

● PERSPECTIVE

Retinal ganglion cell neuroprotection induced by activation of alpha7 nicotinic acetylcholine receptors

Glaucoma is a group of degenerative retinal diseases that damage the eye's optic nerve and can result in vision loss and blindness. It is characterized by optic neuropathy, cupping of the optic disk, and progressive loss of retinal ganglion cells (RGCs) and axons in the optic nerve. Glaucoma patients initially develop loss of visual function in the periphery and slowly develop tunnel vision that becomes worse over time. As the second leading cause of blindness in the world, it is suspected that there are over 60 million cases of glaucoma worldwide. Blindness caused by glaucoma is irreversible. Once adult neurons in the mammalian retina are lost, there is no known treatment that can regenerate new neurons. Although there are known familial genetic causes of glaucoma, there are many more complex cases where the cause is not known. However, the primary risk factor associated with glaucoma is an increase of intraocular pressure (IOP) in the eye and all currently approved treatments are designed to lower IOP. Initial treatments use pharmacological agents to reduce IOP. Topically applied pharmacological treatments include the use of β -blockers, prostaglandins, α -agonists, carbonic anhydrase inhibitors, muscarinic cholinergic agonists or a combination of these. If the use of pharmacological agents to decrease aqueous humor input or decrease aqueous humor outflow is insufficient to reduce IOP, laser therapy or incisional surgery is typically performed. However, these treatments alone can be insufficient to halt the progression of blindness associated with glaucoma. For example, in a subset of patients, RGCs continue to die, even after IOP reduction. In addition, a significant number of glaucoma patients exhibit normal intraocular pressures even though they present typical signs of glaucomatous damage, like optic nerve head excavation and thinning of the retinal nerve fiber layer. Clearly, future glaucoma treatment must involve more than IOP reduction. One avenue of research that addresses this issue involves neuroprotective treatments that work at the level of the retina.

Neuroprotection for glaucoma has been defined as any intervention designed to prevent optic nerve damage or RGC death, but neuroprotective treatment development remains a challenge because of the range of suspected pathological processes involved. As a result, a great deal of neuroprotective research in the retina has been conducted to address potential mechanisms of action involved in glaucoma, including excitotoxicity due to the overproduction of glutamate, nitric oxide production, other forms of oxidative stress, deprivation of neurotrophic factors in the retina, initiation of apoptotic machinery as well as overactive glial cells to name a few.

A relatively recent neuroprotective strategy used to prevent the loss of RGCs associated with glaucoma involves activation of alpha 7 nicotinic acetylcholine receptors ($\alpha 7$ nAChRs) in the retina. In the brain, activation of $\alpha 7$ nAChRs has been linked to neuroprotection against several neurodegenerative diseases. There is strong evidence that $\alpha 7$ nAChRs are neuroprotective,

reducing β -amyloid induced toxicity in Alzheimer's disease (Oz et al., 2013) and that the $\alpha 7$ nAChRs plays a role in the pathophysiology of schizophrenia (Young and Geyer, 2013). In the retina, RGCs contain $\alpha 7$ nAChRs and receive cholinergic input from a well-described population of starburst amacrine cells that are the only source of ACh in the vertebrate retina.

In vitro and *in vivo* mammalian models have been used to demonstrate the neuroprotective effect of activating $\alpha 7$ nAChRs to prevent the loss of RGCs associated with excitotoxicity and glaucoma. Glutamate-induced neurotoxicity is thought to be an important component of glaucoma. Previous pig and rat *in vitro* studies have demonstrated that the loss of RGCs normally associated with glutamate-induced excitotoxicity can be prevented after activation of $\alpha 7$ nAChRs in a dose-dependent manner and that neuroprotection can be blocked with the use of the $\alpha 7$ nAChR antagonist, methyllycaconitine (Wehrwein et al., 2004; Thompson et al., 2006; Iwamoto et al., 2013). ELISA and pharmacological studies were performed using this same *in vitro* model to examine the neuroprotective pathways activated in RGCs after stimulating $\alpha 7$ nAChRs with the potent $\alpha 7$ nAChR agonist, PNU-282987. PNU-282987 was found to provide neuroprotection of RGCs *in vitro* by stimulating the PI3 kinase \rightarrow Akt \rightarrow Bcl-2 survival pathway and inhibiting the p38 MAP kinase \rightarrow Bcl-2 apoptotic pathway (Asomugha et al., 2010). Calcium imaging studies using pig isolated RGCs linked an influx of calcium through $\alpha 7$ nAChR channels with activation of the neuroprotective pathways. However, further studies found that any stimuli that preconditioned cells with a relatively low concentration of intracellular calcium before glutamate insult, induced a neuroprotective effect against glutamate-induced excitotoxicity (Brandt et al., 2011). To determine if $\alpha 7$ nAChRs in the retina prevent the loss of RGCs under glaucoma-like conditions in a physiological state, an *in vivo* rat model was utilized.

2M hypertonic saline was injected into the episcleral vein of rat eyes to induce glaucoma-like conditions. Injection of 2M NaCl saline into the episcleral veins of adult Long Evans rats caused scarring in the trabecular meshwork, decreased the outflow of aqueous humor, and produced a gradual increase of intraocular pressure to mimic glaucoma-like conditions. Animals were sacrificed at different time points between 1 week and 4 months following this procedure and retinas were flat-mounted in one piece to maintain orientation to the optic nerve head. RGCs were fixed, immunocytochemically stained with primary antibodies against Thy 1.1 to label RGCs, secondarily labeled with fluorescent Alexa Fluor antibodies for visualization using a confocal microscope, quantified and RGC counts were normalized to control untreated conditions. Within a month of performing the procedure to induce glaucoma-like conditions, there was significant loss of RGCs compared to control untreated retinas by an average of 27.35% (Iwamoto et al., 2014; Mata et al., 2015). This significant loss of RGCs correlated with an increase of intraocular pressure that averaged 12.6 mmHg before the procedure to induce glaucoma in 3 month Long Evans rats and rose to an average of 21 mmHg 1 month following the procedure. In addition, significant loss of RGCs correlated with defasciculation of axon bundles in the nerve fiber layer and a significant decrease in RGC body circularity (Mata et al., 2015). This rodent model of glaucoma was used to determine if application of the $\alpha 7$ nAChR agonist, PNU-282987, prevented the

loss of RGCs typically associated with the procedure to induce glaucoma-like conditions.

In initial studies, PNU-282987 was intravitreally injected in adult Long Evans rat eyes 2 hours before the procedure to induce glaucoma. Intravitreal injection of PNU-282987 prevented the loss of RGCs in a dose-dependent manner. The EC50 for PNU-282987's effect was calculated to be 50 μ M, while intravitreal injection of 100 μ M PNU-282987 prevented the loss of RGCs compared to internal controls by 100% if 5 μ L of 100 μ M PNU-282987 was injected into the eye 1 hour before NaCl injection (Iwamoto et al., 2014). The neuroprotective effect of PNU-282987 was eliminated if 10 μ L of the α 7 nAChR antagonist, MLA, was injected into the eye 1 hour before introduction of PNU-282987 and 2 hours before the procedure to induce glaucoma supporting the hypothesis that PNU-282987's effect is mediated through activation of α 7 nAChRs.

However, the use of intravitreal injections is invasive and reduces the appeal of developing an α 7 nAChR agonist for potential glaucoma treatment. To address this issue, eye drops of PNU-282987 were applied to adult Long Evans rats before and after the procedure to induce glaucoma-like conditions. Eye drops contained various amounts of PNU-282987 between 100 μ M and 2 mM and were applied to the right experimental eyes of adult Long Evans rats 3 days before the procedure to induce glaucoma-like conditions and for 1 month following the procedure. The left eye in each animal was used as an internal untreated control for comparison. Eye drops (30 μ L) were applied twice a day (Mata et al., 2015). Although the main pathway for absorption of eye drops is through the cornea, a small amount of agent applied directly to the cornea can reach the retina depending on the agent's vehicle, by working its way from the anterior of the eye to the retina, or by drops that are absorbed through the sclera as eye drops follow the curvature of the eye structure. Eye drops of PNU-282987 resulted in neuroprotection against glaucoma-induced RGC loss in a dose-dependent manner using concentrations between 100 μ M and 2 mM PNU-282987 (Mata et al., 2015). Although eye drops containing 100 μ M PNU-282987 had no significant neuroprotective effect on RGC survival, eye drops containing 1 mM PNU-282987 prevented the typical loss of RGCs by 93% (Mata et al., 2015). In addition, the typical axon defasciculation and loss of circularity in RGC bodies that typically resulted after inducing glaucoma-like conditions were eliminated after 1 mM PNU-282987 eye drop treatment (Mata et al., 2015).

Although PNU-282987 has been used in animal schizophrenia models (Young and Geyer, 2013), it has not been found to be suitable for systemic use in humans because of excessive inhibition of a hERG antitarget in the heart. Liquid chromatography mass spectroscopy/mass spectroscopy results demonstrated that PNU-282987 was detected in the retina when applied as eye drops, relatively small amounts of PNU-282987 were measured in blood plasma but no PNU-282987 was detected in cardiac tissue (Mata et al., 2015). In addition, other α 7 nAChR agonists that are not associated with the negative effects on hERG channels in the heart were also found to prevent the loss of RGCs

under glaucoma-like conditions, though to a lesser degree than PNU-282987 (Birkholz et al., 2016).

In conclusion, the neuroprotective effect of α 7 nAChR agonists applied to the retina has been shown to prevent the loss of RGCs in glaucoma-like conditions in a dose-dependent manner. Glaucoma is a multifactorial ocular disease. As there are many different types of glaucoma, it is likely that a variety of treatments in addition to conventional therapies to lower IOP will be needed to delay and even stop the progression of the disease. Alternatively a potential treatment could be devised to protect RGCs directly with an α 7nAChR agonist eye drop in conjunction with current treatments designed to lower IOPs in glaucoma patients. These results have clinical significance for treating glaucoma that directly treats the retina in a non-invasive manner instead of focusing exclusively on IOP measurements.

Cindy L. Linn*

Department of Biological Sciences, Western Michigan University,
Kalamazoo, MI, USA

*Correspondence to: Cindy L. Linn, Ph.D., Cindy.linn@wmich.edu.

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