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Tropisetron as a Neuroprotective Agent Against Glutamate-Induced Excitotoxicity and Mechanisms of Action

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Abstract

The objective of this study was to determine the neuroprotective role of tropisetron on retinal ganglion cells (RGCs) as well as to explore the possible mechanisms associated with $\alpha 7$ nAChR-induced neuroprotection. Adult pig RGCs were isolated from all other retinal tissue using a two-step panning technique. Once isolated, RGCs were cultured for 3 days under control untreated conditions, in the presence of 500 μ M glutamate to induce excitotoxicity, and when tropisetron was applied before glutamate to induce neuroprotection. 500 μ M glutamate decreased RGC survival by an average of 62% compared to control conditions. However, RGCs pretreated with 100 nM tropisetron before glutamate increased cell survival to an average of 105% compared to controls. Inhibition studies using the $\alpha 7$ nAChR antagonist, MLA (10 nM), support the hypothesis that tropisetron is an effective neuroprotective agent against glutamate-induced excitotoxicity; mediated by $\alpha 7$ nAChR activation. ELISA studies were performed to determine if signaling cascades normally associated with excitotoxicity and neuroprotection were up- or down-regulated after tropisetron treatment. Tropisetron had no discernible effects on pAkt levels but significantly decreased p38 MAPK levels associated with excitotoxicity from an average of 15 ng/ml to 6 ng/ml. Another mechanism shown to be associated with neuroprotection involves internalization of NMDA receptors. Double-labeled immunocytochemistry and electrophysiology studies provided further evidence that tropisetron caused internalization of NMDA receptor subunits. The findings of this study suggest that tropisetron could be an effective therapeutic agent for the treatment of degenerative disorders of the central nervous system that involves excitotoxicity.

Keywords

neuroprotection; retina; excitotoxicity; retinal ganglion cells; tropisetron; pig

INTRODUCTION

Excitotoxicity has been implicated in the pathology of a number of neurodegenerative disorders of the brain, such as, Alzheimer's, Parkinson's, Huntington's disease and amyotrophic lateral sclerosis (Romano et al., 1998, Mattson, 2003). Neurodegenerative

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diseases of the eye, including glaucoma, retinal ischemia, and diabetic retinopathy, also have pathologies linked to excitotoxicity (Lipton, 2001; Kim et al., 2007; Schmidt et al., 2008). While not all neurodegenerative diseases occur with elevated concentrations of glutamate, alteration of glutamate receptor activity is a common key in the process of excitotoxic cell death, giving rise to the term glutamate-induced excitotoxicity (Michaelis, 1998; Mattson, 2003). Many recent studies have indicated that excitotoxicity is initiated in response to excessive Ca^{2+} influx (Sattler and Tymianski, 2000; Arundine and Tymianski, 2003; Brandt et al., 2011), which initiates signaling cascades to activate caspases that ultimately destroy the cells (Li et al., 1997; Tenneti et al., 1998; Tenetti and Lipton, 2000). Recent studies have progressed in the direction of determining the specific intracellular signaling pathways that are involved in glutamate-induced excitotoxicity. Several studies have demonstrated that apoptosis associated with excitotoxicity is regulated through the p38 MAP kinase pathway (Dineley, et al., 2001; Pearson et al., 2001; Manabe and Lipton, 2003; Zarubin and Han, 2005; Wang et al., 2007). Asomugha et al., (2010) found chronically stimulated glutamate receptors activates the MAPKKK > MAPKK > p38 MAP kinase intracellular signaling pathway and leads to apoptosis. Using ELISA techniques, they reported that when adult pig RGCs are cultured with 500 μM glutamate for 12 hours, there was an average resulting cell death of 40% from untreated control conditions and an increase in phosphorylation of p38 MAP kinase by an average of 72% over control. When the p38 MAP kinase inhibitor, SB 203580, was applied before excessive glutamate, RGC death and phosphorylation of p38 MAP kinase were significantly decreased. This strengthens the notion that the p38 MAP kinase pathway activation is key in glutamate-induced excitotoxicity in cultured pig RGCs.

With excitotoxicity playing a key role in some of the most widely suffered neurodegenerative disorders, the clinical implications involved with protecting cells from excitotoxicity are immense. A recent line of research has shown that, in various neural tissues, when cells are pre-treated with substances to stimulate nicotinic acetylcholine receptors (nAChR), the toxic effects of excessive glutamate can be prevented (Akaike et al., 1994; Kaneko et al., 1997; Dajas-Bailador et al., 2000; Wehrwein et al., 2004; Thompson et al., 2006). However, the neuroprotective mechanisms by which excitotoxicity is prevented by nAChR activation are not fully understood. Several studies have suggested that Ca influx through activated nAChRs affects phosphorylation level of the p38 MAPK and Akt intracellular signaling pathways resulting in neuroprotection (Asomugha et al., 2010, Brandt et al., 2011).

Another mechanism that has been proposed for neuroprotection from glutamate-induced excitotoxicity is reduction of Ca^{2+} influx through internalization of calcium channels or through internalization of glutamate receptors. Studies by Cristofanilli and Akopian (2006) found that treatment with actin destabilizing agents caused internalization of $\text{Ca}_v1.3$ L-type calcium channels and protected dissociated RGCs from excitotoxicity induced by activation of iGluRs, suggesting a possible mechanism for the regulation of the Ca^{2+} current and neuroprotection (Cristofanilli et al., 2007). These same studies have linked nAChR activation to internalization of receptor proteins. Shen et al. (2010) found that activation of nAChRs in fetal rat cortical neurons by treatment with nicotine and donepezil, an acetylcholinesterase inhibitor, caused internalization of glutamate receptors, resulting in attenuation of the glutamate induced Ca^{2+} influx, reduction in caspase-3 activation, and protection of cells from glutamate-induced excitotoxicity. This tie between nAChR mediated neuroprotection and internalization of glutamate receptors led us to explore glutamate receptor internalization as a possible mechanism of neuroprotection.

In the current study, we investigate the neuroprotective properties of tropisetron, ((1*R*,5*S*)-8-methyl-8-azabicyclo[3.2.1]octan-3-yl 1-methyl-indole-3-carboxylate), against glutamate-induced excitotoxicity in isolated and cultured adult pig retinal ganglion cells. Tropisetron

has long been used as an anti-emetic to help alleviate symptoms associated with chemotherapy and postoperative condition. However, it has been discovered that tropisetron is a unique substance in that it also has a partial $\alpha 7$ nAChR agonist action as well (Papke et al., 2005; Macor et al., 2001). It is this action that has been analyzed in this study. In previous studies, activation of $\alpha 7$ nAChRs has been shown to protect cells from glutamate-induced excitotoxicity in a number of model systems (Kawamata and Shimohama, 2011; Yu et al., 2011; Liu et al., 2012). Previous studies from this lab using pharmacological and immunocytochemical techniques have identified a number of specific nAChR subunits that are involved in neuroprotection against glutamate-induced excitotoxicity in isolated cultured adult pig RGCs (Thompson et al., 2006). In this previous study, it was demonstrated that nAChRs composed of $\alpha 4$ and $\beta 2$ subunits are only found on small pig RGCs and selective agonists for $\alpha 4$ nAChRs produced significant neuroprotection against excitotoxicity. $\alpha 7$ nAChRs were found only on large pig RGCs and ACh or nicotine provided significant neuroprotection against a glutamate insult (Wehrwein et al., 2004; Thompson et al., 2006). As tropisetron also exhibits an agonist action at $\alpha 7$ nAChRs, it may prove to be useful as a neuroprotective agent on RGCs.

As a result, pharmacology studies were designed using isolated adult pig RGCs in culture to investigate the efficacy of tropisetron as a neuroprotective agent. Further studies using competitive agonists and antagonists were performed to determine which receptors elicit tropisetron's neuroprotective effect. Two different approaches were used to further investigate the mechanisms involved in tropisetron's ability to protect adult pig RGCs from glutamate-induced excitotoxicity. ELISA studies were used to look at the role of p38 MAPK and Akt signaling proteins in tropisetron treatments against glutamate. Internalization of NMDA GluRs in response to tropisetron treatment was also investigated using fluorescent immunocytochemistry and electrophysiology techniques. It was hypothesized that if tropisetron protects cells from excitotoxicity, then it may occur through internalization of NMDA receptors. Understanding the mechanisms involved in ACh-induced neuroprotection in the pig retina could ultimately lead to therapeutic treatment for any central nervous system (CNS) disease that involves excitotoxicity.

EXPERIMENTAL PROCEDURES

2.1 Retinal Ganglion Cell Isolation

In order to obtain pure retinal ganglion cells (RGCs) for *in vitro* studies, adult pig eyes were removed from animals at a local slaughterhouse (Pease Slaughterhouse, Scotts, MI) and transported on ice to the laboratory for removal of retinas and isolation of RGCs. To isolate the RGCs, we used a modified two-step panning procedure described in Wehrwein et al. (2004). The retinas were removed from eyes according to the methods described by Wehrwein et al., (2004). Isolated retinas were then placed in a modified CO₂-independent medium (Gibco, Carlsbad, CA) kept at 37°C, containing 4mM glutamine, 10% fetal bovine serum (FBS), 5% antibiotic/antimycotic, and 4 mM HEPES and enzymatically dissociated using papain (27 u/mg) for 20 minutes at 37°C. After 20 minutes in papain, tissue was rinsed with fresh CO₂-independent medium to stop the papain action and 1 mg/ml DNase. Complete dissociation of the retina was obtained using an unpolished Pasteur pipette to gently triturate the tissue.

RGCs were isolated from all other retinal tissue using a two-step panning technique according to methods previously described (Wehrwein et al., 2004; Thompson et al., 2006; Brandt et al., 2011). The first step in this process plated dissociated retinal tissue onto dishes, coated with goat anti-rabbit IgG antibody (Jackson ImmunoResearch, West Grove, PA; 0.5 mg in 10 ml of 20mM Tris buffer) to eliminate nonspecific binding. After 1 hour of incubation on the IgG plates, cells from each dish were transferred onto Petri dishes coated

with mouse anti-rat Thy 1.1 antibody (BD Biosciences, San Diego, CA; 12.5 μ g in 10 ml PBS containing no magnesium chloride and no calcium chloride) bound to goat anti-mouse IgM (Jackson ImmunoResearch; 0.36 mg in 10 ml of 20 mM Tris buffer) for 1 hour at 37°C. This represented the second panning step in the process. After 1 hour, the culture medium was replaced with fresh CO₂-independent medium including supplemental factors consisting of NGF, transferrin and insulin (Wehrwein et al., 2004). Each 4 mls of culture medium contained 50 μ l of 15 μ g/ml nerve growth factor (NGF), 48 μ l of 500 μ g/mL transferrin, and 12 μ l of 10 mg/mL insulin.

2.2 Pharmacology Studies

In pharmacology studies, isolated RGCs were evenly distributed into dishes at a density of 1×10^5 cells/ml. Each dish contained isolated RGCs that were cultured under six different conditions. The first dishes in each experiment always contained isolated RGCs that were untreated. The second condition consisted of dishes containing isolated RGCs treated with 500 μ M glutamate to induce excitotoxicity. The remaining four conditions consisted of dishes containing cultured RGCs that were treated with appropriate concentrations of agonists and/or antagonists. In dose-response studies, conditions 3 – 6 were treated with various concentrations of tropisetron for 1 hour prior to a 500 μ M glutamate insult. Glutamate was obtained from Sigma (St. Louis, MO). Tropisetron was obtained from RBI (Natic, MA). In inhibition studies, the $\alpha 7$ nAChR antagonist, methyllycaconitine (MLA), obtained from Tocris (Bristol, UK) was applied to conditions 3 – 6 for 1 hour before tropisetron application to allow the antagonist time to bind to receptors. Since tropisetron has both $\alpha 7$ nAChR agonist and 5-HT₃ antagonist properties, control experiments using the 5-HT₃ agonist, SR-57227, (Sigma; St. Louis, MO), were performed to determine which receptors are responsible for tropisetron-induced neuroprotection.

After all pharmacological treatments were added to isolated pig RGCs, cells were cultured for three before cell viability was assessed. At the end of the experimental period, cells were loaded with 2 μ M calcein in normal PBS for 1 hour to label living cells when exposed to 495 nm excitation. A Nikon Diaphot epifluorescent research microscope illuminated by a 100-W mercury arc lamp with an excitation filter EX 510 to 590, dichroic mirror DM 580, and barrier filter BA590 was used to examine the cell cultures for RGC survival. Images of fluorescent cells were recorded using a Hamamatsu XC-77 CCD camera, captured and counted using Metamorph Imaging system (Universal Imaging, Downingtown, PA). Cell counts were obtained from four images taken from each dish in a compass rose pattern. Cell counts from each of the four images were averaged to derive the count for each dish. In each experiment, the average count from several dishes under each condition using the same tissue was used to derive an average cell count for each treatment condition. This represented an N of 1. Each experiment using tissue from different pig eyes was repeated a minimum of three times. Untreated control samples were counted and normalized as a baseline to determine cell survival. Cells surviving in other culture conditions were counted and compared to the number of living RGCs in the untreated control condition to determine the percentage of cells surviving in experimental conditions. Large and small RGCs were identified using Metamorph Imaging system software (Universal Imaging). Statistical analyses were performed using an analysis of variance (ANOVA) with Kuskall-Wallis post hoc testing. Results were considered statistically significant if $P < 0.05$.

2.3 Lysate Preparation for ELISA Studies

In order to prepare cell lysates for use in ELISA studies, adult pig RGCs were dissociated and isolated by the same method as in the pharmacology studies with the exception that 12 pig eyes were used for every experiment instead of 4 eyes. Isolated cells were plated onto dishes using the two-step isolation procedure and cultured for 12 hours at 37°C in fresh

modified culture medium under four different pharmacological conditions: 1) untreated control, 2) 500 μ M glutamate, 3) 100 nM tropisetron, 4) pretreatment of 100 nM tropisetron for 1 hour before 500 μ M glutamate. Preliminary time- and dose-dependent studies determined the time of incubation that produced maximal effects and determined what concentrations were used throughout this study.

After culturing for 12 hours, the supernatant fluid was removed and placed in appropriately labeled conical tubes. To release the RGCs from the bottom of the Thy1.1 plates, 0.25% trypsin was added to each plate and plates were incubated at 37°C for 10 minutes. Once cells were loosened from the bottom of the plates, lysates were produced and collected using the procedure outlined by Asomugha et al., (2010). Cell extraction buffer contained 10 mM Tris, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM NaF, 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 2 mM Na_3VO_4 , 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate (SDS), 0.5% deoxycholate (all obtained from Sigma). Protease inhibitor cocktail added to the extraction buffer contained 14 μ M E-64, 130 μ M bestatin, 1 μ M leupeptin, 0.1 mM aprotinin, 2 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) in 1 mM phenylmethylsulfonyl fluoride (PMSF) with 100 mM EDTA (all obtained from Sigma). The resulting liquid lysate was transferred to labeled centrifuge tubes and stored at -80°C until used in ELISA experiments.

2.4 ELISA Studies

ELISA techniques were used to quantify the degree of up- or down-regulation of phosphorylated enzymes in intracellular signaling pathways involved with neuroprotection and glutamate-induced excitotoxicity. The ELISA kits (obtained from Invitrogen) used in this study were designed to detect phosphorylated Akt and phosphorylated p38 MAPK. To measure phosphorylated protein content, prepared lysates representing each experimental condition were thawed at room temperature and kept on ice. ELISAs were performed according to the manufacturer's instructions. After processing for phosphorylated Akt or p38 MAPK, optical density readings were measured using a PowerWave 200 microplate scanning spectrophotometer. A standard curve was constructed using the optical density readings for known concentrations of standards provided in the kit. Using the curve, protein concentrations for unknown protein lysates were derived by comparing optical density readings to readings of known protein concentrations. Results were analyzed using ANOVA and considered significant if $P < 0.05$.

2.5 Receptor Internalization Immunocytochemistry Studies

To examine the idea that treatment with tropisetron triggers internalization of NMDAR1 receptor subunits (GluN1; Collingridge et al., 2009), a procedure was followed to differentially label GluN1 receptor subunits on the cell surface and receptor subunits internalized within the cell. For these experiments, adult pig RGCs were isolated as in previous studies and plated onto IgG dishes for the first panning step. For the second panning step, cells were transferred to 8-welled culture chamber slides that had been coated with IgM and Thy 1.1. Cells were allowed to settle in these chamber slides in culture medium for 1 hour at 37° C. After the hour, the culture medium was replaced with fresh modified culture medium containing NGF, transferrin, and insulin. Prior to pharmacological treatment of cells in the culture chamber slides, cells were treated with a primary monoclonal rabbit antibody (1:500) against GluN1 subunits, obtained from Millipore/Chemicon, Billerica, MA) for 1.5 hours at 37° C. After cells were labeled with the antibody against GluN1, cells were rinsed with culture medium and different pharmacological agents were applied to appropriate chamber well slides. Some wells containing RGCs were not treated with pharmacological agents and were used as a control. Other wells containing RGCs were treated with 100 nM tropisetron for 1 hour. Still other chamber wells on these

slides were treated with 10 nM MLA for 20 minutes before addition of tropisetron to block activation of $\alpha 7$ nAChRs. After culturing with tropisetron, RGCs were fixed for 30 minutes at room temperature using 4% paraformaldehyde. RGCs were subsequently rinsed with PBS and secondarily labeled with an anti-rabbit antibody conjugated to Alexa Fluor 594 donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA), diluted 1:300 in PBS to label the GluN1 subunits on the cell surfaces. The cells were allowed to sit in this fluorescently labeled antibody overnight at 4°C. The next day, cells were rinsed with PBS and then treated with 0.4% Triton-X for 5-minutes to permeabilize the cell membranes so that any internalized receptors could be labeled. After 5 minutes, the Triton-X was removed and PBS containing the same secondary antibody (donkey anti-rabbit IgG diluted to 1:300 in PBS) labeled with a different fluorescent marker (Dylight 405), Jackson ImmunoResearch Laboratories, Inc. West Grove, PA), was applied to already labeled cells and allowed to sit overnight at 4°C. The next day, cells were rinsed, the chamber walls were removed and slides containing labeled RGCs were cover slipped using a mounting medium containing ½ glycerol and ½ PBS. RGCs were examined using a Nikon Diaphot epifluorescence research microscope illuminated by a 100 W mercury arc lamp. Fluorescent cells were recorded by a Hamamatsu XC-77 CCD camera, and captured using Metamorph Imaging system (Universal Imaging, Downingtown, PA). Pictures of cells were compared visually to determine localization of labeled receptors. Receptors labeled with Alexa Fluor 594 were determined to be on the cell surface and receptors labeled with Dylight 405 were determined to be located inside the cell.

Control experiments were conducted to display specificity of the antibodies used. Negative control experiments were performed to examine antibody specificity. In some experiments, large 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 experiments, preabsorption controls were performed where the primary antibody and GluN1 antigen were added together before applying to tissue. No significant epifluorescence was observed under any of these conditions. For double-label experiments, controls performed to ensure specificity of antibodies are discussed in the results section. All experiments were repeated a minimum of 3 times using tissue from different animals.

2.6 Electrophysiology

For electrophysiological studies, isolated RGCs were evenly distributed onto glass coverslips coated with laminin and poly-L-lysine at a density of 1×10^5 cells/ml and cultured for 1 hour, 1 and 3 days with and without 100 nM tropisetron to induce internalization. After each period of time, coverslips containing isolated RGCs were transferred to the stage of an inverted Nikon Diaphot microscope and the culture media was replaced with pig Ringer's solution containing (in mM), 125 NaCl, 5 KCl, 1 CaCl_2 , 10 glucose, 5 HEPES and 10 4-AP to block voltage-gated potassium channels. The Ringer's solution was titrated to pH 7.75 with sodium hydroxide. Magnesium blocks the pore of the NMDA receptor when the cell's membrane potential is hyperpolarized from -30 mV (Mayer et al., 1984). As a result, Mg^{+2} was omitted from all extracellular solutions for electrophysiological experiments. Added to the pig Ringer's solution was tetrodotoxin (10 μ M), to block voltage-gated sodium channels and nitrendipine (10 μ M) to block voltage-gated calcium channels (Davis and Linn, 2003).

Cells were voltage-clamped as described by Hamill et al. (1981). Patch pipettes were pulled from borosilicate glass by a Narishige (Tokyo, Japan) vertical microelectrode puller. Electrodes were uncoated and unpolished and contained (in mM) 120 CsCl_2 and 15 TEA to block contribution of voltage-gated potassium channels, 1 MgCl_2 , 0.5 EGTA, 5 adenosine triphosphate (ATP) and 4 HEPES. The intracellular solution was titrated to pH 7.4 using potassium hydroxide. Electrodes used to voltage-clamp pig isolated RGCs had resistances

between 5 and 8 M Ω . Once an isolated RGC was voltage-clamped, 100 or 200 μ M NMDA was applied to cells via a gravity fed solenoid controlled perfusion system (Warner Instruments) at the rate of 1 ml/min. NMDA was applied to RGCs whose membrane potential was voltage-clamped between -60 and +40 mV in 20 mV step increments. In response to NMDA, the peak current amplitude at each membrane potential was recorded and plotted as well as the membrane potential corresponding to the reversal potential.

All recordings were obtained using an Axon Instrument Axopatch 200A amplifier (Foster City, CA). Series resistance and capacitive artifacts were compensated for using amplifier controls. No data was collected from cells demonstrating leakage currents greater than 0.02 nA. Data collection was controlled using a computer and a Digidata acquisition board. Digitization and analysis were performed with pCLAMP software (Molecular Devices, Sunnyvale, CA). Data was filtered at 1 kHz and sampled at 10 kHz.

RESULTS

3.1 Pharmacology

In this study, we have sought to test the hypothesis that tropisetron is an effective neuroprotective agent against glutamate-induced excitotoxicity in isolated adult pig RGCs. Furthermore, it is hypothesized that this neuroprotection occurs through activation of $\alpha 7$ nAChRs. To test these hypotheses, tropisetron was applied at various concentrations to isolated adult pig RGCs and cultured for one hour prior to a 500 μ M glutamate application and then cultured for 3 days. At the end of 3 days, live cells were labeled with calcein AM, imaged and counted using Metamorph Imaging System. Untreated control samples from the same animals, plated at the same density, were used as a baseline in each experiment to compare cell survival in all studies (Fig 1A). A typical effect of 500 μ M glutamate on isolated RGCs is illustrated in fig. 1B. 500 μ M glutamate decreased the average percent of total RGCs by 62.1% (± 3.1 ; N=8) when applied to cultured RGCs for 3 days compared to untreated control RGCs. Previous results from isolated cultured pig RGCs have demonstrated that glutamate-induced cell loss affects both large and small pig RGCs and is mediated through an apoptotic mechanism (Wehrwein et al., 2004; Asomugha et al., 2010). However, if tropisetron was applied to cultured RGCs for 1 hour before the glutamate insult, neuroprotection of RGCs occurred. Dose response studies showed that tropisetron provided neuroprotection against glutamate-induced excitotoxicity when 50 nM tropisetron was applied before 500 μ M glutamate, but maximal protection occurred using a dose of 100 nM tropisetron (fig 2). The EC₅₀ for tropisetron's neuroprotective effect equaled 62 nM. If isolated RGCs were incubated with 100 nM tropisetron before a glutamate insult, the average survival rate of total RGCs increased to 105.2% (± 20.3) compared to untreated conditions (Fig 1C and Fig 2).

As seen in figure 3, the primary effect of tropisetron was seen in large RGCs (fig. 1C and fig 3, left bars). Each bar graph in fig. 3 represents the average percent of RGC survival compared to control untreated conditions for large and small RGCs. Large retinal ganglion cells exhibited large circular somata (30–40 μ m in diameter) and typically had several fine smooth processes emerging from the cell body. The small RGCs had diameters <20 μ m and were typically monopolar or bipolar with circular bodies, similar to results obtained from Luo et al., (2001) and Wehrwein et al., (2004). 500 μ M glutamate effects both large and small pig RGCs (Wehrwein et al., 2004). However, 100 nM tropisetron provided greater neuroprotection against glutamate-induced excitotoxicity in the larger RGCs. If RGCs were cultured with 100 nM tropisetron for 1 hour before application of 500 μ M glutamate and cultured for 3 days, the mean percent of large RGC survival increased to 122.2% (± 12) compared to control untreated conditions, while the percent of small RGCs survival was calculated to be only 62.4 (± 5.4) compared to control untreated conditions. The

neuroprotective effect of tropisetron primarily on large RGCs could be due to activation of $\alpha 7$ nAChRs, which is found exclusively on large pig RGCs (Thompson et al., 2006).

In order to determine which receptor elicits tropisetron's neuroprotective effect on large pig RGCs, further culture studies using isolated pig RGCs were conducted using the highly selective $\alpha 7$ nAChR antagonist, MLA, to block $\alpha 7$ nAChR action. MLA was applied at concentrations of 1 nM and 10 nM one hour prior to application of 100 nM tropisetron (right two bar graphs). Figure 4 demonstrates that 1 nM MLA did not effectively block the neuroprotective effect of 100 nM tropisetron. In this condition, RGCs survived at a rate of 106.5% (± 9.2) compared to untreated controls. However, with application of 10 nM MLA (right bar graph), the neuroprotective effect of tropisetron was significantly reduced to an average of 47% survival (± 6.1) compared to untreated conditions. Each bar graph represents the mean percentage of RGCs surviving in each treatment condition after 3 days in culture, with untreated controls representing baseline survival rates at 100% (left bar graph). This supports the notion that tropisetron elicits the neuroprotective effect on adult pig RGCs through $\alpha 7$ nAChRs.

In addition to its action as a nAChR agonist, tropisetron also acts on 5-HT₃ receptors as an antagonist. To determine if action at 5-HT₃ receptors has a role in neuroprotection of cultured RGCs, further culture studies were conducted using the 5-HT₃ receptor agonist, SR-57227. Figure 5 demonstrates the effect of various concentrations of SR-57227 on cell viability when applied one hour before a 500 μ M excitotoxic dose of glutamate. Application of SR-57227 was ineffective at protecting RGCs from glutamate assault at all concentrations tested using 1 nM, 10 nM, 100 nM and 1 μ M (right four bar graphs). In the presence of 1 nM SR-57227, the RGC survival rate averages 41.9% (± 5.2) compared to untreated conditions, which was not significantly different from the effect of 500 μ M glutamate. 10 nM SR-57227 application before glutamate produced an average RGC survival of 60.3% (± 22.4), 100 nM SR-57227 produced an average survival of rate of 37.8% (± 6.9), while 1 μ M SR-57227 produced an average survival rate of 58.3% (± 14.9). None of these values were significantly different from the effect of 500 μ M glutamate on isolated pig RGCs, supporting the hypothesis that 5-HT₃ receptors are not involved in neuroprotection.

In another control experiment designed to further support the hypothesis that 5-HT₃ receptors are not involved in neuroprotection of RGCs against glutamate induced excitotoxicity, studies were conducted using SR-57227 (10 and 100 nM) for one hour prior to 100 nM tropisetron treatment and two hours before a 500 μ M glutamate assault. The two right bar graphs in fig 6. demonstrate that SR-57227 pretreatment did not diminish tropisetron's neuroprotection against glutamate induced excitotoxicity when incubated with 100 nM tropisetron. In both conditions, cells survived at rates comparable to controls (94.2% (± 12.1) and 113.3% (± 12.9) respectively). The finding that use of a 5-HT₃ agonist, SR-57227, failed to provide neuroprotection against glutamate-induced excitotoxicity lends support to the hypothesis that tropisetron produces neuroprotection through $\alpha 7$ nAChRs and not through 5-HT₃ receptors.

ELISA studies—Recent pig studies have identified several intracellular signaling proteins that are involved in glutamate-induced excitotoxicity and ACh-induced neuroprotection through activation of $\alpha 7$ nAChRs. Asomugha et al. (2010) found that glutamate-induced excitotoxicity was associated with an increase in phosphorylation of p38 MAPK. Furthermore, pretreatment with ACh or nicotine before a glutamate assault eliminated the increase in p38 MAPK phosphorylation as well as increased phosphorylation of Akt. From these findings it was hypothesized that the partial $\alpha 7$ nAChR agonist, tropisetron, could protect RGCs through activation of pathways similar to ACh. In support of this, pretreatment of isolated pig RGCs with tropisetron should inhibit p38 MAPK

phosphorylation as well as increase Akt phosphorylation if the mechanisms involved in ACh- or nicotine-induced neuroprotection of RGCs against glutamate-induced excitotoxicity are the same.

To test this hypothesis, cell lysates were obtained from adult pig RGCs collected under four different treatment conditions: untreated controls, 500 μ M glutamate alone, 100 nM tropisetron alone, and when 100 nM tropisetron was added 1 hour before 500 μ M glutamate. Lysates collected under each condition were processed using ELISA plates obtained from Invitrogen using the manufacturer's procedure to quantify the concentrations of phosphorylated p38 MAPK and Akt in pig RGCs. Figure 7 displays the mean phosphorylation rates of p38 MAPK obtained after each treatment conditions. Each bar graph in fig. 7 represents the mean level of phosphorylated p38 MAPK measured in ng/ml. The level of phosphorylated p38 MAPK in glutamate treated cells was significantly greater ($15 \text{ ng/ml} \pm 0.76$) than in untreated controls ($6.07 \text{ ng/ml} \pm 0.99$), representing a 247% increase in phosphorylated p38 MAPK. Cells treated with tropisetron alone did not exhibit this increase in p38 MAPK phosphorylation ($5.07 \text{ ng/ml} \pm 0.99$) and was statistically the same as the phosphorylation measured from untreated controls. When cells were treated with tropisetron prior to glutamate application, RGCs also exhibited phosphorylated p38 MAPK levels near untreated control samples ($7.3 \text{ ng/ml} \pm 1.45$).

ELISA studies investigating phosphorylated Akt did not yield significant changes when cells were treated with glutamate or when cells were treated with tropisetron (data not shown). This suggests that although inhibition of the p38 MAPK pathway is likely to be involved in tropisetron-induced neuroprotection, there is no evidence that a pathway involving Akt is involved.

Receptor Internalization—An alternate mechanism of neuroprotection in these cells may involve receptor internalization. Several recent studies have linked internalization of ion channels to neuroprotection (Akopian et al., 2006; Christofanelli and Akopian, 2006; Mizuno et al., 2010; Shen et al., 2010). Shen et al. (2010), found internalization of GluN1 subunits were linked to increased cell survival after treatment with the acetylcholinesterase inhibitor, donepezil. It was therefore hypothesized that tropisetron may protect RGCs by internalization of GluN1s. To determine if receptor internalization is involved in neuroprotection by tropisetron, immunocytochemistry studies were designed to label and image GluN1 subunits within the cells with Dylight 405 and on the cell surface with Alexa Fluor 594. The GluN1 subunit is the most commonly expressed subunit and is necessary for the formation of functional NMDA receptors throughout the body. The typical structure of NMDA receptors is of a bi-lobed heterotetrameric ion channel composed of 2 GluN1 subunits and 2 other types of GluN2 subunits that are found in varying frequency throughout the nervous system (Paoletti and Neyton, 2007). Selecting a primary antibody against GluN1 subunits ensured that any NMDA receptors on cultured RGCs would be labeled.

Figure 8 displays images of control studies that demonstrate specificity of labeling. Fig. 8A illustrates a normal illumination image of a large adult pig RGC that was labeled in a chambered culture well with a 1:500 dilution of primary monoclonal rabbit antibody against GluN1. The RGC was subsequently placed in a 1:300 dilution of secondary anti-rabbit antibody conjugated to Alexa Fluor 594. Fig 8B is the same cell under Alexa Fluor 594 illumination. Figure 8C demonstrates a control cell treated with Alexa Fluor 594 after omitting the primary antibody. No fluorescence was observed under this condition.

In figure 8D, a RGC is shown under normal illumination that was labeled for 1.5 hours with a 1:300 dilution of primary rabbit antibody against GluN1. After labeling with the primary antibody, the RGC was rinsed and secondary labeled with an anti-rabbit antibody conjugated

to Dylight 405 (1:300). Figure 8E shows the same cell under Dylight 405 illumination. Figure 8F is an image of another control cell when the primary antibody against GluN1 subunit was omitted. As seen in fig. 8F, under these conditions, no fluorescence was detected after subsequent treatment with a secondary antibody conjugated to Dylight 405.

Figure 9 displays images of cells fluorescently labeled with Alexa Fluor 594 and Dylight 405 under various treatment conditions. In fig. 9A, illumination that excites Alexa Fluor 594 is shown. Labeled cell surface GluN1 receptor subunits were illuminated by this method. Figure 9B shows the same cells under Dylight 405 illumination. No fluorescence indicates that the Dylight 405-conjugated antibody was unable to bind to GluN1 as no GluN1 subunits were internalized. Figure 9C is an overlay image composed of 9B and 9A displaying that Dylight 405-conjugated antibody was unable to bind to receptors.

In figure 9D, cells were treated with 100 nM tropisetron for 1 hour after labeling GluN1s. Figure 9D illustrates surface GluN1s labeled with Alexa Fluor 594. Figure 9E shows the same cells under Dylight 405 illumination. Internalized receptors as a result of tropisetron treatment are shown labeled with the Dylight 405-conjugated secondary antibody. Figure 9F is an overlay showing surface receptors vs. internalized receptors. As a result of tropisetron treatment, there are different localization of labeled receptors in the same cells.

After isolated pig RGCs were treated with 100 nM tropisetron for one hour and fixed, an average of 11.2% (± 2.22) large pig RGCs labeled for internalized receptors. The number of internalized receptors were quantified using Metamorph software. To determine if a longer treatment with tropisetron would increase internalization of NMDA receptors, cultured isolated pig RGCs containing labeled GluN1 subunits were fixed 1 day and 3 days after 100 nM tropisetron treatment. After culturing cells with tropisetron for 1 day, an average of 38.5% (± 7.22) large pig RGCs labeled for internalized NMDA receptors. After a 3 day treatment with tropisetron, an average of 88.5% (± 10.22) of isolated large pig RGCs contained internalized receptors.

To generate the images shown in figure 9G, cells were labeled with the monoclonal rabbit antibody against GluN1 subunits. Cells were then treated with 10 nM MLA prior to tropisetron treatment, subsequently fixed and secondarily Alexa Fluor 594-conjugated antibody overnight. Labeled cells were then permeabilized and treated with the Dylight 405-conjugated antibody. Under illumination designed to excite Alexa Fluor 594, surface GluN1 subunits fluoresced red (fig. 9G). Figure 9H shows the same cell population under illumination that excites Dylight 405. No internalized receptors are seen as MLA was used to block tropisetron's binding site. Figure 9I is an overlay of 9G and 9H showing that only surface receptors labeled. These results were typical repeated 5 times with similar results. The data from these studies support the hypothesis that tropisetron protects RGCs from glutamate toxicity by internalizing GluN1s.

Electrophysiology—To further support this finding, large pig RGCs were cultured for 3 days under control untreated conditions with or without 100 nM tropisetron. After 3 days in culture, NMDA was applied to voltage-clamped isolated RGCs (fig. 10) in pig Ringer's saline that contained 10 μ M TTX to block the voltage-gated sodium channels, 10 μ M nifedipine to block voltage-gated calcium channels and 4 mM 4-AP to block voltage-gated potassium channels (Boos et al., 1993; Davis and Linn, 2003). The responses to 100 and 200 μ M NMDA shown in fig. 10A represent superimposed current traces obtained under control untreated conditions when large RGCs were voltage-clamped at -60 mV. Each response represents the result of ten superimposed currents obtained from ten different voltage-clamped RGCs. All recordings were made from large pig RGCs with cell body diameters between 30-40 microns that were cultured for three days before recording. Under control

untreated conditions, 100 μM NMDA produced an inward current in voltage-clamped pig RGCs that averaged 75.21 pA (± 6.22), whereas 200 μM NMDA produced a superimposed current averaging 125.36 pA (± 8.76) (fig. 10A). However, if cells were treated and cultured with 100 nM tropisetron for 3 days before recording, 100 and 200 μM NMDA elicited significantly reduced responses (fig. 10B) compared to control untreated conditions (fig. 10A), supporting the hypothesis that tropisetron caused internalization of NMDA receptors. When NMDA receptors were internalized due to tropisetron, the response to 100 μM NMDA significantly decreased from an average response of 75.21 pA (± 6.22) under control untreated conditions to an average response of 39.74 pA (± 4.82), representing a 48% decrease. In response to 200 μM NMDA, control untreated cells produced an inward current averaging 125.36 pA (± 8.76) compared to an average inward current of 65.21 pA (± 11.32) when cells were cultured with tropisetron for 3 days. Again, this represents a 48% reduction in NMDA-induced current when the voltage-clamped pig RGCs were held at -60 mVs.

A significant reduction in NMDA-induced current as a result of culturing isolated pig RGCs with 100 nM tropisetron was apparent if the membrane potential was held at -60 , -40 , -20 , $+20$ and $+40$ mV, as shown in figure 10C. The dotted line illustrated in fig. 10C represents the current/voltage relationship obtained using 200 μM NMDA on voltage-clamped isolated RGCs under control untreated conditions after cells were cultured for 3 days. The solid line illustrated the current/voltage relationship obtained after applying 200 μM NMDA to voltage-clamped RGCs that were treated with 100 nM tropisetron in culture for 3 days. Each data point represents an average of between 5 and 10 voltage-clamp recordings in response to 200 μM NMDA. There were significant differences between control untreated and tropisetron treated currents when NMDA was applied at a holding potential of -60 mV, -40 mV, $+20$ mV and $+40$ mV. Tropisetron treatment did not significantly affect NMDA's reversal potential, which occurred at -3 mVs.

DISCUSSION

The results obtained from these studies support the hypothesis that tropisetron acts on $\alpha 7$ nAChRs on isolated pig RGCs to provide neuroprotection against glutamate-induced excitotoxicity. In this study, several methods were used to address this issue, including pharmacological studies, immunocytochemistry, ELISAs and electrophysiology. Pharmacology studies demonstrated which receptors were responsible for inducing excitotoxicity in isolated pig RGCs and that neuroprotection against excitotoxicity occurs through activation of $\alpha 7$ nAChRs. In studies designed to analyze the mechanism of neuroprotection in RGCs, ELISA studies demonstrated that the p38MAP kinase was involved in triggering apoptosis in RGCs and that tropisetron acted to decrease p38MAP kinase levels to inhibit apoptosis. Besides a decrease in p38MAP kinase, immunocytochemistry and electrophysiological studies supported the hypothesis that neuroprotection also involves internalization of GluN1 subunits found in functional NMDA receptors.

In these studies, 500 μM dose of glutamate applied to isolated adult pig RGCs resulted in an average cell loss of 62% in culture. Studies showed that tropisetron was able to protect RGCs from a glutamate assault in a dose-dependent manner when applied to cultures 1 hour prior to glutamate application, with a maximal effect observed when 100 nM was used. Interestingly, when RGCs were cultured with 100 nM tropisetron for 1 hour before application of 500 μM glutamate and cultured for 3 days, the mean percent of large RGC survival increased to 122.2% (± 12) compared to control untreated conditions, while the percent of small RGCs survival was calculated to be only 62.4 (± 5.4) compared to control untreated conditions. The significant increase of large RGCs compared to control untreated conditions after 3 days in culture is likely due to tropisetron preventing the normal loss of

large RGCs associated with the dissociation and culturing process (Linn et al., 2009). In previous studies by Wehrwein et al., (2004), it was demonstrated that an average of 68% of isolated pig RGCs originally plated at a density of 1×10^5 cells/ml typically survived after 3 days in culture due to the normal process of dissociation. As adult RGCs are neurons that do not typically divide, it is likely that tropisetron acts to prevent the cell death of RGCs that would normally die off due to the dissociation process. As a result, after 3 days in culture with tropisetron, there would be significantly more large RGCs compared to untreated controls, as seen in our results. This suggests that tropisetron may have a dual effect on cell survival; one that is mediated through activation of nAChRs and another effect that does not involve nAChR activation, but prevents cells from dying due to the normal culture procedure. This second aspect of tropisetron needs to be investigated further.

Tropisetron has been shown to exhibit a selectively potent partial agonist action at $\alpha 7$ nAChRs based on previous binding studies and electrophysiology results (Papke et al., 2005; Macor et al., 2001). In pharmacology, partial agonists are drugs that bind to and activate a given receptor, but have only partial efficacy at the receptor relative to a full agonist. However, in these studies tropisetron protected RGCs from glutamate-induced excitotoxicity by activation of nAChRs in a manner similar to effects previously reported for ACh and nicotine (Wehrwein et al., 2004; Thompson et al., 2006). Therefore, although tropisetron is classified as a partial agonist based on binding studies, it acted as a full agonist in these studies to protect against glutamate-induced excitotoxicity. Results from pharmacology and electrophysiology studies provide evidence in support of this hypothesis.

The neuroprotective effect of tropisetron was found to be mediated through activation of $\alpha 7$ nAChRs, which is found exclusively on large pig RGCs (Thompson et al., 2006). Inhibition studies contributed further evidence that tropisetron exhibits neuroprotection through activation of $\alpha 7$ nAChRs. When the $\alpha 7$ nAChR inhibitor, MLA, was applied to RGCs 1 hour prior to tropisetron application, protection from glutamate was significantly reduced. Control studies showed that application of the 5-HT₃ receptor agonist, SR 57227, did not provide protection from a glutamate assault. In addition, if SR 57227 was applied before tropisetron to cultured RGCs, there was no difference in the degree of neuroprotection against glutamate-induced excitotoxicity caused by tropisetron. Taken together, the results from these pharmacology studies provide evidence to support the hypothesis that pre-treatment of adult pig RGCs with tropisetron protects cells from glutamate-induced excitotoxicity by activation of $\alpha 7$ nAChRs.

Another interesting result in this study was that tropisetron's provided a greater degree of neuroprotection to large RGCs over small RGCs. Under cultured conditions, the majority of cells that survived after 3 days in culture with tropisetron were primarily large RGCs; while only a small number of small RGCs survived. This finding is in line with the notion that tropisetron's effect selectively involves the $\alpha 7$ nAChR. Immunocytochemistry studies investigating localization of the $\alpha 7$, $\alpha 4$, and $\beta 2$ nAChR subunits by Thompson et al. (2006) found that $\alpha 7$ nAChR subunits are found exclusively on large RGCs, and that $\alpha 4$ and $\beta 2$ nAChR subunits are found exclusively co-localized on small pig RGCs. Furthermore, it was shown that neuroprotection by ACh in large pig RGCs occurs through $\alpha 7$ nAChRs and occurs in small RGCs through activation of $\alpha 4\beta 2$ nAChRs. Quantifying the differential survival rates between large and small RGCs provided evidence to support this notion. However, there was a small but significant increase in neuroprotection shown in the small RGCs. This may be due to activation of other subunits of the nAChRs by tropisetron in small RGCs. Although tropisetron has affinity for binding to $\alpha 7$ nAChRs, it may also have a lower affinity for other nAChR subunits found on small RGCs that would affect some cell survival (Papke et al., 2005). Future studies using other nAChR subtype inhibitors could further specify the receptor action of tropisetron on small RGCs.

This study was also designed to examine the mechanism associated with tropisetron's neuroprotective effect in the retina against glutamate-induced excitotoxicity. Several studies have suggested involvement of various intracellular signaling pathways (Dineley, et al., 2001; Pearson et al., 2001; Manabe and Lipton, 2003; Zarubin and Han, 2005; Wang et al., 2007; Asomugha et al. 2010). Using ELISA techniques, Asomugha et al. (2010) found that in addition to decreased survival rates, cells subjected to 500 μ M glutamate showed an increase in phosphorylation of p38 MAPK. When cells were treated with ACh or nicotine before the glutamate assault, cells survived at rates comparable to controls and were associated with significant increased phosphorylation of AKT and significant decreased p38 MAPK. This scenario suggests that activation of the p38 MAPK pathway is involved in regulating cell death, whereas the activation of the AKT pathway inhibited the p38 MAPK pathway and promoted cell survival. To test the involvement of these pathways, ELISA studies were conducted to investigate phosphorylation rates of the target proteins.

Results from ELISA studies were only able to indicate involvement of p38 MAPK when tropisetron was used as a neuroprotective agent. Cells in excitotoxic conditions treated with glutamate alone showed decreased survival rates and increased phosphorylation of p38 MAPK when compared with controls. RGCs exposed to tropisetron alone exhibited survival rates comparable to untreated controls and significantly lower levels of phosphorylated p38 MAPK compared to glutamate treated samples. Treatment with a protective dose of tropisetron before glutamate exposure ameliorated cell death and prevented phosphorylation of p38 MAPK to levels found in samples treated with glutamate alone.

ELISA analysis for phosphorylated Akt did not show any significant differences between conditions and the results have not been included in this study. This was surprising as previous studies using ACh and nicotine showed a significant increase in phosphorylation of Akt associated with neuroprotection. These studies using tropisetron, did not show this trend. It is possible that Akt activation associated with ACh in previous studies (Asomugha et al., 2010) occurred through activation of $\alpha 4\beta 2$ nAChRs since studies used lysates prepared from large and small RGC cultures. These findings suggest that tropisetron provides neuroprotection through inhibition of p38 MAPK but does not involve the Akt pathway.

As mentioned previously, both iGluRs and nAChRs are nonspecific cation channels that allow the passage of Na^+ , K^+ , and Ca^{2+} (Michaelis, 1998) when activated. Multiple studies have determined that Ca^{2+} is the key ion involved in excitotoxicity as well as neuroprotection (Sucher et al., 1996; Kihara et al., 2001; Brandt et al., 2010). So calcium ions may have a dual neurotoxic/neuroprotective effect. As a neurotoxic effect, excitotoxicity occurs after a prolonged influx of Ca^{2+} creates a Ca^{2+} overload and activates a cascade of calcium-activated proteases or caspases that initiate apoptosis. However, Brandt et al. (2011) provided strong evidence that neuroprotection is also dependent on Ca^{2+} influx. In cell culture studies, neuroprotection by nicotine and ACh were affected in a dose-dependent manner when the extracellular concentration of Ca^{2+} was manipulated during induced excitotoxic conditions. Lower concentrations of extracellular Ca^{2+} led to lower degrees of neuroprotection. It was further shown that the physiological outcome did not depend on the avenue of Ca^{2+} entry as neuroprotection could be induced by Ca^{2+} changes induced by a variety of methods (Brandt et al., 2011). Even more interestingly, neuroprotection against 500 μ M glutamate was also induced by Ca^{2+} entry through glutamate receptors if a relatively low-dose pretreatment of glutamate was applied. All of this evidence suggests that the amount of Ca^{2+} that enters the cell is what determines the fate in isolated pig RGCs. When stimulation of nAChRs occurs, the receptors characteristically rapidly desensitized allowing only a relatively small amount of Ca^{2+} to enter the cell (Gotti et al., 1997; Giniatullin et al., 2005), initiating signaling pathways that promote cell survival

and inhibit apoptosis. Therefore, it can be reasoned that a method of decreasing Ca^{2+} influx in the presence of excessive amounts of glutamate may be a plausible method of providing neuroprotection in excitotoxic conditions.

Studies have indicated involvement of voltage-gated Ca^{2+} channel internalization in the process of neuroprotection (Schubert and Akopian, 2004; Akopian et al., 2006; Cristofanilli and Akopian, 2006; Cristofanilli et al., 2007; Mizuno et al., 2010). Other studies have demonstrated that internalization of receptors involve ACh, as treatment with the acetylcholinesterase inhibitor, donepezil, internalized NMDA type-1 receptors (Shen et al., 2010). In the receptor internalization studies shown in this study, results were presented to support the hypothesis that GluN1 subunit internalization is involved in neuroprotection of adult pig RGCs with tropisetron using immunocytochemistry and electrophysiology studies. These studies also revealed that internalization of receptors occurred after an hour of tropisetron treatment, but the amount of cells containing internalized receptors significantly increased if cells weren't fixed until 1 and 3 days after tropisetron application. Although some internalization occurred with a short exposure to tropisetron, significantly more large RGCs internalized GluN1 receptor subunits with longer tropisetron treatment, similar to results obtained from Shen et al., (2010). This is likely due to the time it takes to trigger the internal mechanisms associated with internalization. For instance, Christofannili and Akopian (2006) demonstrated that calcium influx through voltage-gated L-type calcium channels triggered actin cytoskeleton reorganization in retinal neurons that led to internalization of calcium channels. Alternatively, dynamic trafficking proteins were found to be involved in surface levels of glutamate receptors (Danielson et al., 2012), while phosphorylation of tyrosine regulated endocytosis of NMDA receptors in other systems (Chowdhury et al., 2013). All of these mechanisms involve multiple intracellular steps and it is likely that a longer time period of incubation in tropisetron allows for amplification of the internal cascades responsible for internalization of GluN1 receptor subunits, resulting in greater internalization of receptors.

Electrophysiological studies were also performed that supported the hypothesis that internalization of GluN1 subunits occurred after tropisetron treatment. In these studies, voltage-clamped large pig RGCs elicited consistent responses to 100 and 200 μM NMDA when Mg^{+2} was omitted from the recording saline. Responses to NMDA in retinal ganglion cells in pig were consistent with those recorded from other vertebrate RGCs (Karschin et al., 1988; Mittman et al., 1990; Cohen et al., 1994). However, when cells were cultured with tropisetron for 3 days in culture before applying NMDA, the responses were reduced by an average of 48%. The significant reduction in the NMDA-induced response compared to control untreated conditions support the hypothesis that internalization of NMDA receptors occurred. Fewer receptors would result in smaller NMDA-induced current. However, alternative explanations that would explain smaller NMDA-induced currents could include inhibition or modulation of NMDA channels through activation of signaling cascades (Chowdhury et al., 2013; Wang and Wang, 2012; Cook et al., 2011; Aramakis et al., 1999). However, the reduction in NMDA-induced current along with the immunocytochemical evidence of internalized receptors does not support a modulation scenario.

CONCLUSIONS

These results support the notion that tropisetron is an effective neuroprotective agent against glutamate-induced excitotoxicity by way of $\alpha 7$ nAChR activation. This effect involves inhibition of the apoptotic p38 MAPK pathway that is stimulated by excessive Ca^{2+} influx through NMDARs. Furthermore, these results suggest that neuroprotection by tropisetron also involves attenuation of Ca^{2+} entry through glutamate receptors by removal of GluN1s from the cell membrane via receptor internalization. Considering the widespread and

devastating effects of diseases in the CNS associated with excitotoxicity, gaining a better understanding of the mechanisms involved in excitotoxicity and in neuroprotection is of great importance from a public health standpoint. The findings of this study suggest that tropisetron could be an effective therapeutic agent for degenerative disorders of the central nervous system that involves excitotoxicity and warrants further exploration as a treatment option for such disorders.

ABBREVIATIONS

ACh	acetylcholine
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
$\alpha 7$nAChR	alpha7 nicotinic acetylcholine receptor
ANOVA	analysis-of-variance
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
GluRs	glutamate receptors
5-HT₃	serotonin
NGF	nerve growth factor
CNS	central nervous system
IgG	immunoglobulin G
IgM	immunoglobulin M
MAPK	mitogen activated protein kinase
MLA	methyllycaconitine
nAChR	nicotinic acetylcholine receptor
NMDA	N-methyl-d-aspartate
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
RGC	retinal ganglion cell
SDS	sodium dodecyl sulfate
TEA	tetraethylammonium chloride
Thy	glycoprotein originally identified in thymus gland
tropisetron	(1 <i>R</i> ,5 <i>S</i>)-8-methyl-8-azabicyclo[3.2.1]octan-3-yl 1-methyl-indole-3-carboxylate

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Tropisetron prevents loss of RGCs normally associated with excitotoxicity.
Tropisetron action is mediated through activation of $\alpha 7$ nAChRs.
Tropisetron significantly decreased levels of phosphorylated p38 MAPK.
Tropisetron's action was linked to internalization of NMDA receptors.
Tropisetron could be an effective agent for excitotoxic diseases in the CNS.

Figure 1. Images displaying cultured isolated pig RGCs in (A) control untreated conditions, (B) in the presence of 500 uM glutamate, and (C) in the presence of 100 nM tropisetron and glutamate. Arrows indicate large RGCs, arrowheads indicate small RGCs.

Figure 2.

Dose response study displaying the survival rates of RGCs compared to control untreated conditions after various concentrations of tropisetron were applied before 500 μ M glutamate. Each data point represents the mean percent of neuroprotection that occurred. Data points were curve fit. Error bars represent SEM. All data points obtained above 1 nM tropisetron provided significant neuroprotection from control untreated conditions. (N = 3-7).

Figure 3.

Bar graphs summarizing the effects of glutamate and 100 nM tropisetron on large (left bars) and small (right bars) RGCs. Each bar graph represents the average percent of RGC survival compared to control untreated conditions. Error bars represent SEM. * represent significance from control untreated conditions. # represents significance from glutamate treated RGCs (N = 8). Data is considered significantly different if $P < 0.05$ in all figures.

Figure 4.

Bar graphs displaying MLA's inhibitory effect on tropisetron-induced neuroprotection. 10 nM MLA significantly blocked the protective effect of a 100 nM dose of tropisetron. * represent significance from glutamate conditions, # represent significance from control. Error bars represent SEM. (N = 5).

Figure 5. Control study showing the effect of the 5-HT₃ agonist, SR 57227, pretreatment on glutamate-induced excitotoxicity. SR 57227 provided no significant protection from glutamate induced excitotoxicity. # represent significance from control. Error bars represent SEM. (N = 4)

Figure 6. Study showing the effect of the 5-HT₃ agonist, SR 57227, treatment prior to tropisetron treatment. Presence of a 5-HT₃ agonist had no significant effect on neuroprotection. * represent significance from glutamate conditions, # represent significance from control. (N = 4). Error bars represent SEM

Figure 7.

ELISA study showing involvement of the p38 MAPK pathway in neuroprotection. Each bar graph represents the average amount of phosphorylated p38 MAPK measured under 4 different conditions. These values were obtained from RGCs that were treated under control conditions, in the presence of 500 μ M glutamate (Glu), in the presence of 100 nM tropisetron (trop) and in the presence of 100 nM tropisetron and 500 μ M glutamate (T/G) for 12 hours before lysates were made according to ELISA procedures. The * represents a significant difference from the other treated conditions. (N=6). Error bars represent SEM.

Figure 8. Antibody labeling control study. (A) illustrates a large RGC labeled with a primary antibody against GluN1 subunit under normal illumination and after visualizing with a secondary antibody conjugated with Alexa Fluor 594 (B). In C, the primary antibody was omitted before application of the Alexa Fluor 594 labeled secondary antibody. In D, 2 large RGCs were labeled with the primary antibody against GluN1 and secondarily labeled with a Dylight 405 conjugated secondary antibody. Normal illumination is shown in D and the fluorescent image due to excitation of Dylight 405 is shown in E. In F, the primary antibody was omitted prior to application of the Dylight 405 labeled secondary antibody. The same results were obtained from 3 different experiments.

Figure 9.

Internalization study. All cultured isolated RGCs were labeled with a primary rabbit antibody against GluN1 prior to any pharmacological treatments. Cells were then subjected to experimental conditions, fixed, labeled with rhodamine conjugated to anti-rabbit antibody, permeabilized to allow antibody labels access to the interior of the cell, then labeled with DAPI conjugated to anti-rabbit antibody. (Top row) RGCs with no pharmacological treatment under (A) Alexa Fluor 594 illumination, (B) Dylight 405 illumination, and (C) overlay of both images. (Middle row) RGCs treated with 100 nM tropisetron, then fixed and labeled with secondary antibody linked to Alexa Fluor 594, permeabilized, then labeled with a secondary antibody linked to Dylight 405 under (D) Alexa Fluor 594 illumination, (E) Dylight 405 illumination, and (F) overlay of both images. (Bottom row) RGCs already bound to antibodies against GluN1 treated with the specific $\alpha 7$ nAChR inhibitor, MLA, prior to tropisetron treatment, then fixed and labeled with the 2 fluorescently labeled secondary antibodies under (G) Alexa Fluor 594 illumination, (H) Dylight 405 illumination, and (I) overlay of both images. Only cells treated with tropisetron displayed GluN1s labeled with Dylight 405, suggesting internalization of GluN1s with tropisetron treatment.

Figure 10.

Tropisetron reduced NMDA-induced current. RGCs were cultured for three days under control untreated conditions and in the presence of 100 nM tropisetron. Large RGCs were held at -60 mV in Mg^{+2} -free saline containing blockers of voltage-gated potassium, sodium and calcium channels before application of NMDA. Each current trace represents a superimposed response obtained from 10 different voltage-clamp recordings. (A) Superimposed current traces obtained when 100 or 200 μ M NMDA were perfused over voltage-clamped large RGCs under control untreated conditions. (B) Superimposed current traces obtained when 100 or 200 μ M NMDA were perfused over voltage-clamped large RGCs that were treated with 100 nM tropisetron. (C) Current-voltage relationship obtained

when 200 μ M NMDA was applied to voltage-clamped large RGCs under control conditions (dotted line) and when cells were treated with 100 nM tropisetron (solid line) for 3 days. Each data point represents an average current response obtained using 200 μ M NMDA when cells were held between -60 and $+40$ mV in 20 mV increments. Error bars represent standard error. * represents significance difference from control untreated responses.