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

David Mata [a], David M. Linn [b], Cindy L. Linn [a, *]

[a] Western Michigan University, Department of Biological Sciences, Kalamazoo, MI 49008, USA
[b] Grand Valley State University, Department of Biomedical Sciences, Allendale, MI 49401, USA

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ABSTRACT

The α7nAChR agonist, PNU-282987, has previously been shown to have a neuroprotective effect against loss of retinal ganglion cells (RGCs) in an *in vivo* glaucoma model when the agent was injected into the vitreous chamber of adult Long Evans rat eyes. Here, we characterized the neuroprotective effect of PNU-282987 at the nerve fiber and retinal ganglion cell layer, determined that neuroprotection occurred when the agonist was applied as eye drops and verified detection of the agonist in the retina, using LC/MS/MS.

To induce glaucoma-like conditions in adult Long Evans rats, hypertonic saline was injected into the episcleral veins to induce scar tissue and increase intraocular pressure. Within one month, this procedure produced significant loss of RGCs compared to untreated conditions. RGCs were quantified after immunostaining with an antibody against Thy 1.1 and imaged using a confocal microscope. In dose −response studies, concentrations of PNU-282987 were applied to the animal's right eye two times each day, while the left eye acted as an internal control. Eye drops of PNU-282987 resulted in neuroprotection against RGC loss in a dose-dependent manner using concentrations between 100 μM and 2 mM PNU-282987. LC/MS/MS 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 and no PNU-282987 was detected in cardiac tissue. These results support the hypothesis that eye drop application of PNU-282987 can prevent loss of RGCs associated with glaucoma, which can lead to neuroprotective treatments for diseases that involve α7nAChRs.

1. Introduction

Glaucoma is characterized as a neuropathic disease that causes damage to the optic nerve and progressive degeneration of retinal ganglion cells (RGCs) in the retina, resulting in irreversible loss of vision (Foster et al., 2002; Guo et al., 2005). All current treatments for glaucoma are focused on reducing an increase of intraocular pressure (IOP), the primary risk factor associated with glaucoma (Chauhan et al., 2002; Levkovitch-Verbin et al., 2002; Damji et al., 2003). Eye drop medications for glaucoma treatments decrease the production of aqueous humor or affect drainage of fluid through the trabecular meshwork. Surgical procedures cut small holes in the eye to drain the aqueous humor or lasers are used to produce holes in the trabecular meshwork to increase outflow of aqueous humor (Cairns, 1968). Eye drop or surgery treatment is also used in normotensive glaucoma patients, even when IOP measurements never exceed 22 mmHg (Mi et al., 2014; Jeong et al., 2014). However, these treatments alone are insufficient to halt the progression of blindness associated with glaucoma (Lickter et al., 2001; Heijl et al., 2002; Kass et al., 2002; Beidoe and Mousa, 2012; Jeong et al., 2014). As a result, new treatment options focused on preventing the loss of neurons from the retina are required.

In previous studies from Iwamoto et al. (2014), the loss of retinal ganglion cells (RGCs) was prevented in an *in-vivo* rat model of glaucoma when intravitreal injections of a specific α7 nicotinic acetylcholine receptor agonist (α7nAChR), N-[(3R)-1-azabicyclo [2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride (PNU-282987) (Bodnar et al., 2005; Hajós et al., 2005), were delivered into the vitreal chamber of the adult Long Evans rat eyes before a procedure to induce glaucoma-like conditions. RGCs contain acetylcholine (ACh) receptors (Keyser et al., 1988; Whiting et al., 1991; Keyser et al., 1993; Kaneda et al., 1995) and receive cholinergic input from a well-described population of starburst amacrine cells

* Corresponding author.
E-mail addresses: David_Mata@rush.edu (D. Mata), linnd@gvsu.edu (D.M. Linn), cindy.linn@wmich.edu (C.L. Linn).

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common to a majority of vertebrate retina (Massey and Redburn, 1987). They are the only source of ACh in the vertebrate retina. A growing body of evidence indicates that nAChRs play a key role in neuroprotection within the brain against several neurodegenerative diseases. Specifically, activation of α7 nAChRs in the brain have been linked to neuroprotection against several neurodegenerative diseases (Conejero-Goldberg et al., 2008; Liu et al., 2012). There is strong evidence that α7 nAChRs are neuroprotective, reducing β-amyloid induced toxicity in Alzheimer's disease (Kawamata and Shimohama, 2011; Oz et al., 2013) and that the α7 nAChRs plays a role in the pathophysiology of schizophrenia (Winterer et al., 2013; Young and Geyer, 2013). In the retina, pig and rat *in vitro* studies have demonstrated that the loss of RGCs normally associated with glutamate-induced excitotoxicity involves activation of α7 nAChRs (Wehrwein et al., 2004; Thompson et al., 2006; Iwamoto et al., 2013). In addition, intravitreal injections of the α7 nAChR agonist, PNU-282987, in adult Long Evans rats *in vivo* prevented the loss of RGCs normally associated with a procedure to induce glaucoma-like conditions (Iwamoto et al., 2014). However, the use of intravitreal injections is invasive and reduces the appeal of developing an α7 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 according to the method described by Morrison et al. (1997) to test the hypothesis that eye drop application of a specific α7 nicotinic acetylcholine receptor agonist can prevent loss of RGCs in an *in-vivo* adult rat glaucoma model. The effect of eye drop application on RGCs in the retinal ganglion cell layer was addressed and its advantages over systemic applications discussed.

2. Materials and methods

2.1. Animals

Adult Long Evans rats (males and females 3 months of age) were used for all *in vivo* studies. Rats were kept at Western Michigan University's animal facility until needed and were cared for in accordance with the approved guidelines of the Institutional Animal Care and Use Committee (IACUC).

2.2. Inducing glaucoma-like conditions

The procedure to induce glaucoma-like conditions in rats was modified from the procedure initially described by Morrison et al. (1997). These modified procedures were fully described in Iwamoto et al. (2014). Briefly, Long Evans rats were anesthetized with 0.1 ml/100 gm KAX via intraperitoneal injections until no reflexes were observed. KAX is a combination of 5 ml of ketamine (100 mg/ml), 2.5 ml xylazine (20 mg/ml), 1 ml acepromazine (10 mg/ml), and 0.5 ml sterile water. A topical anesthetic of 0.5% procaine hydrochloride was applied to the eye before the procedure to induce glaucoma. Once the animal exhibited no reflexes, the episcleral vein of the right eye in each experimental animal was injected with 50 μl of 2 M NaCl using a glass microinjection needle (Iwamoto et al., 2014). Injection of 2 M hypertonic saline caused blanching of the episcleral veins and subsequent scarring of the vascular system (Morrison et al., 1997). Visualization of blanching in the episcleral veins correlated directly with significant loss of RGCs after one month and an increase of intraocular pressure (IOP) (Morrison et al., 1997; Johnson and Tomarev, 2011; Iwamoto et al., 2014). Following the procedure to induce glaucoma-like conditions, animals were closely watched to ensure full recovery before being returned to the animal facility.

2.3. Eye drop application

Previous *in vivo* studies on adult Long Evans rats injected the α7 nAChR agonist, PNU-282987, directly into the vitreal chamber of adult Long Evans rats to gain access to the retina. In this study, the α7 nAChR specific agonist was introduced as eye drops to Long Evans rats prior to the procedure that induced glaucoma-like conditions. Four different concentrations of PNU-282987 were used in a dose-dependent study and included 100 μM, 500 μM, 1 mM, and 2 mM. These concentrations were 10 times the concentration used in studies where PNU-282987 was injected intravitreally (Iwamoto et al., 2014), as only a small percentage of any eye drop reaches the retina through the back of the eye. The ACh agonist was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution and then diluted in PBS. The eye drops were applied for three days before the injection of hypertonic solution, as previous studies demonstrated that the neuroprotective agent needed to be applied 3 days before an insult for neuroprotection to occur (Wehrwein et al., 2004; Iwamoto et al., 2013). Following surgery, eye drops were applied twice a day for one month based on previous LC/MS/MS studies that demonstrated evidence of PNU-282987 in the retina up to 12 h after intravitreal injections (Iwamoto et al., 2014). Animals were sacrificed after one month. Previous studies have demonstrated that significant loss of RGCs in the periphery (4 mM from the optic nerve head) occurred one month following the procedure to induce glaucoma (Iwamoto et al., 2014). Eye drops were delivered to the bulbar conjunctiva, which provided the best access for the eye drops to seep into the eye socket while also retaining consistency in the administration of PNU-282987. In vehicle control experiments, eye drops of sterile PBS containing up to 1% DMSO were delivered to the right eye of experimental rats 3 days before the procedure to induce glaucoma-like conditions and for 1 month following the procedure.

2.4. Labeling RGCs

After rats were exposed to PNU-282987 for a month, they were sacrificed and retinas were removed from the eyes. Rats were euthanized in a carbon dioxide chamber. Both left and right retinas were peeled off the back of eyecups after removal of the cornea, lens and vitreous humor. Care was taken to peel the retina off the back of the remaining eyecup in one piece to maintain anatomical and geographical orientation (Iwamoto et al., 2014). Whole retinas were then flat-mounted, pinned out in a sylgard dish with the RGC layer facing upward using cactus needles, and fixed in 10% formalin overnight at 4 °C. After the samples were fixed, the tissue was rinsed with PBS three times. To block nonspecific binding, the tissue was incubated in 2% BSA in PBS containing 0.02% saponin for 30 min at room temperature before applying the primary antibody (anti-Thy 1.1) to label RGCs. Anti-Thy 1.1 (mouse anti-rat, BD Biosciences) is a monoclonal antibody against glycoproteins found exclusively on the plasma membrane of RGCs in the retina (Barnstable and Drager, 1984). Preliminary serial dilution studies determined that optimal results were obtained when the primary antibody was diluted 1:300 in 0.02% saponin in PBS with 2% BSA. Fixed flat-mounted retinas were kept at 4 °C in a humidified chamber for 5 days. After 5 days, the retina was rinsed 3 times using PBS and incubated in fluorescent secondary antibody (goat anti-mouse IgG), Alexa Fluor 595 (Invitrogen/Molecular Probes), for visualization (1:300) for another 5 days. The retina tissue was then rinsed 3 times with PBS and mounted on glass slides using 50% PBS and 50% glycerol. Preliminary time and dose-dependent studies using the antibodies was performed to select the concentration and incubation period that produced optimal results.

In control studies, experiments were conducted to determine