in accordance with the approved guidelines of the Institutional Animal Care and Use Committee (IACUC) at WMU. IACUC at WMU specifically approved this study and formal approval can be provided upon request. Studies were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

Eye Drop Treatments and Retina Preparation

The right eye of each animal was treated twice daily with 30 μ L PBS containing 1 mg/mL bromodeoxyuridine (BrdU) and various concentrations of PNU-282987. The left eye acted as an internal control and was either untreated or was treated twice daily with 30 μ L PBS containing only 1 mg/mL BrdU. All animals received this treatment for a maximum of 2 weeks and were sacrificed at specific times after the start of treatment by CO₂ asphyxiation (Iwamoto et al., 2014; Mata et al., 2015). In previous control experiments, eye drop treatments were randomized between right and left eyes (Mata et al., 2015). The percent change of new RGCs due to PNU-282987 did not differ whether the right or left eye was treated (Mata et al., 2015). After eye drop treatment, eyeballs were removed, retinas were excised, flat-mounted and fixed in 4% paraformaldehyde overnight at 4°C (Iwamoto et al., 2014; Mata et al., 2015).

Immunocytochemical Analysis

Following fixation, retinas were labeled with specific primary antibodies including; sheep anti-BrdU (7.5 μ L/mL, Abcam ab1894); mouse anti-Thy1.1 (3.4 μ L/mL, BD Pharmingen 554892); rabbit anti-vimentin (7.5 μ L/mL, Abcam ab92547); rabbit anti-nestin (10 μ L/mL Abcam ab6142); rabbit anti-caspase-3 (3.4 μ L/mL Abcam ab44976); rabbit anti-PCNA (7.5 μ L/mL Abcam ab18197) and/or rabbit anti-Sox9 (3.4 μ L/mL bs4077R). Retinas were incubated in primary antibodies overnight at room temperature in 1% bovine serum and 1% goat serum in PBS. Retinas were then rinsed and treated with appropriate secondary Alexa Fluor fluorescent antibodies (1:300, Life Technologies) overnight in PBS (Table 1).

Cell Counting and Normalization

Cells labelled with various antibodies were counted. Stained retinas were flat-mounted or sectioned and visualized using a Nikon C2+ scanning laser confocal microscope. For flat-mounted retinas, four separate 200 μ m² images of the ganglion cell layer, GCL, were captured 4 mm from the optic nerve head (Iwamoto et al., 2014; Mata et al., 2015). The number of RGCs in each image were counted, averaged and normalized to the internal control retina from each animal. This represented an "N" of 1 for flat-mounted retinas. Images from the same retinal locations were analyzed in treated and untreated tissues (Iwamoto et al., 2014; Mata et al., 2015). Normalization of the data occurred by dividing the average number of RGCs obtained from treated retinas by the average number of RGCs from internal control retinas and producing a percentage.

When retinal sections were analyzed, sections were stained with anti-BrdU and counterstained with DAPI to label cell nuclei. Cells stained with anti-BrdU were compared

to the number of cells stained only for DAPI in each image. BrdU positive counts were averaged and normalized from four 200 μm^2 retinal images obtained from each retinal section. This represented an “N” of 1 for retinal sections. Normalization of retinal sections occurred by dividing the average number of BrdU positive cells in a layer of the retina by the average total number of unstained cells in that layer and producing a percentage.

Antibody specificity

In control studies, experiments were conducted to determine the specificity of the antibodies used. All antibodies used in this study originated from rabbit, mouse, goat or sheep. Antibody processing was performed on untreated internal control retinas as well as in experimentally treated retinas from the same animal to determine if positive labelling occurred in untreated controls. In some experiments (N=3), retinas were processed with the primary antibody omitted. In other experiments, preabsorption controls were performed with Thy 1.1 (N=3). No significant epifluorescence was observed under any of these conditions.

Statistical Analysis

For analysis of figure 1 data, a Student's paired T-test was used for single comparisons that used the contralateral eye as a control. For experiments requiring multiple comparisons, one way ANOVA with post hoc analysis using the Holm-Bonferroni correction for multiple comparisons was performed (Holm, 1979). All significant differences represent $P \leq 0.001$. Normal distributions and equal variances were tested for and found in samples distributions. Appendix A lists the statistic parameters used in this study.

Results

Increase of adult retinal ganglion cells

Initial experiments were designed to quantify the increase of RGCs in PNU-282987 treated retinas in adult Long Evans rats. Figure 1 A is a confocal image obtained from an untreated left retina 4 mm from the optic nerve head (ONH) in a flat-mounted preparation stained with anti-Thy1.1 to label RGCs. Figure 1B is the right eye of the same animal that was treated with eye drops containing 1 mM PNU-282987 twice a day. One month after initiating treatment, the animal was sacrificed and the retina was immunocytochemically processed using an antibody against Thy 1.1 to label RGC bodies and RGC axon fascicles. Compared to the untreated internal control retina, an increase in the number of RGCs was apparent. Figure 1C summarizes the average percent change of RGCs after PNU-282987 treatment compared to untreated controls. The left bar indicates baseline in the control untreated eyes and the right bar show the percent change in RGCs relative to the contralateral control. PNU-282987 eye drop treatment caused a significant increase in the number of RGCs compared to internal control retinas by an average of 23% (± 5.2 ; N=8, Degrees of freedom for paired T- test =7, T-stat = -18.0301, $P \leq 0.001$).

Evidence of BrdU Positive Retinal Cells

To determine if the increase in Thy1.1-positive RGCs resulted from mitotically active cells, BrdU was used to label cells in S-phase. Retinas were assayed for BrdU incorporation following unilateral treatment with PBS containing 1 mM PNU-282987 and 1 mg/ml BrdU

for two weeks compared to the contralateral eye treated with BrdU alone. In flat-mounted retinas, anti Thy1.1 antibodies stained the plasma membrane of RGCs (arrows) and their axon fascicles (double-headed arrows, Fig. 2A, C, D, F). In control eyes, no BrdU-positive cells were detected in the GCL (Fig. 2B, C). In contrast, PNU-282987 treated eyes contained numerous BrdU-positive cells that were also Thy1.1-positive (arrowheads in Fig. 2E, F), identifying them as RGCs. The incorporation of BrdU into RGCs is consistent with PNU-282987 induced proliferation in the mature retina. Similar results were obtained from 7 additional PNU-282987/BrdU treated flat-mounted retinas.

Dose dependent effect of PNU-282987

To determine if PNU-282987's neurogenic effect was dose dependent, concentrations of PNU-282987 between 0 μ M and 2 mM were applied as eye drops with BrdU for two weeks. One month after eye drop treatment, BrdU positive cells in the RGC layer from flat-mounted retinas were quantified and summarized in the bar graph shown in figure 3. PNU-282987 elicited a dose-dependent effect on the percent of BrdU positive cells in the RGC layer. The maximal neurogenic effect was seen using 1 mM PNU-282987, but BrdU positive cells were found when concentrations as low as 10 μ M were used. No BrdU positive cells were identified in the retina if concentrations less than 10 μ M PNU-282987 were used. For dose-response analysis, one way ANOVAs were performed with post-hoc Holm-Bonferroni correction for multiple comparisons. N's between 4 and 8 were used for each dosage of PNU-282987 used in these studies. See appendix B.

In addition, relatively few BrdU positive cells were identified if eyes were treated with the α 7 nAChR antagonist, MLA, before initiation of PNU-282987/BrdU eye drop treatment. Figure 4A shows BrdU positive cells (green) in the INL that were observed after eye drop treatment with PNU-282987 and BrdU for 1 week. To obtain figure 4B, 5 μ l of 10 μ M MLA was injected into the posterior portion of the vitreous chamber before application of eye drops containing 1 mM PNU-282987/1 mg/ml BrdU. Eye drops were delivered to the right eye of the same animal that produced results shown in figure 4A. This demonstrates that the presence of the α 7 nAChR antagonist, MLA, significantly reduced PNU-282987's effect to induce BrdU positive cells, supporting the hypothesis that PNU-282987's effect in the retina is mediated through α 7 nAChRs.

Figure 4C summarizes the percent of BrdU positive cells in PNU-282987 treated retinal sections compared to BrdU negative cells. When retinas were treated with PNU-282987 and BrdU for one week, 18.42% ($\pm$ 5.2 N=4) of all cells in the INL labelled with antibodies against BrdU. If MLA was injected directly into the vitreous chamber before initiating PNU-282987/BrdU eye drop treatment, the percent of BrdU positive cells in retinal sections significantly dropped to 2.51% ($\pm$ 1.8; N=4).

As BrdU was delivered topically twice a day for two weeks, it could potentially accumulate in cells undergoing DNA repair, producing a false positive result. To address this issue, experiments were performed using a short-term incorporation of BrdU. The confocal images shown in figure 5 were obtained from 3 different retinas treated with 1 mM PNU-282987 for 14 days. To generate the image in figure 5A, a single dose of BrdU was applied with the PNU-282987 at day three. In figure 5B, a single dose of BrdU was applied with

PNU-282987 to another eye at day 7 and in figure 5C, a single application of BrdU was applied as eye drops with PNU-282987 to a third eye at day 10. At day 14, animals were sacrificed and the retinas were immunostained with anti-BrdU antibody and DAPI. As seen in all three images, BrdU positive cells were seen in the INL and ONL at all three time points after only receiving a single day of BrdU eye drop application. No BrdU positive cells are seen in the GCL as they do not typically appear until 21–28 days (see figure 7).

Experiments for each time point were conducted 3 times, producing similar results.

Absence of apoptotic cells after treatment

In some previous studies, limited regenerative effects of rod, bipolar and amacrine cells have been demonstrated to repair injured retinal tissue in mammals (Li and Zhou; 2015; Gallina et al., 2014; Gorsuch and Hyde, 2014; Luz-Madrigal et al., 2014). To support the hypothesis that eye drop application of PNU-282987 did not induce apoptosis to trigger regenerative effects, immunocytochemistry studies were performed using an antibody against caspase-3 in control retinas after two weeks of eye drop treatment with 1 mM PNU-282987. Caspase-3 is a caspase protein involved in sequential activation of caspases that play a central role in the execution-phase of cell apoptosis (Wyllie, 1997). Figure 6 illustrates confocal images of retinas processed for antibodies against caspase-3. A typical retinal section obtained from the left untreated eye of a Long Evans rat is shown in figure 6A after PNU-282987 treatment and subsequent processing of the tissue with an antibody against caspase-3. No caspase-3 positive cells were seen. The image shown in figure 6B was obtained from the right eye of the same animal that was treated with PNU-282987 for 2 weeks. Retinas were removed and processed for caspase-3 staining after PNU-282987 treatment at the two-week time point. No evidence of caspase 3 positive cells was found in PNU-282987 treated retinas. To generate the caspase-3 positive staining shown in figure 6C, the episcleral vein in another mature Long Evans rat was injected with 2M hypertonic saline to induce glaucoma-like conditions. This procedure has been previously shown to increase IOP and cause death of RGCs due to apoptosis (Morrison et al., 1997; Iwamoto et al., 2014; Mata et al., 2015). No PNU-282987 or BrdU was applied in this positive control. The animal was sacrificed two weeks after the procedure and immunostained with antibodies against caspase-3 at the two-week time point. The caspase-3 positive cell shown in figure 3B (arrow) was found in the RGC layer. Similar caspase-3 positive results were obtained from 2 other glaucomatous retinas. Each experiment under the three different conditions was repeated 3 times. These results support the hypothesis that eye drop application of PNU-282987 does not trigger apoptosis in retinal tissue.

BrdU positive cells are born from Müller glia

Although adult mammalian retinas are not known to generate new neurons, retinas of several other vertebrates are capable of regenerative effects (Gallina et al., 2014; Gorsuch and Hyde, 2014; Luz-Madrigal et al., 2014). To identify the origin of the BrdU positive RGCs, retinal sections were assayed for BrdU incorporation after eye drop treatments containing PNU-282987 and BrdU for various amounts of time between 1 and 28 days. After eye drops treatments, retinas were immunocytochemically processed for the presence of BrdU positive cells in the INL as early as 1.5 days following initiation of eye drop treatment. By 3 days, a significant number of BrdU positive cells were identified in the ONL and a significant

amount of BrdU positive cells were not identified in the GCL until 21 days. No BrdU positive cells were found in control retinas treated only with BrdU. As seen in figure 7, once the first cells emerged in each layer, there was a steady increase in the number of BrdU positive cells. Surprisingly, a relatively small number of BrdU positive cells were also found in the interplexiform layer (IPL) after treatment for 14 or 21 days (3 and 2 BrdU positive cells/retinal slice respectively). These experiments were repeated at each time point 3–8 times with similar results. Appendix B lists the data that was used to generate figure 7. These results suggest that the origin of BrdU positive RGCs in PNU-282987 treated retinas are likely from the INL and migrate through to the different retinal layers.

The cellular source of regeneration in teleost is Müller glia (Gorsuch and Hyde, 2014). Müller cell bodies sit in the INL and project irregularly thick and thin processes in either direction to the outer and inner limiting membranes of the retina. To determine if Müller glia also play a role in PNU-282987 induced proliferation in a vertebrate mammal, retinal sections were immunostained with antibodies against BrdU (figures 8A, B, C), PCNA (figure 8D), to label mitotically active cells, vimentin to label Müller glia (figure 8C,E,F), nestin (figures 8E,F) to label neuronal precursor progenitor cells expressed in dividing cells, and cell nuclei were stained with DAPI (blue). BrdU-positive staining was seen in the ONL, INL and some Müller glia. In addition, nestin and PCNA positive cells were found in the INL. PCNA labeled a smaller pool of BrdU positive cells, as it is expressed in cells during S-phase of the cell cycle. The arrow in figure 8E, focused in the IPL, provides evidence that nestin is associated with vimentin. This data supports the hypothesis that proliferation of adult rat retinal neurons is the result of Müller glia derived progenitor cells after application of an $\alpha 7$ nAChR agonist. Each of the experimental conditions described above were repeated 3–8 times with similar results.

To further demonstrate that some Müller glia are BrdU positive after PNU-282987 treatment, retinal sections were treated with the $\alpha 7$ nAChR agonist for one week and immunostained with antibodies against BrdU and Sox9. Sox9 regulates transcription of the anti-Müllerian hormone gene localized in Müller glia (Fisher et al., 2010) instead of the type III intermediate filament protein in Müller glia that is labeled with anti-vimentin (Lewis et al., 1989). Figure 9 illustrates two confocal images obtained from a mature rat. Figure 9A was obtained from the left untreated control eye. Figure 9B was obtained from the right eye of the same animal that was treated with eye drops containing 1 mM PNU-28297 and 1 mg/ml BrdU for 1 week. After the week, the retinas were double stained with antibodies against BrdU (green) and Sox9 (red). No evidence of BrdU or Sox9 label was seen under control untreated conditions (fig. 9A). However, in figure 9B, there is evidence of a BrdU positive cell in the INL (arrow), a Sox9 positive cell (star) as well as a double-labeled cell (arrowhead). These experiments were repeated 3 times with similar results and demonstrate that some BrdU positive cells in the INL also label with the Müller glia marker, Sox9.

Discussion

This is the first study to look at the neurogenic effect of an $\alpha 7$ nAChR agonist in the adult mammalian retina. The results from this study provide evidence that application of an $\alpha 7$ nAChR agonist to the mature rat retina leads to BrdU positive cells throughout the retina and

that new neurons may originate from Müller glia in the INL and then migrate. Retinal sections immunostained with antibodies against BrdU and vimentin, or with antibodies against BrdU and Sox9, reveal co-labeled Müller glia to support the evidence that Müller glia undergo division in the presence of PNU-282987. In addition, it takes 4 weeks for the maximal number of BrdU positive cells to be seen in the GCL. This result would dispute the possibility that BrdU positive cells are initiated from RGCs that contain $\alpha 7$ nAChRs (Thompson et al., 2006). However, it does not rule out the possibility that the $\alpha 7$ nAChR agonist binds to receptors in the retinal pigment epithelium (Maneu et al., 2010; Matsumoto et al., 2012; Fuhrmann et al., 2014), which then secrete unknown signaling factors that initiates the division of Müller glia. Further experiments are required to examine this possibility.

An increase of 20% BrdU positive RGCs is a remarkable increase. Previous studies have demonstrated limited neurogenesis of new retinal neurons, but only after insult or injury, and none of these models elicit new RGCs. In the adult rat retina, there are over 97,000 RGCs (+/- 3,903) (Danas et al., 2002). Neurogenesis that increase RGCs by 20% could generate up to 19,000 new RGCs. In one *in vivo* rat glaucoma model, as many as 25% of RGCs are lost within a month of injecting hypertonic saline into the episcleral veins of the retina to increase IOP and trigger glaucoma-like conditions (Morrison et al., 1997; Iwamoto et al., 2014; Mata et al., 2015). 20% new functional RGCs could potentially replace lost RGCs due to glaucoma to restore normal vision. Functional studies to determine if PNU-282987-induced new RGCs send out axons through the optic nerve are currently underway.

As stated previously, adult mammalian neurons are not known to possess regenerative capabilities. Recent studies suggest the regenerative process is blocked in mammals (Todd and Fischer, 2015). However, proliferation and regenerative effects of neurons in adults can occur in other vertebrates (Gallina et al., 2014; Gorscuh and Hyde, 2014; Luz-Madrigal et al., 2014) though typically triggered as a result of injury. For instance, Müller glia have been found to respond to damage in teleost fish (Lenkowiak and Raymond, 2014; Wan et al., 2014). However, the study presented here demonstrates that PNU-282987 was able to elicit a significant neurogenic response through Müller glia without triggering apoptosis, as indicated by the lack of caspase 3 activity. Interestingly, recent work in adult mice has shown that modulation of Wnt signaling can also induce cell cycle reentry in Müller glia (Yao et al., 2016).

Cellular mechanisms underlying the PNU-282987 stimulated proliferative and neurogenic response remains to be determined and studies are underway. However, previous studies have demonstrated that an $\alpha 7$ nAChR agonist upregulates neurogenesis in the subventricular zone in the brains of adult mice by activation of FGF1R signaling (Narla et al., 2013) and the selective $\alpha 7$ nAChR receptor antagonist, MLA, blocks nicotine-mediated increases in FGF2 expression in cortical microglia (Morioka et al., 2015). FGF2 and insulin promote proliferative and neurogenic responses of Müller glia in the chick retina in the absence of overt retinal injury (Fischer et al., 2002). Combined with Notch inhibition, FGF2 stimulates human Müller glia to generate RGC precursors *in vitro* (Singhal et al., 2012). As FGF2 binds to the FGF1R to activate intracellular signaling (Lundin et al., 2003), a potential mechanism for PNU-282987 induced retinal neurogenesis is offered. Other studies

supporting the role of FGF in regeneration include a study in zebrafish that demonstrates how FGF signaling regulates rod photoreceptor cell maintenance and regeneration in zebrafish (Qin et al., 2011). In the chick embryo, retinal regenerative effects require FGF/FGFR/MEK/Erk dependent upregulation of Pax6 (Spence et al., 2007). Further characterization of the mechanisms of $\alpha 7$ nAChR agonist activation of neurogenic and regenerative responses could reveal new strategies to restore depleted retinal neuron populations through *de novo* generation of new neurons to reverse vision loss. This has implications for treatment of traumatic injuries, degenerative retinal disease or normal age related changes.

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Appendix A

Statistics associated with dose-response results that generated figure 3. One-way ANOVAs were performed with post hoc Holm-Bonferroni corrections for multiple comparisons. df = degree of freedom. t(df) = total degrees of freedom. F values have two dfs.

Concentration of PNU-282987	df between groups	df within groups	t(df)	F-value	p-value
2 mM	1	54	55	F(1,54)=50.96	p<0.001
1 mM	1	54	55	F(1,54)=317.29	p<0.001
500 μ M	1	54	55	F(1,54)=174.71	p<0.001
250 μ M	1	54	55	F(1,54)=80.63	p<0.001
100 μ M	1	30	31	F(1,30)=49.06	p<0.001
10 μ M	1	30	31	F(1,30)=3.09	p<0.09

Appendix B

Data used to generate Figure 7.

Retinal layer	Days of treatment	Average % BrdU cells	SEM
ONL	1	0	0.11
	1.5	0.53	0.27
	3	5.42	2.52
	7	9.22	3.53
	14	10.37	3.01
	21	12.21	2.08
	28	15.39	2.75
INL	1	0.33	0.16
	1.5	2.54	1.03

Retinal layer	Days of treatment	Average % BrdU cells	SEM
	3	11.22	2.52
	7	10.53	3.54
	14	17.12	3.25
	21	22.17	1.54
	28	29.04	6.21
GCL	1	0	0
	1.5	0	0.11
	3	0.42	0.26
	7	0.81	1.21
	14	0.32	0.22
	21	8.12	2.27
	28	21.34	2.25

References

- Beidoe G, Mousa SA. Current primary open-angle glaucoma treatments and future directions. *Clin Ophthalmol.* 2012; 6:1699–1707. [PubMed: 23118520]
- Birkholz PJ, Gossman CA, Webster MK, Linn DM, Linn CL. Prevention of glaucoma-induced retinal ganglion cell loss using alpha7 nAChR agonists. *J Ophthalmol Vis Sci.* 2016; 1(1):1–8.
- Bucolo C, Platania CB, Reibaldi M, Bonfiglio V, Longo A, Salomone S, Drago F. Controversies in Glaucoma: Current Medical Treatment and Drug Development. *Curr Pharm Des.* 2015; 21:4673–4681. [PubMed: 26350532]
- Bodnar AL, Cortes-Burgos LA, Cook KK, Dinh DM, Groppi VE, Hajos M, Higdon NR, Hoffmann WE, Hurst RS, Myers JK, Rogers BN, Wall TM, Wolfe ML, Wong E. Discovery and structure-activity relationship of quinuclidine benzamides as agonists of alpha7 nicotinic acetylcholine receptors. *J Med Chem.* 2005; 48(4):905–908. [PubMed: 15715459]
- Chauhan BC, Pan J, Archibald ML, LeVatte TL, Kelly ME, Tremblay F. Effect of intraocular pressure on optic disc topography, electroretinography, and axonal loss in a chronic pressure-induced rat model of optic nerve damage. *Invest Ophthalmol Vis Sci.* 2002; 43:2969–2976. [PubMed: 12202517]
- Damji KF, Behki R, Wang L. Canadian perspectives in glaucoma management: Setting target intraocular pressure range. *Can J Ophthalmol.* 2003; 38(3):189–197. [PubMed: 12733686]
- Danias J, Shen F, Goldblum D, Chen B, Ramos-Esteban J, Podos SM, Mittag T. Cytoarchitecture of the retinal ganglion cells in the rat. *Invest Ophthalmol Vis Sci.* 2002; 43(3):587–594. [PubMed: 11867571]
- Fischer AJ, McGuire CR, Dierks BD, Reh TA. Insulin and fibroblast growth factor 2 activate a neurogenic program in Müller glia of the chicken retina. *J Neurosci.* 2002; 22:9387–9398. [PubMed: 12417664]
- Fisher AJ, Scott MA, Zelinka C, Sherwood P. A novel type of glial cell in the retina is stimulated by insulin-like growth factor 1 and may exacerbate damage to neurons and Müller glia. *Glia.* 2010; 58(6):633–649. [PubMed: 19941335]
- Foster PJ, Buhrmann R, Quigley HA, Johnson GJ. The definition and classification of glaucoma in prevalence surveys. *Brit J Ophthalmol.* 2002; 86:238–242. [PubMed: 11815354]
- Fuhrmann S, Zou C, Levine EM. Retinal pigment epithelium development, plasticity, and tissue homeostasis. *Exp Eye Res.* 2014; 123:141–150. [PubMed: 24060344]
- Gallina D, Todd L, Fischer A. A comparative analysis of Müller glia-mediated regeneration in the vertebrate retina. *Exp Eye Res.* 2014; 123:121–130. [PubMed: 23851023]

- Gorsuch RA, Hyde DR. Regulation of Müller glial dependent neuronal regeneration in the damaged adult zebrafish retina. *Exp Eye Res.* 2014; 123:131–140. [PubMed: 23880528]
- Guo L, Moss SE, Alexander RA, Ali RR, Fitzke FW, Cordeiro MF. Retinal ganglion cell apoptosis in glaucoma is related to intraocular pressure and IOP-induced effects on extracellular matrix. *Invest Ophthalmol Vis Sci.* 2005; 46(1):175–185. [PubMed: 15623771]
- Holm S. A simple sequential rejective method procedure. *Scand J Statistics.* 1979; 6:65–70.
- Iwamoto K, Birkholtz P, Schipper A, Mata D, Linn DM, Linn CL. A nicotinic acetylcholine receptor agonist prevents loss of retinal ganglion cells in a glaucoma model. *IOVS.* 2014; 55:1078–1087.
- Lenkowski JR, Raymond PA. Müller glia: Stem cells for generation and regeneration of retinal neurons in teleost fish. *Prog Retin Eye Res.* 2014; 40:94–123. [PubMed: 24412518]
- Lewis GP, Ericksom PA, Guerin CJ, Anderson DH, Fisher SK. Changes in the expression of specific Müller cell proteins during long-term retinal detachments. *Exp Eye Res.* 1989; 49:93–111. [PubMed: 2503391]
- Levkovitch-Verbin H, Quigley HA, Martin KR, Valenta D, Baumrind LA, Pease ME. Translimbal laser photocoagulation to the trabecular meshwork as a model of glaucoma in rats. *Invest Ophthalmol Vis Sci.* 2002; 43:402–410. [PubMed: 11818384]
- Li Y, Zhou GM. Interneuron regeneration after ouabain treatment in the adult mammalian retina. *Neuroreport.* 2015; 26:712–717. [PubMed: 26164459]
- Lundin L, Rönstrand L, Cross M, Hellberg C, Lindahl U, Claesson-Welsh L. Differential tyrosine phosphorylation of fibroblast growth factor (FGF) receptor-1 and receptor proximal signal transduction in response to FGF-2 and heparin. *Exp Cell Res.* 2003; 287:190–198. [PubMed: 12799194]
- Luz-Madrigal A, Grajales-Esquivel E, McCorkle A, DiLorenzo AM, Barbosa-Sabanero K, Tsonis PA, Del Rio-Tsonis K. Reprogramming of the chick retinal pigmented epithelium after retinal injury. *BMC Biol.* 2014; 12:12–28. [PubMed: 24528630]
- Maneu V, Geron G, Fernandez L, Cuenca N, Lax P. Evidence of alpha7 nicotinic acetylcholine receptor expression in retinal pigment epithelial cells. *Vis Neurosci.* 2010; 27(5-6):139–147. [PubMed: 20932358]
- Mata D, Linn DM, Linn CL. Retinal ganglion cell neuroprotection induced by activation of alpha7 nicotinic acetylcholine receptors. *Neuropharm.* 2015; 99:337–346.
- Matsumoto H, Shibasaki K, Uchigashima M, Koizumi A, Kurachi M, Moriwaki Y, Misawa H, Kawashima K, Watanabe M, Kishi S, Ishizaki Y. Localization of acetylcholine-related molecules in the retina: implication of the communication from photoreceptor to retinal pigment epithelium. *PLoS One.* 2012; 7:e42841. [PubMed: 22880119]
- Morioka N, Harano S, Tokuhara M, Idenoshita Y, Zhang FF, Hisaoka-Nakashima K, Nakata Y. Stimulation of alpha7 nicotinic acetylcholine receptor regulates glutamate transporter GLAST via basic fibroblast growth factor production in cultured cortical microglia. *Brain Res.* 2015; 1625:111–120. [PubMed: 26327163]
- Morrison JC, Moore CG, Deppmeier LM, Gold BG, Meshul CK, Johnson EC. A rat model of chronic pressure-induced optic nerve damage. *Exp Eye Res.* 1997; 64:85–96. [PubMed: 9093024]
- Narla ST, Klejbor I, Birkaya B, Lee YW, Morys J, Stachowiak EK, Prokop D, Bencherif M, Stachowiak MK. Activation of developmental nuclear fibroblast growth factor receptor 1 signaling and neurogenesis in adult brain by alpha7 nicotinic receptor agonist. *Stem Cells Transl Med.* 2013; 2:776–788. [PubMed: 24014683]
- Qin Z, Kidd AR 3rd, Thomas JL, Poss KD, Hyde DR, Raymond PA, Thummel R. FGF signaling regulates rod photoreceptor cell maintenance and regeneration in zebrafish. *Exp Eye Res.* 2011; 93(5):726–734. [PubMed: 21945172]
- Thompson SA, Smith O, Linn DM, Linn CL. ACh neuroprotection against glutamate-induced excitotoxicity in adult pig RGCs is partially mediated through alpha4 nAChRs. *Exp Eye Res.* 2006; 83(5):1135–1145. [PubMed: 16928373]
- Todd L, Fischer AJ. Hedgehog signaling stimulates the formation of proliferating Müller glia-derived progenitor cells in the chick retina. *Development.* 2015; 142:2610–2622. [PubMed: 26116667]
- Rossetti L, Digiuni M, Giovanni M, Centofanti M, Fea AM, Iester M, Frezzotti P, Figus M, Ferreras A, Oddone F, Tanga L, Rolle T, Battaglini V, Posarelli C, Motolese I, Mittica P, Bagaglia SA,

- Menicacci C, De Cilla' S, Autelitano A, Fogagnolo P. Blindness and Glaucoma: A Multicenter Data Review from 7 Academic Eye Clinics. *PLoS One*. 2015; 10(8):e0136632. [PubMed: 26302445]
- Saaddine JB, Narayan KM, Vinicor F. Vision loss: a public health problem? *Ophthalmol*. 2003; 110(2): 253–254.
- Singhal S, Bhatia B, Jayaram H, Becker S, Jones MF, Cottrill PB, Khaw PT, Salt TE, Limb GA. Human Müller glia with stem cell characteristics differentiate into retinal ganglion cell (RGC) precursors in vitro and partially restore RGC function in vivo following transplantation. *Stem Cells Transl Med*. 2012; 1:188–199. [PubMed: 23197778]
- Spence JR, Madhavan M, Aycinena JC, Del Rio-Tsonis K. Retina regeneration in the chick embryo is not induced by spontaneous *Mitf* downregulation but requires FGF/FGFR/MEK/Erk dependent upregulation of *Pax6*. *Mol Vis*. 2007; 13:57–65. [PubMed: 17277739]
- Walker DP, Wishka DG, Piotrowski DW, Jia S, Reitz SC, Yates KM, Myers JK, Vetman TN, Margolis BJ, Jacobsen EJ, Acker BA, Groppi VE, Wolfe ML, Thornburgh BA, Tinholt PM, Cortes-Burgos LA, Walters RR, Hester MR, Seest EP, Dolak LA, Han F, Olson BA, Fitzgerald L, Staton BA, Raub TJ, Hajos M, Hoffmann WE, Li KS, Higdon NR, Wall TM, Hurst RS, Wong EH, Rogers BN. Design, synthesis, structure-activity relationship, and in vivo activity of azabicyclic aryl amides as $\alpha 7$ nicotinic acetylcholine receptor agonists. *Bioorg Med Chem*. 2006; 14(24): 8219–8248. [PubMed: 17011782]
- Wan J, Zhao XF, Vojtek A, Goldman D. Retinal injury, growth factors, and cytokines converge on β -catenin and pStat3 signaling to stimulate retina regeneration. *Cell Rep*. 2014; 9(1):285–297. [PubMed: 25263555]
- Wyllie AH. Apoptosis: an overview. *Brit Med Bull*. 1997; 53(3):451–465. [PubMed: 9374030]
- Yao K, Qui S, Tian L, Snider WD, Flannery JG, Schaffer DV, Chen B. Wnt regulates proliferation and neurogenic potential of Müller glia via a *Lin28/let-7* miRNA-dependent pathway in adult mammalian retinas. *Cell Rep*. 2016; 17:165–178. [PubMed: 27681429]

Highlights

The alpha7 nAChR agonist, PNU-282987, induces BrdU positive cells in adult mammalian retina.

Alpha7 nAChR agonist-induced BrdU positive cells are derived from Müller glia.

Cells in the ganglion cell layer label for anti-BrdU and anti-Thy 1.1 after application of PNU-282987.

Eye drop treatment of PNU-282987 induces BrdU positive cells in adult mammalian retina.

Results of this study have broad implications for reversing degenerative diseases associated with alpha7 nACh receptors.

Figure 1. PNU-282987 induced an increase of RGCs

Figure 1A shows a confocal image obtained 4 mm from the ONH in an untreated control retina. Figure 1B shows an image obtained from the other eye of the same animal that was treated with 1mM PNU-282987 for two weeks. One month following treatment initiation, the animal was sacrificed and the retinas were processed with anti-Thy 1.1 antibody to label RGC bodies (arrow) and their axon fascicles (double arrows). Alexa Fluor 594 was used for visualization. The same region of the retina is shown in both images. Figure 1C contains a bar graph summarizing the average percent of RGCs after treatment compared to untreated control conditions. The left bar indicates baseline in the control untreated eyes and the right bar show the mean percent change in RGCs relative to the contralateral control. Error bars in all figures represent standard error. * represents significance from untreated internal controls. Significance represents $P = 0.001$; $N=8$. Scale bar= 50 μm .

Figure 2. BrdU positive RGCs in the rat retina

Confocal photomicrographs of representative retinal flat-mounts showing BrdU labeled cells in the ONL of rats that received eye drops containing 1 mg/mL BrdU (A-C) or 1 mM PNU-282987 and 1 mg/mL BrdU (D-F) for 2 weeks, then sacrificed at 4 weeks. RGCs immunostained with antibodies against Thy 1.1 fluoresced green (figures 2A, D) when secondarily labeled with fluorescent Alexa Fluor 488. BrdU positive cells using anti-BrdU (figures 2B, E) fluoresced red when secondarily labeled with Alexa Fluor 594. Figures 2C and 2F show superimposed images. The arrows indicate RGC bodies, double headed arrows indicate RGC axon fascicles and arrowheads point to BrdU positive RGCs. This experiment was repeated a total of 8 times with similar results. Scale bar = 50 μ m.

Fig. 3. Dose response of BrdU positive cells

Different concentrations of PNU-282987 were applied to the right eyes of experimental animals for two weeks in PBS containing 1 mg/ml BrdU. The left eyes only received BrdU and acted as an internal control. Four weeks after initiation of eye drop treatment, the animals were sacrificed and the retinas were processed with anti-Thy 1.1 and anti-BrdU and visualized with secondary fluorescent antibody. Each bar represents the average percent of BrdU positive RGCs that were found in a $200 \mu\text{m}^2$ confocal image obtained from flat-mounted retinas. Bars that have the same symbol (, *, •) are statistically similar to each other and statistically different ($P < 0.001$) from all other symbols. N's between 4 and 8 were used for each dosage of PNU-282987 used.

Fig. 4. MLA blocks PNU-282987's effect

Figure 4A shows a retinal section obtained from a rat eye treated with PBS eye drops containing 1 mM PNU-282987 and 1 mg/ml BrdU for 1 week. After 1 week, the retina was processed for BrdU positive cells in the outer nuclear layer (ONL) and inner nuclear layer (INL). BrdU positive cells in the INL fluoresced green in a field of DAPI stained nuclei (blue). Figure 4B shows a confocal image obtained from the opposite eye of the same animal. Before initiating eye drop treatment with 1 mM PNU-282987 and 1 mg/ml BrdU, the eye was intravitreally injected with 5 μ l of 10 μ M MLA into the posterior vitreous chamber. MLA significantly reduced the number of BrdU positive cells in the INL. The bar graph in figure 4C summarizes the percent of BrdU positive cells compared to BrdU negative cells from retinal sections. Error bars represent standard error. * represents a significant mean percent difference from eyes that received PNU/BrdU eye drop treatment without an injection of MLA (P = 0.001; N=4). Scale bar represents 30 μ m.

Fig. 5. BrdU pulse chase

1 mM PNU-282987 was applied as eye drops for 2 weeks. In addition, 1mg/ml BrdU was applied in a single pulse at day 3 (figure 5A) in some rats, at day 7 (figure 5B) and 10 (figure 5C) in other rats. Fourteen days following the initiation of PNU-282987 treatment, animals were sacrificed and the retinas were processed for BrdU staining and DAPI. Retinal sections were viewed to identify BrdU positive cells in the INL and ONL. N=3 for each experimental condition at each time point with similar results. Scale bar represents 50 μ m.

Fig. 6. Absence of caspase-3 activity after treatment with PNU-282987

The confocal image shown in figure 6A was obtained from an adult Long Evans rat under control untreated conditions and after eye drop application of 1 mM PNU-282987 for two weeks (figure 6B). Both images were obtained from the same animal and from the same retinal region. After two weeks of eye drop treatment, the animal was sacrificed and immunostained with anti-caspase-3 and secondarily labeled with Alexa Fluor 594. No caspase-3 positive cells are seen after PNU-282987 treatment. To obtain the image shown in fig. 6C, hypertonic saline was injected into the episcleral veins of a mature Long Evans rat. Two weeks after the procedure to induce glaucoma-like conditions, the animal was sacrificed and the retina was processed for caspase-3 staining. The arrow points to a caspase-3 positive cell (red). Each experiment was repeated a total of 3 times with similar results. Scale bar represents 60 μm .

Fig. 7. Migration of BrdU positive cells

Each bar represents the average percent of BrdU positive cells compared to BrdU negative cells obtained from the ONL, INL, and GCL from confocal images at different treatment and sacrifice times in 200 μm^2 retinal sections. In each retinal layer, bars that have the same symbol (, *, ●, ◇) are statistically similar to each other and statistically different from all other days of treatment ($P < 0.001$; $N=3-8$ depending on the time point corresponding to when animals were sacrificed).

Fig. 8. BrdU positive retinal neurons and Müller glia

Figure 8A shows a confocal image obtained from a rat retina treated only with 1mg/ml BrdU for two weeks. After one month, the retina was stained with anti-BrdU (green) and counterstained with DAPI (blue). The retinal sections shown in figure 8B-F were treated with 1 mM PNU-282987 and 1mg/ml BrdU in PBS eye drops for two weeks. In figure 8B, the retinal section was stained for anti-BrdU (green) and DAPI. BrdU positive cells are seen in the ONL and INL after PNU-282987 treatment (arrow heads). In figure 8C, the treated retina stained for anti-BrdU (green), anti-vimentin (red) and DAPI. Several examples of BrdU positive Müller glia in the INL are demonstrated (arrows). In figure 8D, treated retina are stained for anti-PCNA (green) and DAPI (blue). PCNA is localized in the INL. In figure 8E, the PNU-282987 treated retina stained for anti-nestin (green; arrow) and anti-vimentin (red), while cell bodies stained blue. A nestin stained fiber extends out of the INL into the IPL along a Müller glia (red). Nuclei can be seen located along the nestin filament (arrow heads). In figure 8F, nestin stained cells (green) are present in the INL and Müller glia fibers are present in the OPL. Scale bars= 30 μm. All images show typical results obtained when repeated 4–8 times.

Fig. 9. Double-stained Müller glia

Figure 9A shows a confocal photomicrograph of a control untreated retina. The retina shown in Figure 9B was treated with eye drops containing 1 mM PNU-282987 and 1 mg/ml BrdU for 1 week from the same animal. Both retinas were immunostained with antibodies against BrdU (green) and Sox9 (red). The arrow points to a BrdU positive cell in the INL (inner nuclear layer), the star indicates a Sox9 positive cell and the arrowhead (orange cell) indicates a double-labeled Müller glia cell in the INL. Images show typical results obtained from 3 experiments. Scale bar = 50 μ m.

Table 1

A list of antibodies used in this study with corresponding fluorescent secondary antibodies.

Primary Antibody	Supplier	Secondary Antibody	Supplier
Mouse anti-Thy1.1; 3.4 $\mu\text{L/mL}$	BD Pharmingen 554892	Goat anti-Mouse Alexa Fluor 594 or 488; 3.4 $\mu\text{L/mL}$	Thermo A11005
Sheep anti-BrdU; 7.5 $\mu\text{L/mL}$	Abcam ab1894	Donkey anti-Sheep Alexa Fluor 488 or 594; 3.4 $\mu\text{L/mL}$	Thermo A11015
Rabbit anti-vimentin; 7.5 $\mu\text{L/mL}$	Abcam ab92547	Donkey anti-Rabbit Alexa Fluor 594; 3.4 $\mu\text{L/mL}$	Thermo A21207
Rabbit anti-nestin; 10 $\mu\text{L/mL}$	Abcam ab6142	Donkey anti-Rabbit Alexa Fluor 488; 3.4 $\mu\text{L/mL}$	Thermo A21206
Rabbit anti-PCNA; 7.5 $\mu\text{L/mL}$	Abcam ab18197	Donkey anti-Rabbit Alexa Fluor 488; 3.4 $\mu\text{L/mL}$	Thermo A21206
Rabbit anti-caspase-3; 3.4 $\mu\text{L/mL}$	Abcam ab44976	Donkey anti-Rabbit Alexa Fluor 594; 3.4 $\mu\text{L/mL}$	Thermo A21207
Rabbit anti-Sox9; 3.4 $\mu\text{L/mL}$	Bioss bs4177R	Goat anti-Rabbit Alexa Fluor 594; 3.4 $\mu\text{L/mL}$	Thermo A11037