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Neuroprotection of rat retinal ganglion cells mediated through alpha7 nicotinic acetylcholine receptors

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Abstract

Glutamate-induced excitotoxicity is thought to play an important role in several neurodegenerative diseases in the CNS. In this study, neuroprotection against glutamate-induced excitotoxicity was analyzed using acetylcholine, nicotine and the alpha7 specific nicotinic acetylcholine receptor agonist, PNU-282987, in cultured adult rat retinal neurons. Adult Long Evans rat retinas were dissociated and RGCs were isolated from all other retinal tissue using a two-step panning technique. Once isolated, RGCs were cultured under various pharmacological conditions to demonstrate excitotoxicity and neuroprotection against excitotoxicity. After three days, RGCs were immunostained with antibodies against the glycoprotein, Thy 1.1, counted and cell survival was assessed relative to control untreated conditions. 500 μ M glutamate induced excitotoxicity in large and small RGCs in an adult rat dissociated culture. After three days in culture with glutamate, the cell survival of large RGCs decreased by an average of 48.16% while the cell survival of small RGCs decreased by an average of 42.03%. Using specific glutamate receptor agonists and antagonists, we provide evidence that the excitotoxic response was mediated through AMPA/KA and NMDA glutamate receptors through an apoptotic mechanism. However, the excitotoxic effect of glutamate on all RGCs was eliminated if cells were cultured for an hour with 10 μ M ACh, 100 μ M nicotine or 100 nM of the α 7 nAChR agonist, PNU-282987, before the glutamate insult. Inhibition studies using 10 nM MLA or α -Bgt supported the hypothesis that neuroprotection against glutamate-induced excitotoxicity on rat RGCs was mediated through α 7 nAChRs. In immunocytochemical studies, double-labeled experiments using antibodies against Thy 1.1 and alpha7 nAChR subunits demonstrated that both large and small RGCs contained alpha7 nAChR subunits. The data presented in this study supports the hypothesis that ACh and nAChR agonists provide neuroprotection against glutamate-induced excitotoxicity in adult rat RGCs through activation of α 7 nAChR subunits. These studies lay the groundwork required for analyzing the effect of specific α 7 nAChR agonists using *in vivo* models of excitotoxicity. Understanding the type of ACh receptors involved in neuroprotection in the rat retina could ultimately lead to therapeutic treatment for any CNS disease that involves excitotoxicity.

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Conflict of interest:

The authors state no conflict of interest.

Keywords

neuroprotection; acetylcholine; excitotoxicity; retinal ganglion cells; retina; rat

Excitotoxicity is defined as neuronal cell death caused by over stimulation of receptors for the amino acid transmitter glutamate (Olney and de Gubareff, 1978; Michaelis, 1998). In the retina, diseases associated with excitotoxicity include retinal ischemia, diabetic retinopathy, and glaucoma (Quigley, 1998; Lafuente et al., 2001). Glaucoma is one of the leading causes of blindness in the world, and is characterized by optic neuropathy, cupping of the optic disk, degeneration of RGCs and eventual visual field loss. The primary risk factor associated with glaucoma is an increase in intraocular pressure. However, reduction in intraocular pressure is frequently insufficient to prevent progression of visual field loss (Baltmr et al., 2010). Rather, glutamate-induced excitotoxicity has been suggested to play an important role in glaucoma by some investigators (Vickers et al., 1995; Brooks et al., 1997; Dkhisssi et al., 1999; Nucci et al., 2005), while others have pointed out that the role of glutamate excitotoxicity in glaucoma remains elusive (Lotery, 2005). Glutamate is the major excitatory neurotransmitter released from bipolar cells onto ganglion cells in the vertebrate retina (Massey and Redburn, 1987; Copenhagen, 1991). However, relatively high concentrations of glutamate has been used to induce excitotoxicity in a variety of *in vivo* and *ex vivo* preparations (Romano et al., 1998; Saitoh et al., 1998; Luo et al., 2001; Kawasaki et al., 2002; Xin et al., 2007; Putkonen et al., 2011; Liu et al., 2011). Previous pharmacological studies from our lab have demonstrated that chronic exposure of 500 μ M glutamate to adult cultured porcine RGCs significantly reduced the survival of total RGCs compared to untreated conditions. Time-course and dose-response studies demonstrate that maximal excitotoxicity occurred when cells were cultured for 3 days in 500 μ M glutamate (Wehrwein et al., 2004). Using a combination of specific glutamate receptor agonists and antagonists for glutamate receptors, evidence was provided that the glutamate-induced excitotoxic effect in adult porcine RGCs was mediated through both NMDA and non-NMDA glutamate receptors. Based on these porcine studies and other labs that use glutamate to induce excitotoxicity, excessive chronic glutamate exposure was used in this study to induce excitotoxicity in cultured adult rat retinal neurons.

Using this glutamate-induced excitotoxic model, the neuroprotective effect of ACh and nicotinic agonists on RGCs in the rat retina will be examined. Besides glutamate receptors, RGCs contain ACh receptors and receive cholinergic input from a well-described population of starburst amacrine cells common to a majority of vertebrate retina. (Famigletti, 1983; Masland, 1988). Both ionotropic nicotinic ACh receptors (nAChR) as well as muscarinic ACh receptors that activate second messenger systems (Whiting et al., 1991; Keyser et al., 1993; Kaneda, 1995) have been described in the vertebrate retina. Nicotinic ACh receptors (nAChRs) are linked to the opening of non-specific cation channels, including calcium and sodium (Rogers and Dani, 1995; Burnashev, 1998; Ragozzino et al., 1998) and act to depolarize RGCs. A growing body of evidence indicates that nAChRs modulate actions within the CNS other than mediating fast cholinergic transmission. In particular, $\alpha 7$ nAChRs have been linked to neuroprotection against glutamate-induced excitotoxicity in the brain (Marin et al., 1994; Shimohama et al., 1996; Kaneko et al., 1997; Dajas-Bailador et al., 2000).

Results from our lab have also demonstrated that activation of nAChRs in adult porcine RGCs is linked to neuroprotection against glutamate-induced excitotoxicity. In the presence of 5 μ M ACh or 1 μ M nicotine, the excitotoxic effect of 500 μ M L-glutamate was significantly reduced. This neuroprotective effect of ACh was significantly reduced if isolated RGCs were pretreated with the $\alpha 7$ specific inhibitor, MLA (10 nM), or α -Bgt (10

nM) supporting the hypothesis that ACh's neuroprotective effect is at least partially mediated through $\alpha 7$ nAChRs (Wehrwein et al., 2004). In this current study, the neuroprotective effect of ACh and nicotine in rat retina was investigated to determine if agents that provided neuroprotection against glutamate-induced excitotoxicity in the porcine have a similar effect in rat retina. In addition, these studies analyze the role of $\alpha 7$ nAChRs in this system using a specific $\alpha 7$ nAChR agonist.

Neuronal AChRs are composed of multiple subunits that have been designated either α or β (Sargent, 1993; McGehee and Role, 1995). Different combinations of these subunits produce receptors with distinct ion conducting properties and distinct pharmacological properties. Previous immunocytochemistry studies have demonstrated that only $\alpha 7$ nAChR subunits are located on cultured large porcine RGCs (Thompson et al., 2006). The combination of pharmacology and immunocytochemistry results suggested that $\alpha 7$ nAChR subunits were responsible for ACh-induced neuroprotection on large RGCs in adult porcine. In the rat retina, nAChRs are abundant and at least seven heteromeric subtypes have been detected as well as homomeric pentamers containing $\alpha 7$ nAChR subunits (Marriott et al., 2005; Cox et al., 2008). These studies will be repeated in rat cultured cells using ACh, nicotine and a specific $\alpha 7$ nAChR agonist, PNU-282987, to confirm that ACh induces neuroprotection against glutamate-induced excitotoxicity in the rat model and that neuroprotection involves $\alpha 7$ nAChRs.

Binding studies in rat chimera cells using PNU-282987 (Pharmacia & Upjohn) have demonstrated that PNU-282987 is a potent specific agonist against the $\alpha 7$ nAChR. PNU-282987 displaced MLA, a specific $\alpha 7$ nAChR antagonist (Bodnar et al., 2005). 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). This specific drug was used in these studies to understand the role of the $\alpha 7$ nACh receptors on rat RGCs without worrying about cross reactivity or cross binding with other nAChRs that may make the analysis and interpretation of data difficult. Immunocytochemistry studies were conducted to support the hypothesis that rat RGCs contain $\alpha 7$ nAChR subunits.

In summary, the objective of this study was to confirm that ACh, nicotine and the $\alpha 7$ nAChR agonist, PNU-282987, provided neuroprotection against glutamate-induced excitotoxicity in adult rat RGCs. Characterization of excitotoxicity and neuroprotection was required before future studies could determine if these agents can provide neuroprotection against cell loss in an *in vivo* rat model of glaucoma or any disease of the retina associated with excitotoxicity. Identifying ACh as a neuroprotective agent in the retina has implications in understanding how the retina works and can lead to understanding mechanisms associated with disease states. Lastly, using PNU-282987 and MLA, we tested the hypothesis that neuroprotection on rat RGCs is mediated through $\alpha 7$ nAChRs. Understanding the type of ACh receptors responsible for neuroprotection in the rat retina can ultimately lead to therapeutic treatment for glaucoma in the retina that target a subtype of nAChRs and other diseases associated with excitotoxicity in the CNS.

Experimental Procedures

Animals

Adult male and female Long Evans rats (Charles River, Portage, MI USA), between the ages of 3–6 months, were used for these experiments. Long Evans are docile by nature and have prominent eyeballs, thus making the surgical manipulations easier to perform. In addition, visual deficits are not prominent in outbred Long Evans rats compared to other rat strains

(Jeffery et al., 1997). Each animal was kept in an animal colony, treated and euthanized according to IACUC protocol.

Preparation

Long Evans rats were euthanized by CO₂ asphyxiation followed by decapitation. After decapitation, eyes were removed, dipped in 70% alcohol and the cornea and lens were carefully removed from the eye to create an eyecup. The retina was then scraped off from the back of the eyecup using a flattened spatula and detached from the optic nerve. The remaining euthanized rat carcass was placed in a freezer until disposed of through an IACUC approved animal care facility.

Solutions and Materials

CO₂-independent culture media (Invitrogen) containing magnesium (complete formula is confidential) was used to for all experiments. The culture media was modified to contain 4mM glutamine (Sigma), 5% fetal bovine serum (FBS, Sigma), 5% antibiotic/antimycotic (Invitrogen), and 4mM HEPES (Sigma). All drugs were purchased from Sigma/Aldrich (St. Louis, MO USA) except for papain, which was obtained from Worthington Biochemicals (Lakewood, NJ), antibody against Thy 1.1, which was obtained from Pharminogen (San Diego, CA, USA), secondary antibodies conjugated to Alexa Fluor 595 or Alexa Fluor 488, which were obtained from Invitrogen/Molecular Probes (San Diego, CA, USA), and non-immune mouse immunoglobulin antibodies from Jackson ImmunoResearch (West Grove, PA, USA). All drugs were dissolved in culture medium.

Dissociation, isolation and culture

Retinas were dissociated using an enzymatic treatment that consisted of a papain solution (Worthington Biochemicals) (27 units/ mg) in modified CO₂-independent culture media. Retinas were enzymatically treated for 20 minutes in a 37° C water bath, inverted every 2–3 minutes to ensure proper reaction. To stop the enzymatic reaction after 20 minutes, fresh culture media containing DNase solution (Sigma) was substituted for the papain solution. Following papain treatment, the retinas were dissociated by gentle titration using sterile Pasteur pipettes.

Retinal tissue was then processed using a modified two-step panning technique to isolate the RGCs from other retinal cells (Wehrwein et al., 2004). Initially, enzymatically dissociated retinal cells were placed onto petri dishes containing goat anti-rabbit IgG antibody (Jackson ImmunoResearch; 0.5 mg in 48 ml of 20 mM Tris buffer) for one hour in a 37°C incubator to eliminate nonspecific binding. Afterwards, retinal tissue was transferred to petri dishes containing mouse anti-rat Thy 1.1 antibody (BD Biosciences; 10 µg of .5 mg/ml in 10 ml PBS containing zero calcium and zero magnesium) bound to goat anti-mouse IgM (Jackson ImmunoResearch; 0.3 mg in 48 ml of 20 mM Tris buffer). In the retina, the Thy 1.1 antibody selectively binds to glycoproteins found exclusively on RGCs (Barnstable and Drager, 1984). After an hour on the Thy 1.1-IgM plates, the supernatant in each of the petri dishes was discarded and the isolated RGCs that remained bound to Thy 1.1 in the petri dishes were released using 0.25% trypsin (Sigma) for 10 minutes at 37°C. Trypsin activity was terminated using 1 mg/ml soybean trypsin inhibitor (Sigma) and cells were strained. The cell density of the dissociated RGCs was calculated using a hemocytometer and cells were subsequently plated evenly at a density of 1×10^5 cells/ml in modified CO₂-independent medium into petri dishes for pharmacology studies or on coverslips placed at the bottom of petri dish wells for immunocytochemistry studies.

Pharmacological Treatments

For pharmacological studies, isolated rat RGCs were allowed to settle for 2 hours before replacing the culture medium with fresh modified CO₂-independent media containing additional supplements that enhanced cell survival and growth of processes (15 µg/ml NGF (nerve growth factor), 10 mg/ml insulin and 500 µg/ml transferrin (Wehrwein et al., 2004)). Cells were cultured and treated for 3 days, based on previous studies from this lab (Wehrwein et al., 2004; Thompson et al., 2006; Asomugha et al., 2010; Brandt et al., 2011) using porcine tissue and based on preliminary studies using cultured rat RGCs (Linn et al., 2011).

Acetylcholine (ACh), nicotine and the $\alpha 7$ nicotinic AChR agonist, PNU-282987, were added one hour prior to glutamate treatment. The $\alpha 7$ specific antagonist, methyllycaconitine (MLA), or α -bungarotoxin (α -Bgt) was added 30 minutes prior to the ACh or ACh agonists, and 1 ½ hours prior to 500 µM glutamate application. The timing of these agents has been previously worked out through preliminary studies and represents the time that produced the greatest effects (Wehrwein et al., 2004; Linn et al., 2011). The concentrations of agonists and antagonists reported in this study were obtained from previous dose-response experiments. In each case, the dose of agonist or antagonist that produced the maximal effect was used.

After three days in culture, cell viability was determined by incubating cells with 2µM calcein-AM (Molecular Probes, Carlsbad, CA) for one hour. Calcein labels the cell bodies of living viable cells through their esterase activity (Bozyczko-Coyne et al., 1993). Cells were photographed under a Nikon Diaphot epifluorescent research microscope illuminated by a 100-W mercury arc lamp with an excitation filters (EX 488–595), dichroic mirror (DM 580) and barrier filter (BA590).

A minimum of four pictures were taken from different identified quadrants in each culture dish containing fluorescently labeled RGCs using a Hamamatsu XC-77 CCD camera attached to a Nikon Diaphot epifluorescent research microscope illuminated with a 100-W mercury arc lamp with an excitation filter (EX 488–595), dichroic mirror (DM 580) and barrier filter (BA 590). Images were obtained using Metamorph Imaging system software (Universal Imaging). The number of surviving cells from each of the 4 images obtained from each dish were counted, averaged and compared to the internal untreated control counts. The average of 4 images obtained from each culture well represented an “N” of 1.

Identification of apoptotic and necrotic cells

Apoptosis of RGCs was examined using the ApoTag Red *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA) to assess terminal deoxynucleotidyl transferase-mediated dUTP nick-ending labeling (TUNEL) according to the manufacturer’s instructions. Isolated RGCs were plated for culture and labeled first with calcein and secondly for TUNEL staining immediately after plating (time 0), and then again after 1 and 3 days in culture. Isolated RGCs were visualized using the Nikon Diaphot epifluorescent microscope using filters for calcein fluorescent that labeled viable cells and for rhodamine fluorescence that labeled apoptotic RGCs. The number of calcein-positive and TUNEL-positive cells were counted from 4 identified sections from each culture well and averaged to represent an “N” of 1. The cell counts obtained from the 3 different time points were statistically compared.

Necrotic cells were imaged using ethidium homodimer to determine plasma membrane integrity in cultured RGCs under excitotoxic conditions. Ethidium is excluded by live cells, but enters dead cells and stains their nucleus red, which was visualized using rhodamine fluorescence. Cultured RGCs were labeled with ethidium using the LIVE/DEAD® Viability/Cytotoxicity Kit according to the manufacturer’s instructions (Molecular Probes).

RGCs were cultured in excitotoxic conditions for the same periods of time listed above in the apoptotic studies (0, 1 and 3 days), and counted for comparison in the same manner as the apoptotic studies.

Double-label immunocytochemistry

For immunocytochemistry studies, isolated RGCs cultured on coverslips were fixed with 10% formalin at room temperature for 30 minutes after 3 days in culture. Following fixation, RGCs were rinsed and blocked with 1% BSA in PBS. Fixed cells were then incubated in a primary monoclonal mouse anti-rat antibody against Thy 1.1 in PBS containing 0.1% BSA at a final dilution of 1:500. The slides were placed in a humidified chamber overnight at 4°C. The following day, the slides were rinsed 3 times in PBS. To visualize the cells, the RGCs were incubated with a secondary goat anti-mouse secondary antibody at 1:300 dilution that was conjugated to Alexa Fluor 488. The RGCs were incubated in secondary antibody overnight at 4°C. The following day, the RGCs already labeled with Thy 1.1 antibody were labeled with a second monoclonal antibody against the rat $\alpha 7$ nAChR subunit (1:3000, Sigma; mAb 319 subunit) for one day at 4°C., then secondarily labeled with goat anti-mouse Alexa Fluor 595 (1:300) overnight at 4°C. Labeled RGCs on coverslips were mounted on glass slides using a mixture of 50% glycerol/PBS before epifluorescent images were taken using the Nikon Diaphot epifluorescent microscope.

Large and small RGCs were identified using Metamorph Imaging system software (Universal Imaging). Negative control experiments were performed to examine antibody specificity. In some experiments, slides containing retinal tissue including RGCs were processed with the primary antibody omitted, while other experiments substituted non-immune mouse immunoglobulin (dilution: 0.1 – 1.0 μ g/ml) for the monoclonal antibody. In other control experiments, preabsorption studies were performed where the primary antibody was incubated with primary antigen before applying to tissue. No significant epifluorescence was observed in these control experiments. In other control experiments designed to test the specificity of the secondary antibodies, isolated rat RGCs were stained with a primary antibody against $\alpha 7$ nAChR subunits or against Thy1.1. The cells were subsequently stained with a secondary antibody labeled with Alexa Fluor 595. After the tissue was incubated overnight in Alexa Fluor 595, it was washed out and another secondary antibody labeled with Alexa Fluor 488 was applied. Under these conditions, cells were fluorescent only when using excitation filters for Alexa Fluor 595.

Data analysis and statistical procedures

The number of surviving cells treated under the various pharmacological conditions was compared to the number of cells incubated under untreated controls obtained from the same animals in each experiment. A two-way ANOVA was used to measure statistically significant cellular loss with Tukey posthoc tests using Prism Graphpad statistical software. On all normalized data, statistical analysis was performed using Kruskal-Wallis non parametric analysis of variance with post hoc comparisons (Dunn's test). $P < 0.05$ was considered statistically significant.

Results

Glutamate Excitotoxicity

Excessive glutamate application (between 100 μ M and 1 mM) has been used to induce apoptotic cell death of RGCs in a variety of preparations, including rat (Kitano et al., 1996; Fang et al., 2010; Seki et al., 2010). Figure 1 represents images obtained after isolated RGCs were treated and cultured with 500 μ M glutamate for 3 days. As shown in figure 1B and summarized in Table 1, the addition of 500 μ M glutamate to the culture medium

significantly decreased the total number of surviving RGCs by an average of 45.21% ($\pm$ 2.10) when compared to control untreated RGCs (fig. 1A and Table 1). Glutamate excitotoxicity affected both large and small RGCs in this culture system. Large and small RGCs could be distinguished based on the diameter of their cell bodies. The diameter of the soma in large RGCs averaged 24.22 μ m ($\pm$ 3.02), similar to results demonstrated by Huxlin and Goodchild (1997). These large RGCs are likely homologues to alpha cells described by previous authors (Boycott and Wässle, 1974; Perry, 1979; Dreher et al., 1985; Peichl, 1991). The diameter of the soma in small RGCs averaged 15.15 μ m ($\pm$ 3.14) and are likely the morphological equivalents of beta as well as gamma cells previously described (Boycott and Wässle, 1974; Rowe and Stone, 1977; Stone and Clarke, 1980; Stanford and Sherman, 1984; Purpura, 1990). After three days in culture with glutamate, the cell survival of large RGCs decreased by an average of 48.16% ($\pm$ 2.70, N=16) while the cell survival of small RGCs decreased by an average of 42.03% ($\pm$ 1.83, N=16). Since 500 μ M glutamate significantly affected both large and small RGCs, the remaining experiments counted and evaluated effects on the total number of RGCs.

Rat RGCs contain both AMPA/KA and NMDA receptors. The glutamate agonists, KA and NMDA, also produced excitotoxic effects when retinal cells were incubated with the glutamate ionotropic agonists. 100 μ M KA reduced cell survival by an average of 32.33% ($\pm$ 2.19), while 100 μ M NMDA decreased cell survival by an average of 5.28% ($\pm$ 4.03) in culture medium containing magnesium. As the majority of NMDA channels may be blocked by magnesium under culture conditions, some retinal tissue was incubated in culture medium containing a combination of 100 μ M KA and 100 μ M NMDA to determine if a combination of KA and NMDA would account for all of glutamate's excitotoxic effect, (Table 1). Under these conditions, cell survival decreased by an average of 46.23% ($\pm$ 3.54), thus mimicking the excitotoxic effect of glutamate and supports the hypothesis that KA and NMDA receptors are involved in glutamate's excitotoxic effect on RGC survival. To further support this, inhibition studies were performed where cultured rat retinal tissue was incubated with NMDA and KA glutamate receptor antagonists before application of 500 μ M glutamate. KA's partial excitotoxic effect was virtually eliminated in the presence of 10 μ M CNQX, while NMDA's partial excitotoxic effect was significantly reduced in the presence of 10 μ M MK-801 (Table 1). In addition, as seen in fig. 1C and summarized in Table 1, when the cultured cells were incubated with a combination of the KA receptor antagonist, CNQX (10 μ M), and the NMDA antagonist, MK801 (10 μ M), for 30 mins before applying glutamate agonist, the toxic effect of applying glutamate or a combination of KA/ NMDA was eliminated. These results support the hypothesis that the excitotoxic effect of glutamate in isolated adult rat RGCs is mediated through both AMPA/KA and NMDA glutamate receptor subtypes.

Apoptosis, not necrosis

To understand whether the decrease of RGCs induced by exogenous glutamate is due to cell death through an apoptotic pathway, isolated RGCs exposed to 500 μ M glutamate were examined using calcein to label living viable cells and TUNEL technique to assess apoptosis. Fig. 2A and 2B represent RGC images obtained from the same culture dish immediately after plating. In Fig 2A, the isolated RGCs labeled with calcein are viewed. In fig. 2B, the same cells were processed using TUNEL technique but no TUNEL positive cells were seen. In Fig. 2C and 2D, isolated RGCs from another culture dish were allowed to culture in 500 μ M glutamate for 1 day before staining cells with calcein and ApoTag Red. After one day in culture with 500 μ M glutamate, TUNEL positive cells are present (Fig. 2D). RGCs labeled with calcein (fig. 2C) were not the same cells that labeled with the ApoTag Red detection system as calcein labels viable healthy cells, whereas the TUNEL assay labels apoptotic cells. The percent of TUNEL positive cells increased after 3 days in

culture with 500 μ M glutamate. As shown in fig. 2E, 500 μ M glutamate caused a significant loss of isolated RGCs. This correlated with a significant increase in the number of TUNEL positive cells identified in the same dish. Table 2 summarizes the number of TUNEL positive cells counted at the 3 different time points described above.

To determine if application of 500 μ M glutamate also triggered necrosis in cultured rat RGCs, cells cultured for 0, 1 and 3 days were labeled with ethidium homodimer to label necrotic cells using the Viability/Cytotoxicity Kit (Molecular Probes) and visualized using rhodamine fluorescence. Unlike the results obtained using ApoTag Red, there was no evidence of significantly labeled nuclei using ethidium homodimer. Table 2 summarizes the number of necrotic cells counted at 0, 1 and 3 days in culture.

ACh Neuroprotection

The next experiments were designed to determine if ACh, nicotine and the $\alpha 7$ nAChR agonist, PNU-282987, provided neuroprotection against glutamate-induced excitotoxicity in cultured rat retina and to determine the distribution of $\alpha 7$ nAChRs on rat RGCs. To determine if ACh or nicotine provided neuroprotection against glutamate-induced excitotoxicity in rat, retinas were dissociated and isolated RGCs were cultured for 3 days under the various pharmacological conditions summarized in figure 3 as bar graphs. Each bar graph represents the average percent of RGC survival compared to control untreated conditions. 500 μ M glutamate decreased total RGC survival by an average of 45.21% (+/- 2.10) compared to control untreated conditions. However, if the dissociated retinal tissue was pretreated with 100 μ M nicotine or 10 μ M ACh for 1 hour before application of 500 μ M glutamate, glutamate's excitotoxic effect was eliminated. RGCs pretreated with nicotine resulted in an average of 102.22% (+/- 6.23) cell survival against glutamate-induced excitotoxicity compared to control untreated conditions while RGCs pretreated with 10 μ M ACh resulted in an average of 106.29% (+/- 5.78) cell survival compared to untreated conditions. This neuroprotection did not occur if ACh or nicotine was applied at the same time as 500 μ M glutamate or if agents were applied after the glutamate insult. Neuroprotection in this model only occurred if cells were preconditioned with ACh or nicotine before the glutamate insult.

The neuroprotective effect of nicotine and ACh was dose dependent. Significant neuroprotection against glutamate-induced excitotoxicity was demonstrated if RGCs were pretreated with 10 μ M nicotine before glutamate insult. Pretreatment of RGCs with 10 μ M nicotine before the glutamate insult resulted in an average increase of 14.68% (+/- 3.39) in cell survival compared to the internal control. The EC_{50} value calculated for nicotine's effect equaled 56.88 μ M. As shown in figure 3, 100 μ M nicotine produced a maximal neuroprotective effect against glutamate-induced excitotoxicity. ACh's neuroprotective effect was significant when RGCs were cultured with 1 μ M ACh before the glutamate insult, providing an average increase of 12.35% (+/- 4.2) compared to untreated conditions. The EC_{50} for ACh's neuroprotective effect measured 6.44 μ M and ACh produced a maximal neuroprotective effect if cells were pretreated with 10 μ M ACh before glutamate insult (fig. 3).

In previous porcine studies, when the $\alpha 7$ nAChRs antagonist, MLA, was applied to porcine RGCs before ACh or nicotine, 10 nM MLA only provided a partial block of ACh's neuroprotective effect (Wehrwein et al, 2004). However, in rat, preincubation of RGCs with 10 nM MLA or α -Bgt blocked the ACh or nicotine-induced neuroprotection by 80 - 98% (right bars in fig. 3), suggesting that ACh and nicotine-induced neuroprotection is primarily mediated through $\alpha 7$ nAChRs in rat RGCs.

Effects of PNU-282987 *in vitro*

If neuroprotection against glutamate-induced excitotoxicity in the rat is mediated through $\alpha 7$ nAChRs, a specific $\alpha 7$ nAChR agonist should mimic the effect of ACh and nicotine. For these studies, isolated RGCs were evenly plated into culture wells and incubated with various concentrations of the specific $\alpha 7$ nAChR agonist, PNU-282987 for an hour before application of 500 μ M glutamate. After 3 days in culture, the RGCs were fluorescently labeled with 2 μ M calcein AM and the degree of neuroprotection was assessed. Fluorescent images obtained after 3 days in culture are shown in fig. 4 and the results of using various concentrations of PNU-282987 are summarized in fig. 5. In figure 4, panel A represents an image obtained from fluorescently labeled RGCs after 3 days in culture under control untreated condition. In panel B, the effect of 500 μ M glutamate is shown and demonstrates a significant loss of RGCs. However, if the dissociated retina is preincubated with 100 nM PNU-282987 for an hour before the glutamate insult, the excitotoxic effect of 500 μ M glutamate was significantly reduced (fig. 4C and fig. 5) and RGC counts returned to control untreated levels. The effect of PNU-282987 was dose-dependent. 1nM PNU-282987 had no significant neuroprotective effect on RGC survival. However higher concentrations of PNU-282987 between 10 and 200 nM led to a dose-dependent increase of RGCs against glutamate-induced excitotoxicity (fig. 5). The EC₅₀ for PNU 282987's neuroprotective effect was calculated to be 55.3 nM and maximal effects were recorded when RGCs were incubated in 100 nM for an hour before application of the glutamate insult (fig. 5).

To further demonstrate that the effects of PNU-282987 on rat RGCs were mediated through $\alpha 7$ nAChRs, in some experiments, the isolated retinal cells were pretreated with 10 nM MLA after RGCs were evenly distributed into culture wells for culture. The typical result of experiments using 10 nM MLA are illustrated in fig. 4D. If 10 nM MLA was applied 30 minutes before 100 nM PNU-282987, neuroprotection against glutamate-induced excitotoxicity was reduced by an average of 83.5% (+/- 6.2; N=6) compared to control untreated conditions. These results supports the hypothesis that PNU-282987 mimics neuroprotection against glutamate-induced excitotoxicity through $\alpha 7$ nAChRs.

The effect of PNU-282987 on large and small RGCs is demonstrated in fig. 6. In previous porcine studies, $\alpha 7$ nAChRs were only found to be localized on large RGCs (Thompson *et al.*, 2006). However, as seen in the line graphs illustrated in fig. 6 A and B, PNU-282987 provided significant neuroprotection against glutamate-induced excitotoxicity in both large and small RGCs when cultured for 3 days with the $\alpha 7$ agonist. However, the neuroprotective effect of PNU-282987 on large RGC (fig. 6A) was significantly different than the neuroprotective effect on small RGCs (fig 6B). When large RGCs cells were cultured with 100 nM PNU-282987 for three days, the PNU-282987 provided complete neuroprotection against glutamate excitotoxicity compared to control untreated conditions (fig. 6A) (103.3% +/- 3.2). On small RGCs, the maximal neuroprotective effect of PNU-282987 was significantly different compared to glutamate's effect, but did not provide protection back to control untreated conditions no matter what concentration of PNU-282987 was used (fig. 6B) (79.4% +/- 6.5). In fig. 6C, the effect of PNU-282987 against glutamate-induced excitotoxicity was summarized for all RGCs. If RGCs are treated with PNU 282987 before 500 μ M glutamate and cultured for 3 days, total RGC survival increases to an average of 93.2% (+/- 5.1) compared to control untreated conditions. These results provide evidence that both large and small RGCs contain $\alpha 7$ nAChRs, as the specific $\alpha 7$ nAChR agonist, PNU-282987, provided significant neuroprotection against glutamate-induced excitotoxicity.

Results obtained from immunocytochemistry studies supported the evidence that $\alpha 7$ nAChR subunits are located on both large and small rat RGCs. In these double-label experiments, monoclonal antibodies against $\alpha 7$ nAChR subunits and against the glycoprotein, Thy 1.1,

were used to label $\alpha 7$ receptors and RGCs respectively. The result from one experiment is shown in figure 7. Panel 7A, B and C demonstrate that all RGCs isolated using the panning technique labeled with antibodies against $\alpha 7$ nAChR subunits and RGCs. Panel A represents a view of isolated RGCs that were labeled with a primary antibody against $\alpha 7$ nAChR subunits, then secondarily labeled with Alexa Fluor 595. The same group of cells were also labeled with an antibody against Thy 1.1 to label RGCs and secondarily labeled with Alexa Fluor 488 (fig. 7B). Figure 7C demonstrates the co-localization $\alpha 7$ nAChR subunits and the Thy 1.1 glycoprotein on the same cells. This supports the hypothesis that all panned RGCs contain $\alpha 7$ nAChR subunits. These results were repeated 6 times.

To demonstrate the specificity of the primary antibodies in another experiment, the entire retina was dissociated and cultured for 3 days without isolating the RGCs. After 3 days, the cultured retinal tissue was double-labeled with the antibodies against $\alpha 7$ nAChR subunits and Thy 1.1. Panel 7D represents a view of the cultured tissue after labeling with the primary antibody against $\alpha 7$ nAChR subunits and secondarily labeled with Alexa Fluor 595. Only a subset of cells labeled. In panel 7E, the same view of the culture tissue is shown after being double-labeled with the primary antibody against Thy 1.1 to label RGCs. In panel 7F, the co-localization of these fluorescent images is shown. Although some of the tissue double-labeled with both antibodies, a number of cells that labeled with the antibody against $\alpha 7$ nAChR subunits did not label with the antibody against Thy 1.1 (fig. 7F). This would be expected as other cells in the retina besides RGCs have been shown to contain $\alpha 7$ nAChR subunits. On the other hand, all cells that labeled with the antibody against Thy 1.1 labeled with the antibody against $\alpha 7$ nAChR subunits. These experiments were repeated 3 times with the same results.

Discussion

Excitotoxicity

This study was designed to test the neuroprotective properties of ACh, nicotine and a specific nAChR agonist using a cell culture model of excitotoxicity. The excitotoxic effect of glutamate on cultured RGCs was previously examined using porcine RGCs (Wehrwein et al., 2005, Luo et al., 2001). In these studies, experiments were replicated to determine if rat RGCs were affected by excessive glutamate in a similar manner. The *in vitro* results demonstrate that 500 μ M glutamate causes an average of ~45% cell loss in 3 days in dissociated rat retinal cell culture. Significant cell loss due to excessive glutamate concentrations occurred in both large and small RGCs, though the small RGCs were more resistant to toxic conditions.

Both NMDA and non-NMDA glutamate receptors have been identified on rat RGCs (Muller et al., 1992, Hamassaki-Britto et al., 1993; Brandstatter et al., 1994; Zhang et al., 2006). Here we demonstrate that glutamate's excitotoxic effect was mediated through both AMPA/KA and NMDA type glutamate receptors. When excitotoxicity was induced using KA alone, RGC survival decreased by an average of ~34%. Although this represented a significant decrease in RGC survival, it did not mimic the effect of glutamate. NMDA also induced significant excitotoxicity of RGCs by an average of ~23% from control untreated conditions, but again, NMDA alone did not completely mimic the effect of glutamate on RGC survival. However, glutamate's excitotoxic effect was mimicked if a combination of KA and NMDA was applied to cultured retinal cells. When cells were cultured in a combination of NMDA and KA, cell survival decreased by an average of ~43% compared to an average of ~45% due to 500 μ M glutamate. In addition, if the AMPA/KA and NMDA receptor antagonists were applied to cells before KA and NMDA, the excitotoxic effect was eliminated. This supports the hypothesis that glutamate's excitotoxicity in rat cultured RGCs is mediated through multiple ionotropic glutamate receptors, similar to results obtained in porcine retina

(Wehrwein et al., 2004) and that the amount of RGC death associated with glutamate-induced excitotoxicity in our system is similar to the percent of RGC death in excitotoxic rat cultures reported in other studies (Pang et al., 2007).

The exact mechanism of cellular death due to glutamate in rat RGCs is still unknown. Previous studies have demonstrated that excessive glutamate release in the eye leads to prolonged influx of non-specific cations in retinal ganglion cells (RGCs) of the retina and triggers intracellular signaling cascades leading to apoptosis and loss of the visual field (Lam et al., 1999; Quigley, 1998). A variety of signaling pathways leading to apoptosis in other systems have been identified. (Tenneti et al., 1998; Pavlovic et al., 2000; Okamoto et al., 2000; Kirkland et al., 2007; Fang et al., 2008; Boosani et al., 2009). However, previous porcine studies have linked overstimulation of glutamate receptors to apoptosis (Asomugha et al., 2010). These studies found that excessive glutamate triggered p38 MAP kinase to initiate an apoptotic cascade by inhibiting Bcl₂ and the anti-apoptotic role of Bcl₂ (Asomugha et al., 2010). Other studies have analyzed the glutamate excitotoxic pathway in rats after the release of mitochondrial cytochrome c and have demonstrated that glutamate-induced apoptotic cell death in adult rat retina is mediated by a specific activation of cysteine proteases related to the caspase-3 family (Chen et al., 2001).

Still other studies have demonstrated that excitotoxic cell death occurs by both necrotic and apoptotic mechanisms (Lipton, 2004; D'Orsi et al., 2012; Henry et al., 2013) in a variety of preparations including disease models, sliced cortical neurons and under culture conditions. However, in the isolated rat RGC culture system used in this study, there was no evidence of necrotic cell death associated with inducing excitotoxicity through AMPA/KA or NMDA channels. The lack of necrotic cell loss due to excitotoxicity under these culture conditions has also been shown using the same isolation technique using panned porcine RGCs (Wehrwein et al., 2004) and is likely due to the nourishing environment provided by the supplemented culture media.

Neuroprotection

In previous studies using adult cultured porcine RGCs, ACh and nicotine were found to provide neuroprotection against glutamate-induced excitotoxicity (Wehrwein et al., 2004; Thompson et al., 2006). We wanted to replicate these results in adult rat tissue to verify that the neuroprotective effect previously recorded was not species specific. Both ACh and nicotine provided complete neuroprotection in the rat model though the affinity to nicotine differed from porcine results. In the porcine studies, 1 μ M nicotine provided maximal neuroprotection against 500 μ M glutamate. To obtain 100% neuroprotection in adult rat RGCs, retinal tissue had to be incubated with 100 μ M nicotine before glutamate treatment. The concentrations to elicit maximal responses with ACh were also different between the two species. To obtain maximal neuroprotection in porcine, only 5 μ M ACh was needed. However, in adult rats, 10 μ M ACh was needed. Therefore, although ACh and nicotine provided significant neuroprotection against glutamate-induced excitotoxicity in adult rat RGCs, there were differences in concentrations needed to produce the effect. Although the literature on nAChRs demonstrate that it is more likely for nicotine to have a higher potency than ACh, many studies analyzing the rank order of potency have found ACh to be more potent, or equal, to nicotine's potency (Fischer et al., 2005; Amtage et al., 2004; Zhou et al., 2002; Evans et al., 2003; Colquhoun et al., 1991).

The endogenous neurotransmitter released from starburst amacrine cells is ACh. However, choline is a product of ACh hydrolysis and has been shown to have affinity for nAChRs (Strang et al., 2007). In this study, it is improbable that choline is directly activating ACh-induced neuroprotection through nAChRs, due to the concentrations of ACh, nicotine and PNU-282987 found to induce neuroprotection. Previous studies using isolated porcine RGCs

demonstrated that choline has relatively high affinity but low potency for RGCs compared to ACh or nicotine. Significant neuroprotection occurred only when cells were incubated with 1 mM choline (Wehrwein et al., 2004). In this current study, isolated rat RGCs were cultured with neuroprotective agents in the nM range. Choline would therefore likely have no significant neuroprotective effect on isolated RGCs.

ACh and nicotine neuroprotective effects were eliminated if cells were pretreated with the $\alpha 7$ nAChR antagonist, MLA (10 nM), suggesting that neuroprotection was mediated through $\alpha 7$ nAChRs. To provide evidence of this, we used the specific $\alpha 7$ nAChR agonist, PNU-282987, to determine if PNU-282987 would mimic the neuroprotective effect of ACh and nicotine. Our studies using PNU-282987 support the hypothesis that ACh's neuroprotective effect is mediated by activation of $\alpha 7$ nAChRs on rat RGCs. Previous radioligand binding studies and studies using immunoprecipitation with subunit-selective antibodies have demonstrated that rat RGCs contain multiple nAChR subtypes (Cox et al., 2008). These include mixed heteromeric subtypes, all of which contain the $\beta 2$ nAChR subunit, as well as homomeric $\alpha 7$ nAChR subtypes. The results using PNU-282987 on adult cultured rat RGCs suggest that ACh-induced neuroprotection is mediated entirely through $\alpha 7$ nAChRs, as 100 nM PNU-282987 mimics the effect of 10 μ M ACh. This is in contrast to results obtained from previous porcine studies, where activation of $\alpha 7$ nAChRs as well as $\alpha 4\beta 2$ subunits provided neuroprotection against glutamate-induced excitotoxicity. In these previous porcine immunocytochemical studies, large RGCs exclusively immunostained with antibodies against $\alpha 7$ subunits, whereas the smaller RGCs double-labeled with antibodies against $\alpha 4\beta 2$ subunits (Thompson et al., 2006). It still remains to be determined how stimulation of any other nicotinic subunits leads to neuroprotection against glutamate-induced excitotoxicity. However, as stimulation of $\alpha 7$ nAChRs with PNU-282987 mimics the effect of ACh completely, it is likely that the other mixed types of nAChR subunits found on rat RGCs do not significantly contribute to the response.

In previous studies using isolated porcine RGCs, ELISA studies demonstrated that ACh activation of $\alpha 7$ nAChRs triggered activation of the PI3 $\rightarrow$ Akt $\rightarrow$ Bcl₂ signaling cascade to provide neuroprotection against glutamate induced excitotoxicity (Asomugha et al. 2010). This previous study hypothesized that calcium influx through ionotropic $\alpha 7$ nAChR channels was involved in triggering this intracellular cascade to induce neuroprotection. Other studies have demonstrated that the link between glutamate receptor activation and signaling cascade initiation is calcium permeation through glutamate channels (Lam et al. 1999; Quigley et al., 1995). Therefore, it is likely that permeation of extracellular calcium through nAChR channels is the key to inducing neuroprotective pathways in isolated rat RGCs. In this scenario, calcium is either triggering activation of neuroprotective signaling cascades or may induce neuroprotection through other mechanisms, such as internalization of NMDA receptors (Schubert and Akopian, 2004; Akopian et al., 2006; Christofanelli and Akopian, 2006). As extracellular calcium is also required to trigger glutamate-induced excitotoxicity, further calcium imaging studies performed in isolated porcine RGCs demonstrated that relatively low levels of calcium permeate quickly desensitizing $\alpha 7$ nAChRs to precondition the RGCs and initiate activation of the cell's neuroprotective pathways; whereas 500 μ M glutamate induced a significantly larger intracellular larger calcium response to trigger apoptosis (Brandt et al., 2011). Although the present studies in rat RGCs support the hypothesis that $\alpha 7$ nAChRs are involved in ACh-induced neuroprotection, further studies are currently underway to analyze the specific mechanisms that lead to neuroprotection in rat RGCs.

Neuroprotection in Alzheimer's disease

Excitotoxicity and apoptosis has been associated with glaucoma in the eye and other neurodegenerative disease in the brain, such as Alzheimer's (AD) disease. Because of this, a

great deal of research has focused on neuroprotective approaches to prevent cell death. For example, in the retina, to prevent excitotoxicity, inhibition of NMDA receptors with specific antagonists (Pang et al., 1999), neuromodulation of the NMDA receptor with $\alpha 2$ agonists (Dong et al., 2008) and inactivation of caspase enzymes (Seki et al., 2010) have all been linked to neuroprotection against glutamate insult. In our study, we demonstrated that activation of $\alpha 7$ nAChRs before the glutamate insult provided neuroprotection against a glutamate insult and excitotoxicity.

The link between $\alpha 7$ nAChRs and neuroprotection has also been shown before in the brain. In Alzheimer's disease (AD), neurodegeneration is caused by apoptosis of the neuronal cells (Niikura et al., 2006). One of the characteristics associated with the disease is the development of plaque formation in the brain due to β -amyloid proteins (Selkoe and Schenk, 2003; Mattson, 2004). Formation of plaques are neurotoxic and result in loss of cognitive functions. However, early epidemiological studies demonstrated that nicotine decreased the risk for AD (Fratiglioni and Wang, 2000). This negative association was demonstrated in postmortem studies showing significant reduction of amyloid plaque deposits in former smokers with AD (Hellstrom-Lindahl et al., 2004a). Nicotine treatment appears to interfere with the formation of the amyloid plaques *in vitro* and *in vivo* (Hellstrom-Lindahl et al., 2004a; Ono et al., 2002; Utsuki et al., 2002) and to reduce the accumulation of insoluble A β peptides (Nordberg et al., 2002) through a mechanism mediated by $\alpha 7$ nAChRs. Inhibition studies in neuroblastoma cells used the $\alpha 7$ nAChR antagonist, mecamylamine, to block nicotine's effect in transgenic mice containing Alzheimer's disease (Hellstrom-Lindahl et al., 2004b). Thus, the $\alpha 7$ nAChR is linked to neuroprotection against diseases associated with excitotoxicity in the brain and retina.

Implications

This study provides evidence that ACh and nAChR agonists activate neuroprotection against excitotoxicity in a culture system. In the retina, cholinergic amacrine cells release ACh onto RGCs. Cholinergic amacrine cells are known as starburst amacrine cells due to their unique morphology, resembling exploding fireworks (Famiglietti, 1983; Masland, 1988) and are common to the vertebrate retina. Cholinergic starburst neurons are equally divided in the retina, with one group in the inner nuclear layer of the retina and the other group lying in the ganglion cell layer. Physiologically, these starburst amacrine cells receive strong excitatory input from bipolar cells and synapse onto RGCs (Masland et al., 1984; Mariani and Hersh, 1988). They are the only source of ACh in the vertebrate retina. Previous studies of these starburst amacrine cells have demonstrated that they are involved in directional selectivity of ganglion cell responses and play a role in information processing that stabilizes image motion (Barlow and Levick, 1965; Famiglietti, 1992; Yoshida et al., 2001). Could ACh released from these amacrine cells also act to provide neuroprotection against excitotoxicity in the retina under physiological conditions? If so, this would represent a previously undescribed function associated with cholinergic amacrine cells.

If ACh normally acts as a neuroprotective agent *in vivo*, what happens to this ACh-induced neuroprotection in retinal diseases associated with excitotoxicity, such as glaucoma? To address this issue, ACh release from starburst cholinergic amacrine cells needs to be analyzed in the intact retina. In these *in vivo* studies, ACh release from the cholinergic amacrine cells in control untreated rat eyes would be compared to ACh release from amacrine cells in glaucomatous eyes to determine if ACh release is significantly different under the disease state. Even though previous reports have demonstrated that cholinergic amacrine cells aren't lost in glaucoma (Kielczewski et al., 2005), it is possible that ACh release is compromised, that the neuroprotective defense mechanism is overwhelmed by other mechanisms occurring in the glaucoma state, or that ACh receptors in the RGC layer are altered. For instance, a study using the AChE inhibitor, galantamine, has been linked to

neuroprotective effects in glaucoma models (Almasieh, 2010). All of these scenarios are currently being analyzed in our lab using in an in vivo rat model of glaucoma.

Potential application

The main risk factor associated with glaucoma is an increase in IOP. All current therapies, whether they are pharmaceutical or surgical, are designed to decrease the IOP. However, other characteristics of the disease involve loss of RGCs and likely excitotoxicity. The results from this study provide evidence that the specific $\alpha 7$ nAChR agonist, PNU-282987, can be used to prevent excitotoxicity and loss of RGCs. If this agent is found to have similar effects *in vivo*, future treatments for glaucoma could include use of this agent to address the excitotoxic characteristics of the disease instead of concentrating exclusively on controlling IOP. This would represent a drastic change from current conventional treatments. One advantage of using PNU-282987 in this manner is that it could potentially benefit patients regardless of IOP. For instance, some patients have IOP measurements that fall within a normal range, however they develop a typical glaucomatous disc and visual field changes that represent an open angle form of glaucoma (Levene, 1980; Caprioli and Spaeth, 1984; Chauhan et al., 1989; Thonginnetra et al., 2010). In the United States, approximately 15–25% of people with open angle glaucoma have normal IOP measurements. As a result, treatments that reduce IOP can have little effect on these patients. An alternate treatment that targets excitotoxicity and loss of RGCs in the retina may be more beneficial.

However, in our studies, we found that ACh, nicotine and PNU-282987 had to be applied before an excitotoxic insult to provide neuroprotection. If these agents were applied with 500 μ M glutamate or after the glutamate insult, no neuroprotection occurred with rat or porcine RGCs (Wehrwein et al., 2004). The maximal neuroprotective effect occurred when the agonists were applied 1 hour before the glutamate insult. Although nAChRs desensitize relatively quickly (Liu and Berg, 1999; Alkondon and Albuquerque, 1993), the hour preincubation with agonist was required to maximally activate the intracellular neuroprotective mechanisms associated with this process (Asomugha et al., 2010). As a result, these agents would not likely be used to treat patients that already have glaucoma, but could potentially be used to precondition RGCs before any glaucoma-like conditions occur. Therefore, use of the $\alpha 7$ nAChR agonist, PNU-282987, would be best used for a patient with a family history of glaucoma and as a preventative measure for people at high risk based on age, genetics or race.

In conclusion, the pharmacological data presented in this study supports the hypothesis that ACh and nAChR agonists provide neuroprotection against glutamate-induced excitotoxicity in adult rat RGCs. In addition, pharmacology studies using PNU-282987 and MLA as well as immunocytochemistry results demonstrate that the neuroprotective effect is likely mediated through activation of $\alpha 7$ nAChR subunits. Understanding the type of ACh receptors involved in neuroprotection in the rat retina could ultimately lead to therapeutic prevention of glaucoma in the retina or for any CNS disease that involves excitotoxicity.

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ABBREVIATIONS

ACh	acetylcholine
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

α-Bgt	alpha-bungarotoxin
α7nAChR	alpha7 nicotinic acetylcholine receptor
ANOVA	analysis-of-variance
Bcl₂	B-cell lymphoma protein
NGF	nerve growth factor
CNS	central nervous system
IACUC	institutional animal care and use committee
IgG	immunoglobulin G
IgM	immunoglobulin M
IOP	intraocular pressure
KA	kainic acid
MAP	mitogen activated protein
MLA	methyllycaconitine
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-d-aspartate
PI3	phosphatidylinositol 3-kinase
PNU-282987	N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride
RGC	retinal ganglion cell
Thy	glycoprotein originally identified in thymus gland
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-ending labeling

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Highlights

- Activation of alpha7 nAChR induces neuroprotection
- PNU 282987 protects RGCs from glutamate-induced excitotoxicity
- Rat RGCs contain alpha7 nAChR subunits
- Implications for diseases of the CNS associated with excitotoxicity

Figure 1. Effects of glutamate on adult cultured RGCs

Glutamate causes significant cell loss in cultured adult rat RGCs, but the neurotoxic effects are blocked by CNQX and MK-801. (A) represents cultured immunostained RGCs under control untreated conditions that were cultured for 3 days. (B) represents cells cultured for 3 days with 500μM glutamate, and (C) depicts labeled RGCs pretreated with both MK-801 and CNQX before addition of 500 μM glutamate.

Figure 2. Evidence of apoptosis in isolated adult rat RGCs

Cultured adult rat RGCs were plated at 1×10^5 cells/ml and cultured for various time points in the presence of 500 μ M glutamate. After days 0, 1 and 3, cells were labeled with calcein AM (left column) to label living cells and processed for TUNEL staining (right column) to label cells undergoing apoptosis. Figs. A and B represent images of the same cells obtained at Day 0. Figs. C and D represent images of the same cells after 1 day in culture and figs E and F represent images of the same cells after 3 days in culture with 500 μ M glutamate.

Figure 3. ACh and nicotine provide neuroprotection against glutamate-induced excitotoxicity
 Each bar graph represents the average percent of RGCs survival compared to untreated conditions after cells were cultured for 3 days under various pharmacological treatments. MLA and α -Bgt were applied 30 minutes before ACh or nicotine. ACh or nicotine was applied to culture for 1 hour before 500 μ M glutamate. Averages were obtained from between 9–20 animals. The star represents significance from control. The solid circle represents significance from glutamate's effect. Experiments were repeated between 3 and 8 times. Error bars represent SEM.

Figure 4. Effects of PNU-282987 on isolated rat RGCs

PNU-282987 provides neuroprotection against glutamate-induced excitotoxicity. Images of labeled RGCs were obtained after 3 days in culture under the following conditions: (A) under control untreated conditions. (B) in 500μM glutamate. (C) pretreated with 100 nM PNU-282987 for 1 hour prior to 500 μM glutamate and (D) when the specific $\alpha 7$ antagonist, MLA (10nM), was applied 30 minutes prior to PNU-282987 and 1 1/2 hours prior to 500 μM glutamate. After 3 days in culture, cells were labeled with 2 μM calcein AM for 1 hour to label viable RGCs.

Figure 5. Dose dependent effects of PNU-282987 on rat RGCs

Each solid circle represents the average percent of PNU-induced neuroprotection against glutamate-induced excitotoxicity compared to control untreated conditions using various concentrations of PNU-282987 that were introduced to cultured RGCs before glutamate insult. PNU-282987 was applied one hour before the glutamate insult. 100nM PNU-282987 showed the highest degree of neuroprotection. The dose response data points were curve fit. Error bars represent SEM. Averages were obtained from N's of 3 to 20.

Figure 6. PNU-282987 provides neuroprotection in large and small RGCs

PNU-282987 caused an increase in the percent of RGC survival in both large and small RGCs if applied to cultures one hour before 500 μ M glutamate. RGCs were cultured for 3 days before quantification processing. Each bar graph represents the average percent of RGC survival compared to control untreated conditions. Bar graphs shown in fig. 6A represent the effect of glutamate and PNU-282987 on large RGCs, fig 6B represent pharmacological effects on small RGCs while fig. 6C represent the effect of glutamate and PNU-282987 on the total RGC population. The star represents significance from control untreated conditions, while the solid circle represents significance from glutamate. Normalized bar graphs were obtained from between 9–20 rats. Error bars represent SEM.

Figure 7. $\alpha 7$ nAChRs on large and small rat RGCs

Panels A, B and C represent images obtained from the same isolated RGCs double-labeled with primary antibodies against $\alpha 7$ nAChR subunits (left panels) and RGCs (center panels). Panel A illustrates RGCs secondarily labeled with Alexa-Fluor 595, panel B illustrates the same RGCs secondarily labeled with Alexa-Fluor 488, while panel C illustrates the co-localization of the two markers. Panels D, E and F represent images obtained from a dissociated cultured retina where RGCs were not isolated using the panning technique. Panel D illustrates an image obtained after the dissociated tissue was labeled with an antibody against $\alpha 7$ nAChR subunits and secondarily labeled with Alexa-Fluor 595, panel E represents the same cultured retinal tissue double-labeled with an antibody against Thy 1.1 and secondarily labeled with Alexa-Fluor 488 and panel F represents the co-localization of the two panels. Note that not all cells double-labeled under these conditions.

Table 1

Glutamate-induced excitotoxicity of cultured rat RGCs.

Treatments	% decrease from control	SEM (+/- %)	N
Untreated	0	0	45
500 μ M glutamate (total RGCs)	45.21 *	+/-2.10	8
500 μ M glutamate (large RGCs)	48.16 *	+/-2.70	8
500 μ M glutamate (small RGCs)	42.03 *	+/-1.83	8
100 μ M KA	32.33 *	+/-2.19	5
100 μ M NMDA	8.28 ^{*^}	+/-2.03	5
100 μ M KA/NMDA	46.23 *	+/-3.54	5
10 μ M CNQX/100 μ M KA	3.33 ^	+/-2.89	5
10 μ M MK801/100 μ M NMDA	2.22 ^	+/-2.87	5
CNQX/MK801/KA/NMDA	3.45 ^	+/-2.69	5

* represents significance from untreated controls,

^ represents significance from glutamate treatments, KA treated tissue and KA/NMDA treated tissue.

Table 2

Comparison of apoptotic, necrotic and living RGCs. Day 0, 1 and 3 represents the length of time isolated RGCs were in culture before cells were labeled with ApoTag Red (to identify apoptotic cells), ethidium homodimer (to identify necrotic cells), calcein (to identify living healthy cells) and counted.

	Day 0	Day 1	Day 3
Average number of apoptotic labeled RGCs in mm ²	0.25 +/- 0.25 (N=10)	4.15 [#] +/- 1.05 (N=8)	12.45 [*] +/- 2.81 (N=12)
Average number of necrotic labeled RGCs in mm ²	0.33 +/- 0.13 (N=4)	0.29 +/- 0.21 (N=4)	0.24 +/- 0.25 (N=4)
Average number of calcein labeled RGCs in mm ²	56.22 +/- 4.11 (N=8)	49.62 [#] +/- 4.11 (N=10)	27.44 [*] +/- 3.25 (N=12)

[#] represents significant difference from Day 0 and Day 3,

^{*} represents significant difference from Day 0 and Day 1. +/- represents SEM.