Tropisetron as a Neuroprotective Agent Against Glutamate-Induced Excitotoxicity: Potential Mechanisms of Neuroprotection

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TROPISETRON AS A NEUROPROTECTIVE AGENT AGAINST GLUTAMATE-INDUCED EXCITOTOXICITY: POTENTIAL MECHANISMS OF NEUROPROTECTION

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Glutamate-induced excitotoxicity has been shown to play a key role in a number of neurodegenerative diseases of the central nervous system. Activation of nicotinic acetylcholine receptors (nAChRs) significantly protects various types of neuronal tissues from excitotoxic cell death (Kaneko et al., 1997; Dajas-Bailador et al., 2000; Wehrwein et al., 2004; Thompson et al., 2006). Tropisetron is a highly selective 5-HT₃ class serotonin receptor antagonist that is also a partial acetylcholine (ACh) agonist of the α₇ nAChR (Papke et al., 2005). In the current study we aim to determine if tropisetron offers protection to adult pig RGCs. We further aim to determine if protection occurs through activation of α₇ nAChRs. We used cell culture methods to isolate adult pig RGCs using the two-step panning technique first described by Wehrwein et al. (2004). Our results show that tropisetron protects adult pig RGCs from glutamate-induced excitotoxicity in a dose-dependent manner. ELISA studies suggest involvement of the p38 MAPK intracellular signaling pathway. Fluorescent immunocytochemistry studies show evidence that receptor internalization occurs during neuroprotection by tropisetron. The results of this study suggest tropisetron warrants further investigation as a therapeutic agent against neurodegenerative disorders involving dysfunction in glutamate activity.
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by

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INTRODUCTION

Glaucoma and Cell Death

Glaucoma is one of the leading causes of blindness worldwide. It is a progressive neurodegenerative disorder of the retina that occurs through death of retinal ganglion cells (RGCs) resulting in irreversible loss of the visual field. Although a definitive cause of glaucoma is not known, the most common indicator leading to a glaucoma diagnosis is increased intraocular pressure (Quigley et al., 1995). However, increased intraocular pressure alone is not the cause of glaucoma (Dreyer & Lipton, 1999). Studies have shown that relieving intraocular pressure associated with glaucoma does not always protect the ganglion cells and prevent loss of vision (Dreyer & Lipton, 1999). Along with increased intraocular pressure, it has been found that the pathology of glaucoma also includes an elevated concentration of the neurotransmitter glutamate in the vitreous humor of the eye (Vickers et al., 1995; Dreyer et al., 1996; Quigley & Vitale, 1997; Weinreb & Khaw, 2004). Although glutamate is one of the most abundant neurotransmitters in the body, exposure to high concentrations has been shown to produce apoptosis of cells in a variety of model systems through a process that has been termed excitotoxicity (Michaelis, 1998; Mattson, 2003).

The steps leading from the binding of a neurotransmitter molecule to the resultant cell death are not fully understood, however an increase of the intracellular concentration of Ca$^{2+}$ has shown to be an important factor (Olney, 1982). 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 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 factor in the process of excitotoxic cell death, giving rise to the term glutamate-induced excitotoxicity (Michaelis, 1998; Mattson, 2003). Ironically, in addition to being the main neurotransmitter involved in excitotoxicity in mammals, glutamate is also the most prevalent neurotransmitter in the mammalian brain, being utilized in varying degrees by nearly all neurons of the vertebrate central nervous system.

Many recent studies have indicated the process is initiated in response to excessive Ca\(^{2+}\) influx (Choi, 1985; Sattler & Tymianski, 2000; Arundine & Tymianski, 2003; Brandt et al., 2011). In glaucoma, increased concentrations of glutamate in the vitreous humor leads to excessive Ca\(^{2+}\) influx, apoptotic death of retinal ganglion cells and loss of the visual field. When glutamate binds to ionotropic glutamate receptors the immediate result is the opening of ion channels and an influx of Ca\(^{2+}\) and Na\(^{+}\) ions. Previous studies have shown that Ca\(^{2+}\) is a key component in the apoptotic pathway (Sattler & Tymianski, 2000; Arundine & Tymianski, 2003). When too much glutamate binds to receptors, it leads to intracellular Ca\(^{2+}\) overload and triggers the apoptotic pathway. Cells cultured in the presence of a high glutamate concentration without Ca\(^{2+}\) do not exhibit the cell death associated with cultures containing Ca\(^{2+}\) pointing to the
notion that it is Ca$^{2+}$ influx that ultimately leads to cell death (Brandt et al., 2011).

Apoptosis is a type of programmed cell death that is utilized in various ways throughout the body to eliminate cells. The process can be activated by a number of intracellular signaling mechanisms, including excessive Ca$^{2+}$ influx, that initiate an irreversible signaling cascade that activates a family of proteases called caspases that are responsible for the destruction of the cell (Li et al., 1997; Tenneti et al., 1998; Tenetti & Lipton, 2000).

**Neuroprotection**

A recent line of research has shown that, in various neural tissues, when cells are pre-treated with substances that 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). This is known as neuroprotection. However, the mechanisms by which excitotoxicity is prevented by nAChR activation are not fully understood. Several studies have suggested that Ca$^{2+}$ influx through activated nAChRs affects phosphorylation level of the p38 MAPK and AKT intracellular signaling pathways resulting in neuroprotection of RGCs (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. Several studies have shown that substances that destabilize the cytoskeleton attenuate the inward Ca$^{2+}$ current by removal of voltage-gated Ca$^{2+}$ channels from the cell membrane resulting in protection from glutamate induced excitotoxicity (Schubert & Akopian, 2004; Cristofanilli et al., 2007; Mizuno et al., 2010). Another study suggests that stimulation of nAChRs causes internalization of
NMDA receptors, leading to increased cell survival by attenuating the inward Ca\(^{2+}\) current and decreased glutamate activity (Shen et al., 2010).

In the current study, we investigate the neuroprotective properties of tropisetron, a 5-HT\(_3\) (serotonin) antagonist that has long been utilized as an antiemetic that has also been shown, to have a selective partial agonist action at the \(\alpha7\) nAChR (Papke et al., 2004; Macor et al., 2001). 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 through which receptors elicit tropisetron’s neuroprotective effect. Two different approaches were used to further investigate the possible mechanisms involved in tropisetron’s ability to protect adult pig RGCs from glutamate-induced excitotoxicity. ELISA studies were used to examine the role of p38 MAPK and AKT signaling proteins in tropisetron treatments against glutamate. The possibility of internalization of NMDA GluRs in response to tropisetron treatment was also looked into using fluorescent immunocytochemistry techniques.

**BACKGROUND AND SIGNIFICANCE**

*Visual System and Eye Anatomy*

Vision is one of the five senses used by animals to sense external stimuli from the surrounding environment. The visual system is designed to detect light in the form of photons, transduce that energy into action potentials, and generate a visual perception of the environment by processing the specific pattern of stimulation through a complex
network of circuitry from the retina to the visual cortex. A malfunction at any point along this chain results in massive cognitive deficit and an inability to render an accurate visual perception of the surrounding environment.

The eye is the major sensory organ of the mammalian visual system. The general structure of the eye is a fluid filled sphere, the outer skin of which is organized into three distinct layers called tunics that comprise the specific structures of the eye. The most external layer, or fibrous tunic, consists of the sclera and cornea. The sclera, commonly referred to as the “white,” is composed of dense irregular connective tissue and provides most of the structural strength of the eye. The cornea forms the anterior surface of the eye and aids in refracting light as it enters the eye. The layer just deep to the fibrous tunic is the vascular tunic. It includes the choroid, iris, and ciliary body. These structures supply the eye with oxygen and nutrients and regulate the amount and aid in focusing of light that enters the eye. The most internal layer of the tissue surrounding the eye, deep to the vascular tunic, is the neural tunic. It forms the internal surface of the eye and is composed of the pigment epithelium and retina. It surrounds the vitreous humor, the clear jelly-like substance that fills the greater part of the interior of the eye. The pigment epithelium contains pigmented melanocytes that provide vitamin A to photoreceptors and contributes to a clearer image by absorbing extraneous photons of light. The retina is the neural tissue deep to the pigment epithelium covering the inner surface of the posterior portion of the eye. The organized pattern of light energy that enters the eye is focused by the lens and cornea onto the retina, where it is transduced into a coded pattern of action potentials that is used by the brain to derive an image of the environment.
Anatomy of the Retina

The retina is recognized as the most peripheral structure of the central nervous system. It is a complex sensory tissue composed of a network of neural circuitry used to transduce light energy into a signal of action potentials and process the signals before they are sent to the brain. The retina consists of five neuronal cell types (photoreceptors, horizontal cells, bipolar cells, amacrine cells, and ganglion cells) that are connected in a highly organized and specific manner. There are five distinct layers in the retina, three layers of neuron cell bodies (outer nuclear, inner nuclear, and ganglion cell layers) and two layers of synapses (inner and outer plexiform layers).

The photoreceptors are the most peripherally located class of retinal cells. They are responsible for detecting light and transducing the energy into a signal that can be recognized by other neuronal cells. There are two types of photoreceptors, rods, tuned for light and motion detection in low light situations, and cones, tuned for detail and color detection in environments with an abundance of light. The distribution of these different types of photoreceptors has a great effect on how the world is perceived visually. There are more than 100 million rod cells in the human eye distributed primarily throughout the periphery of the retina to gather light from the entire visual field. They are highly sensitive and widely tuned to detect contrast between light and less light, but do not facilitate perception of fine detail. Cone cells occur with much lower frequency, there are roughly 10 million cone cells in the human retina, concentrated at a high density at the center of the visual field in the macula lutea. This high concentration of cone cells forms the fovea centralis, the area of the retina that corresponds with the area of the visual field where acuity is highest. This is the area of the visual field that we use to read and allows
us to focus on fine details of the visual world. Cone cells are further separated into three subtypes (red, green, and blue in mammals) based on the tuning to specific wavelengths of light energy that elicit maximal responses when absorbed. Phototransduction at first seems counterintuitive in vertebrates, as the photoreceptors are designed to release glutamate in the absence, rather than presence of photo stimulation. In the presence of light, photopigment proteins are activated, maintaining photoreceptors in a hyperpolarized state through metabotropic mechanisms. This leads to a decreased inward \( \text{Ca}^{2+} \) current at the synaptic terminal and results in decreased release of glutamate. In the absence of light, photopigment proteins are inactive, leading to depolarization of photoreceptors and release of glutamate at the synapse.

Horizontal cells are involved in modifying the visual signal as it travels from the photoreceptor to the bipolar cells. They contribute to the generation of receptive fields in bipolar cells through a process called lateral inhibition. Lateral inhibition occurs when a photoreceptor is depolarized, releases glutamate onto a horizontal cell that has a synapse with an adjacent photoreceptor, and the horizontal cell releases GABA onto the adjacent photoreceptor causing it to hyperpolarize.

Bipolar cells relay and process the signal between photoreceptors and ganglion cells. They receive input from photoreceptors, horizontal cells, and amacrine cells and release glutamate onto retinal ganglion cells. Bipolar cells are divided into two subtypes, hyperpolarizing “H”-type and depolarizing “D”-type cells, based on their net response to illumination of input photoreceptors. The different responses to illumination are achieved through different types of glutamate receptors that occur on each bipolar cell subtype. H
bipolar cells have ionotropic glutamate receptors that produce a net excitatory effect when bound by glutamate and D bipolar cells have metabotropic glutamate receptors that produce a net inhibitory effect when bound by glutamate. In light, when glutamate release by photoreceptors decreases H bipolar cells hyperpolarize and D bipolar cells depolarize. This provides another level of processing for the visual signal and contributes to the generation of retinal ganglion cell receptive fields.

There are around 40 different subtypes of amacrine cells identifiable in the retina based on morphology, location or neurotransmitter specificity. Amacrine cells receive synaptic input from bipolar cells and help modify the visual signal before it is passed on to ganglion cells. The different subtypes of amacrine cells release a number of neurotransmitters, including dopamine, acetylcholine, GABA and many others. One particular subset of amacrine cells, known as starburst amacrine cells based on their starburst appearance, are known to release ACh onto ganglion cells. Considering that ACh has been shown to protect retinal ganglion cells from excitotoxicity, there are multiple roles that starburst amacrine cells could play in the progression of glaucoma and neuroprotection. It could be possible that glaucoma begins with a defect in these supporting cells leading to increased susceptibility of ganglion cells to excitotoxicity, or that stimulating release of ACh from these cells could be used as a protective therapy against glaucoma.

Ganglion cells are the most downstream cells of the retina. They receive input from bipolar and amacrine cells. Their axons compose the optic nerve and relay the visual signal from the retina in a highly conserved orientation to the lateral geniculate
nucleus (LGN) of the thalamus via glutamate release onto cells of the LGN. The visual signal then travels from the LGN to the visual cortex in the occipital lobe, where visual perception occurs. The information contained in the visual signal travels from the retina to the visual cortex in two distinct parallel pathways. In mammals, the magnocellular or “M” pathway arises from large RGCs and carries visual information regarding high-contrast, black and white and mainly peripheral vision. The parvocellular or “P” pathway arises from small RGCs and carries information about high-acuity, color vision, mainly from the center of the visual field. These pathways are oriented in multiple layers in the LGN containing information about different aspects from different areas of the visual field. The visual cortex also has multiple layers with cells responding to many varied and specific receptive fields that process highly complex aspects about the visual field and give rise to the rich visual perception that most of us are able to experience during consciousness in lighted environments.

The visual system is an invaluable tool that we use nearly every second of our waking life to gather information about the world around us. Without it our ability to function and adapt to our surrounding environment is greatly impaired. The phenomenon of visual perception comes about through a series of complicated physiological processes involving highly sophisticated biological machinery that many take for granted during the course of their day. When one stops to reflect on how we come to derive a visual representation of the world it is really “seen” as an awe-inspiring wonder. Therefore, neurological disorders that cause damage to the visual system such as glaucoma pose a great threat to the quality of life for individuals affected by them and finding ways to treat and cure such disorders is of great importance from a public health standpoint.
Excitotoxicity and Glutamate

Glutamate is the main excitatory neurotransmitter in the central nervous system (Michaelis, 1998). Many studies have established that over-activity of ionotropic glutamate receptor cells causes excessive influx of $\text{Ca}^{2+}$ ions leading to excitotoxic cell death in many model systems (Glovinsky et al., 1991; Lam et al., 1999; Lipton, 2001; Kawasaki et al., 2002; Wehrwein et al., 2004; Thompson et al., 2006; Kim et al., 2007). Quigley et al. (1995) demonstrated that, in glaucoma, excessive levels of glutamate in the eye cause nonspecific cation channels to remain open for extended periods of time allowing various cations to enter the RGCs and trigger intracellular cascades that lead to apoptosis. Wehrwein et al. (2004) found that when adult pig RGCs were cultured using ligands that bind to specific glutamate receptor subtypes the cell survival rates varied depending on which receptor subtypes were activated. They found that activation of KA and NMDA receptors separately produced significant cell death, though not to the level of glutamate. However, when KA and NMDA receptors were activated together RGCs exhibited death rates comparable to those observed with glutamate.

Neuroprotection and Nicotinic Acetylcholine Receptors

Another finding that came about from the Wehrwein et al. (2004) study was that ACh is able to reduce glutamate-induced excitotoxicity in cultured adult pig RGCs. In their study, RGCs were cultured in the presence of 500 $\mu$M glutamate to produce an excitotoxic environment. When the cells were not treated with any pharmacological agent prior to glutamate exposure, the cultures exhibited death of an average 40% of the RGCs. However, when 5$\mu$M ACh was added to the excitotoxic preparations before
glutamate, cultured cells were able to survive almost completely (counts were comparable to the level of untreated control samples). When the highly selective ACh antagonist, MLA, was added to culture prior to ACh treatment, neuroprotection was blocked in a dose dependent manner, supporting the idea that the neuroprotection occurs through ACh activation. Furthermore, the study went on to distinguish that the neuroprotective effect is mediated through the nAChR, not the mAChR. When nicotine was applied, instead of ACh, before glutamate, RGCs survived at a rate comparable to those cultured under ACh. However, when muscarine was used instead of ACh or nicotine before glutamate application, RGCs died off at nearly the same rate as those cultured with glutamate alone. These findings suggest that ACh neuroprotection is mediated through nAChRs, not mAChRs. Results from Thompson et al. (2006) showed that neuroprotection by ACh is mediated through both α7 nAChRs and α4β2 nAChRs, however these receptors are not found on both types of RGCs. Large RGCs were found to have mainly α7 nAChRs and small RGCs were found to have mainly α4β2 nAChRs. This suggests multiple possible mechanisms of action for neuroprotection by nAChR activation.

*Intracellular Signaling Pathways*

Recent studies have progressed in the direction of determining the specific intracellular signaling pathways that are involved in glutamate-induced excitotoxicity. Many studies suggest that apoptosis associated with excitotoxicity is regulated through the p38 MAP kinase pathway (Dineley, et al., 2001; Pearson et al., 2001; Manabe & Lipton, 2003; Zarubin & Han, 2005; Wang et al., 2007). Asomugha and colleagues (2010) found that Ca^{2+} influx through chronically stimulated glutamate receptors in adult
pig RGCs under excitotoxic conditions leads to increased phosphorylation of p38 MAPK and cell death. When the p38 MAPK inhibitor, SB 203580 was applied before excessive glutamate, RGC death and phosphorylation of p38 MAP kinase were significantly decreased. This suggests involvement of the MAPKKK > MAPKK > p38 MAP kinase intracellular signaling pathway in glutamate-induced excitotoxicity.

Many have suggested that neuroprotection occurs through activation of the PI3-K > Akt pathway (Kenedy et al., 1999; Zhou et al., 2000; Kihara et al., 2001; Shaw et al., 2002; Mai et al., 2003). Others have suggested that inactivation of the p38 MAP kinase pathway is involved (Dineley et al., 2001; Shaw et al., 2002; Manabe & Lipton, 2003). These suggestions are not mutually exclusive. Results from Asomugha et al. (2010) suggest that ACh acts through both mechanisms. It was found that when ACh is applied prior to excitotoxic conditions Ca$^{2+}$ influx through activated nAChRs increases Akt phosphorylation, reduces p38 MAPK activity and is associated with increased cell survival. Inhibition of the PI3-K > Akt pathway by wortmannin prior to ACh and glutamate blocked the effect of ACh on Akt and p38 MAPK phosphorylation and cell survival, suggesting involvement of both p38 MAPK and Akt activity in neuroprotection and that Akt plays a role in inhibiting p38 MAPK activity. Perhaps one of the more interesting findings is that Ca$^{2+}$ influx through activated receptors is necessary for both excitotoxicity and neuroprotection (Asomugha et al., 2010; Brandt et al., 2011).

Receptor Internalization

Several recent studies have exposed a link connecting Ca$^{2+}$ influx through voltage- and ligand-gated Ca$^{2+}$ channels with stability of actin filaments in the
cytoskeleton and glutamate receptor activity in salamander RGCs (Schubert & Akopian, 2004; Akopian et al., 2006; Christofanelli & Akopian, 2006). It is suggested that Ca\(^{2+}\) influx causes destabilization of the actin cytoskeleton, internalization of Ca\(^{2+}\) channels and leads to increased cell survival in salamander RGCs under excitotoxic conditions. Another study has 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\(^{2+}\) current, 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 using tropisetron as a neuroprotective agent.

**HYPOTHESES EXAMINED**

*Tropisetron*

Tropisetron has long been used as an anti-emetic to help alleviate symptoms associated with chemotherapy and postoperative condition. This effect is regulated through tropisetron’s action as a 5-HT\(_3\) antagonist. Recent studies show that tropisetron also exhibits a potent selective agonist action at α7 nAChRs (Papke et al., 2004; Macor et al., 2001). In previous studies, activation of α7 nAChRs has been shown to protect cells from glutamate-induced excitotoxicity in a number of model systems, suggesting that tropisetron may also be useful as a neuroprotective agent. It is this action that led us to hypothesize that tropisetron will protect isolated adult pig RGCs against glutamate-
induced excitotoxicity when applied before glutamate. It is also hypothesized that if tropisetron treatment protects RGCs then the action occurs through activation of α7 nAChRs. Pharmacological experiments are used to determine protective concentrations of tropisetron. A number of experiments were also conducted using agonists and antagonists of specific receptor subtypes to differentially counteract each of tropisetron’s actions in order to determine if protection occurs through activation of α7 nAChRs and whether action at 5-HT₃ receptors plays a role.

Mechanisms of Action

To investigate involvement of intracellular signaling pathways, it is hypothesized that if tropisetron protects cells from glutamate-induced excitotoxicity through its agonist action at nAChRs, then tropisetron will also stimulate AKT action and block p38 MAPK action. ELISA studies were used to determine if action of the p38 MAPK and AKT intracellular signaling pathways play a role in either neuroprotection or excitotoxicity of isolated adult pig RGCs. Additionally, in an attempt to further probe mechanisms of neuroprotection, it is hypothesized that if tropisetron protects cells from excitotoxicity, then it may possibly occur through internalization of NMDA receptors. Fluorescent immunocytochemistry studies were performed to look into the possible involvement of NMDAR internalization in protection of RGCs via nAChR activation.

METHODS

Retinal Ganglion Cell Isolation

Adult pig eyes were acquired from a local slaughterhouse (Pease Slaughterhouse,
Scotts, MI). To isolate the RGCs, we used a modified two-step panning procedure described in Wehrwein et al. (2004). Only eyes from freshly slaughtered pigs were used to ensure viability of the samples. The eyes were kept on ice during transport to the laboratory and immediately dissected to remove the retina. To do this, a circular incision was made in the area surrounding the cornea and lens. These were then removed from the eye. The remaining portion of the lens and vitreous humor were then removed to expose the eyecup. To loosen the retina from the eyecup, the space left vacant by the vitreous humor was gently refilled with our culture medium, a modified CO\textsubscript{2}-independent medium (Gibco, Carlsbad, CA; cat # 18,045-088) kept at 37°C, containing 4mM glutamine, 10% fetal bovine serum (FBS), 5% antibiotic/antimycotic, and 4 mM HEPES. Once the retina had separated from the remaining tissue it was peeled out with forceps and snipped from the optic disc with scissors. The retinal tissue from each eye was then cut into 8 smaller pieces and placed into centrifuge tubes for enzymatic treatment with 500 µl of activated papain (Worthington, New Jersey, Cat # 3126, 27 units/mg). The tissue was incubated at 37°C in a water bath for 20 minutes to allow the papain to dissolve the intercellular connections. Tubes were inverted every 2-3 minutes for proper mixing of the papain. After 20 minutes, tissue was rinsed with fresh CO\textsubscript{2}-independent medium containing 1 mg/ml DNase to stop the papain action. After enzymatic treatment, complete dissociation of the RGCs was obtained by using an unpolished Pasteur pipette to gently triturate the tissue several times. Cells were then transferred to 15 ml conical tubes and diluted with culture medium up to 10 ml and plated onto 150 x 15 mm Petri dishes, coated with goat anti-rabbit IgG antibody (Jackson ImmunoReseach, West Grove, PA; Cat # 111-005-003; 0.5 mg in 10 ml of 20mM Tris buffer) at 1 x 10\textsuperscript{5} cells/ml. The
petri dishes were coated with IgG the night before ganglion cell harvest and kept at 4°C overnight. On the morning of culture preparation, the Petri dishes were rinsed three times with PBS (without magnesium chloride and calcium chloride) and allowed to incubate at 37°C with PBS containing 0.2% BSA for 1 hour prior to application of the RGCs.

Dissociated cells were incubated for one hour at 37°C on the IgG coated plates. After 1 hour of incubation on the IgG plates, the cells were transferred onto Petri dishes coated with mouse anti-rat Thy 1.1 antibody (BD Biosciences, San Diego, CA; cat # 554898; 12.5 µg in 10 ml PBS containing no magnesium chloride and no calcium chloride) bound to goat anti-mouse IgM (Jackson ImmunoResearch; cat # 115-005-075; 0.36 mg in 10 ml of 20 mM Tris buffer). The IgM dishes, excluding Thy 1.1, were prepared the night before by the same method as the IgG dishes. On the morning of retinal cell harvest, the solution containing IgM was removed from the dish and replaced with a solution of Thy 1.1 in PBS. Plates were incubated for 1 hour at 37°C with Thy 1.1 solution, then rinsed with PBS three times and allowed to incubate at 37°C for 1 hour with PBS containing 0.2% BSA before use in the second panning step.

After transfer of the cells to the IgM plates, cells were allowed to incubate on the IgM/Thy 1.1 dishes for 1 hour at 37°C. During this time we further modified the CO₂-independent medium to include supplemental factors that increase cell viability in culture. Each 4 ml of culture medium contained 50 µl of 15 µg/ml nerve growth factor (NGF), 48 µl of 500 µg/ml transferrin, and 12 µl of 10 mg/ml. After 1 hour of incubation at 37°C, the medium was replaced with the fresh CO₂-independent medium including supplemental factors.
Pharmacology Studies

In pharmacology studies, isolated RGCs were evenly distributed into twelve 60 x 15 mm dishes grouped into 6 conditions with 2 dishes per condition. The first condition was always used as an untreated control group. The second condition always contained cells treated with 500 µM glutamate to induce excitotoxicity. The remaining four conditions were used as the experimental groups treated with various 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 application of 500 µM glutamate to determine the concentrations required for tropisetron to protect RGCs from glutamate-induced excitotoxicity. Glutamate was obtained from Sigma (St. Louis, MO). Tropisetron was obtained from RBI (Natic, MA). In inhibition studies the antagonists alpha-bungarotoxin (α-Bgt) and methyllycaconitine (MLA) obtained from Tocris (Bristol, UK) were applied to conditions 3 – 6 for 1 hour before tropisetron application to allow the antagonists to occupy the receptors. Since tropisetron has both α7 nAChR agonist and 5-HT3 antagonist properties, control experiments using the 5-HT3 agonist SR-57227 and the 5-HT3 antagonist with no nAChR affinity, ondansetron, obtained from Sigma (St. Louis, MO), were performed to determine which receptors are responsible for the neuroprotection exhibited by tropisetron.

All conditions were allowed to remain in culture for three days before cell viability was assessed. At the end of the experimental period, cells were loaded with 2 µM calcein in normal PBS for 1 hour to label the living cells. After 1 hour of exposure to calcein, living cells fluoresce intensely when exposed to 495 nm excitation. Background
fluorescence is not an issue because calcein does not fluoresce before interacting with cells. 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 well in a compass rose pattern. Cell counts from each of the four images were averaged to derive the count for each well. In each experiment, the average count from two wells in each condition using the same tissue was used to derive an average cell count for each treatment condition, this represented N = 1. Each experiment using tissue from new animals 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. Statistical analyses were performed using an analysis of variance (ANOVA) with Kuskall-Wallis post hoc testing. Results were considered statistically significant if p-values of <0.05 were reached.

**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 used in this study were designed to assay phosphorylated Akt and phosphorylated p38 MAPK. Kits were
obtained from Invitrogen. Phosphorylated protein content was determined from a curve generated from protein standards of known concentrations. Results were analyzed using ANOVA and considered significant if p-values of <0.05 were reached.

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 pharmacology studies with the exception that 12 eyes were used instead of 4 and cells were plated onto 12 150 x 15 mm dishes coated with IgM/Thy 1.1 instead of 60 x 15 mm dishes. Cells were allowed to culture for 12 hours at 37°C in fresh modified culture medium in four different pharmacological treatment conditions with three plates of each condition: untreated control, 500 µM glutamate, 100 nM tropisetron, and 100 nM tropisetron pre-treatment with 500 µM glutamate added after 1 hour.

After 12 hours in culture, the supernatant fluid was removed and placed in appropriately labeled conical tubes. Then 4 ml of .25% trypsin was added to each plate, which were then incubated at 37°C for 10 minutes to release the RGCs from the Thy1.1. Once cells were loosened from the bottom of the plates, they were removed and placed in the conical tubes containing the matching supernatant. Then 1 mg of trypsin inhibitor was added for each ml of trypsin in each conical tube to stop the trypsin activity. Conical tubes were then spun in a clinical centrifuge for 10 minutes at 6000 rpm until the cells formed a pellet at the bottom of the tubes. Culture medium was then removed and each pellet was washed twice with cold normal PBS. The pellets were then combined into 1 conical tube per condition. 1 ml of cell extraction buffer (10 nM Tris, pH 7.4, 100 nM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF (Sigma # S 7920), 20 mM Na4P207
(Sigma P 8010), 2 mM Na3V04 (S 6508), 1% Triton X-100 (T-9284), 10% glycerol (G 5516), 0.1% SDS (L 4522), 0.5% deoxycholate (D4297)) with added protease inhibitor cocktail (14 µM E-64 (Sigma E 3132), 130 µM Bestatin (Sigma B 8385), 1 µM Leupeptin (Sigma L 2884), .1 mM Aprotinin (Sigma A 1153), 2 mM AEBSF (Sigma A 8456)) in 1 mM PMSF with 100 mM EDTA, was then distributed among the tubes and vigorously mixed with the cells. The tubes were then put on ice for 30 minutes and vortexed several times throughout the 30-minute time span. After 30 minutes, the liquid was transferred from the conical tubes to labeled 1.5 ml centrifuge tubes and spun in the centrifuge at 13,000 rpm for 10 minutes. The liquid lysate was then removed from a centrifuge tube and transferred to another labeled 1.5 ml centrifuge tube, leaving behind and discarding any pellet that had accumulated at the bottom of the tube. Lysates were then stored at -80°C until they were needed for use in ELISA experiments.

**Protein Phosphorylation.** To measure phosphorylated protein content, prepared lysates for each condition were thawed at room temperature and kept on ice. Each kit contained twelve strips of 8 wells for a total of 96 wells uniformly coated with an antibody against the targeted protein, protein standard of known concentration, anti-target protein detection antibody, SAV-HRP, stabilized chromogen, wash buffer and stop solution. Each experiment used half of one kit, requiring 6 strips of 8 wells for a total of 48 wells. Serial dilutions of protein standards or lysates were added to each pre-coated well to 100 µl and allowed to sit at room temperature for 2 hours. An example of a 48 well experiment processed for phosphorylated p38 MAPK is shown in Table 1.
Table 1
Distribution of ELISA samples

<table>
<thead>
<tr>
<th>Standard</th>
<th>Standard</th>
<th>Control</th>
<th>Glutamate</th>
<th>Tropisetron</th>
<th>Tropisetron/Glutamate</th>
</tr>
</thead>
<tbody>
<tr>
<td>20ng/ml</td>
<td>20ng/ml</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
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<td>10ng/ml</td>
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<td>100%</td>
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</tr>
<tr>
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<td>50%</td>
<td>50%</td>
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<tr>
<td>2.5ng/ml</td>
<td>2.5ng/ml</td>
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</tr>
<tr>
<td>1.25ng/ml</td>
<td>1.25ng/ml</td>
<td>25%</td>
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<tr>
<td>0.6ng/ml</td>
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</tr>
<tr>
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<td>12.5%</td>
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<tr>
<td>0 ng/ml</td>
<td>0 ng/ml</td>
<td>12.5%</td>
<td>12.5%</td>
<td>12.5%</td>
<td>12.5%</td>
</tr>
</tbody>
</table>

Table 1. An example of the sample distribution pattern used in ELISA studies. Serial dilutions of cell lysates from different pharmacological treatment conditions were applied to wells in ELISA kits. Optical density readings from these wells were compared with readings from wells treated with serial dilutions of protein standards of known concentrations.

After 2 hours, wells were rinsed 4 times with wash buffer and 100 µl of detection antibody was applied to each well and allowed to incubate for 1 hour at room temperature. After 1 hour, cells were rinsed with wash buffer and 100 µl of SAV-HRP was applied to each well and allowed to incubate for 30 minutes at room temperature. After 30 minutes, wells were washed again with wash buffer and 100 µl of stabilized chromogen was applied to each well. Wells were then incubated in the dark for 15-30 minutes before stop solution was added. 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 in unknown protein lysates were derived by comparing optical density readings to readings of known protein concentrations.
Immunocytochemistry Studies

To examine the hypothesis that treatment with tropisetron triggers internalization of NMDA GluRs we differentially labeled NMDA GluR1 receptors on the cell surface and internalized receptors. Adult pig RGCs were isolated as in previous studies and plated onto 150 x 15 mm 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 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 wells, some wells on each slide were treated with a 0.2% solution of primary monoclonal rabbit antibody against the NMDA type 1 receptor (NMDAR1 obtained from Millipore/Chemicon, Billerica, MA) for 1.5 hours at 37° C, while other wells were left without addition of a primary antibody. After a subset of cells had been labeled with antibody against NMDAR1, cells were rinsed with culture medium and pharmacological agents were applied to appropriate slides. One slide was left untreated by pharmacological agents and was used as a control. Some wells on other slides were treated with 10 µM ACh or 100 nM tropisetron for 1 hour. Some wells on these slides were also treated with 10 nM MLA for 20 minutes before addition of ACh or tropisetron to block activation of α7 nAChRs. After 1 hour in culture with ACh or tropisetron, slides were removed from the incubator and fixed for 30 minutes using 4% paraformaldehyde. After cells were fixed, they were rinsed with PBS and then labeled with a secondary anti-rabbit antibody conjugated to rhodamine (Dylight 594 donkey anti-rabbit IgG Jackson ImmunoResearch Laboratories, Inc. West Grove, PA), diluted at 1% in PBS. 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 4% Triton-X for 5-minutes to permeabilize the cell membranes (so that internalized receptors could be labeled). After 5 minutes the Triton-X was removed and PBS containing the same antibody labeled with a different fluorescent marker (DAPI, 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, and coverslipped and 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, Downingon, PA). Pictures of cells were compared visually to determine localization of labeled receptors. Receptors labeled with rhodamine were considered to be on the cell surface and receptors labeled with DAPI were considered to be located inside the cell.

Control experiments were conducted to display specificity of the antibodies used. In these experiments, for both fluorescent antibodies used, one set of wells were left untreated by the primary antibody against NMDAR1 and compared to wells treated with primary antibody to determine specificity of receptor binding. Cells that fluoresced after treatment with fluorescently labeled antibodies were considered to have bound with fluorescently labeled antibodies, those that did not fluoresce were considered to not have bound with the fluorescently labeled antibodies. All experiments were repeated a minimum of 3 times using tissue from different animals.
RESULTS

Pharmacology Studies

Previous studies from our lab have demonstrated that activation of α7 nAChRs prior to an excitotoxic glutamate concentration can protect cells from a dose of glutamate that normally induces apoptosis (Wehrwein et al., 2004; Thompson et al., 2006; Asomugha & Linn, 2009; Brandt et al., 2010). Tropisetron has recently been found to exhibit potent selective α7 nAChR partial agonist activity in addition to being a known 5-HT3-receptor antagonist (Macor et al., 2001). This suggests that tropisetron may be useful as a neuroprotective agent against glutamate-induced excitotoxicity. 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 α7 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 µM glutamate concentration and then incubated in culture for two days. At the end of two days, live cells were labeled with calcein AM, imaged and counted using Metamorph Imaging System. Untreated control samples were used as a baseline in each experiment to compare cell survival in all studies (Fig 1a). 500 µM glutamate effectively killed an average of 62% (± 3.1) of the RGCs when applied without protective pretreatment (Fig 1b). Dose response studies showed that tropisetron provided maximal protection from glutamate-induced excitotoxicity at a dose of 100 nM with an average survival rate of 108% (±20.3) (Fig 1c and Fig 2). Each data point represents the mean survival rate of RGCs for each dose of tropisetron.
Figure 1. Cultured Retinal Ganglion Cells

Images displaying cultured RGCs in (A) control, (B) excitotoxic 500 uM glutamate, and (C) neuroprotective 100 nM tropisetron conditions. Arrows indicate large RGCs, arrowheads indicate small RGCs.

Figure 2. Dose Response

Dose response study displaying the survival rates of RGCs in tropisetron treatment conditions after application of 500 µM glutamate. Tropisetron had a maximal effect at 100 nM. * represent significance from glutamate conditions # represent significance from control. (n = 3-7, p < .05)

In order to determine which receptor elicits tropisetron’s neuroprotective effect, further culture studies using isolated pig RGCs were conducted using the highly selective α7 nAChR antagonist, MLA, to block α7 nAChR action. MLA was applied at concentrations of 1 nM and 10 nM for one hour prior to application of 100 nM
tropisetron. Figure 3 demonstrates that 1 nM MLA did not block the neuroprotective effect of 100 nM tropisetron. In this condition cells survived at a rate of 106.5% (±9.2) compared to controls. Figure 3 also shows that with application of 10 nM MLA, the neuroprotective effect of tropisetron was significantly reduced to an average of 47% (±6.1). 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% (N=3). This supports the notion that tropisetron elicits the neuroprotective effect on adult pig RGCs through α7 nAChRs.

![Figure 3. MLA vs. Tropisetron](image)

**Inhibition study displaying MLA inhibition of neuroprotection by tropisetron.** 10 nM MLA significantly blocked the protective effect of a 100 nM dose of tropisetron. * represent significance from glutamate conditions, # represent significance from control (p < .05, n = 3).

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-HT\(_3\) receptor agonist, SR-57227, and another 5-HT\(_3\) receptor antagonist with no affinity to nAChRs, ondansetron. Figure 4 illustrates the effect of various concentrations of SR-57227 on cell viability when applied one hour before a 500 µM excitotoxic dose of glutamate. Application of SR-57227 was ineffective at protecting RGCs from glutamate-induced excitotoxicity at all concentrations tested from 1 nM to 1 µM with survival rates of 41.9% (±5.2), 60.3% (±22.4), 37.85% (±6.9), 58.3% (±14.9).

![Figure 4. SR 57227 vs. Excitotoxicity](image)

**Figure 4. SR 57227 vs. Excitotoxicity**

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 glutamate conditions) # represent significance from control. (p < .05, n = 4)

In another experiment to further support the hypothesis that 5-HT\(_3\) receptors are not involved in neuroprotection of RGCs by tropisetron against glutamate-induced
excitotoxicity, studies were conducted using SR-57227 applied at concentrations of 10 nM and 100 nM for one hour prior to a tropisetron treatment of 100 nM and two hours before application of 500 µM glutamate. Figure 5 shows 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), 113.3% (±12.9)).

Figure 5. SR 57227 vs. Tropisetron

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. (p < .05, n = 3)

Ondansetron is classified as a 5-HT3 receptor antagonist, along with tropisetron. However, binding studies (Macor et al., 2001) have indicated that ondansetron does not share the affinity for the α7 nAChR that is found with tropisetron. Because of this,
various concentrations, from 1 nM to 1 µM, of ondansetron were applied to adult pig RGCs for one hour before a application of 500 µM glutamate. Figure 6 shows that RGCs in all conditions treated with ondansetron before glutamate showed no better survival rates than cells treated only with a toxic dose of glutamate. In fact, some concentrations of ondansetron resulted in significantly more cell death than glutamate alone (1 nM = 18.3% ± 3.8 and 100 nM = 20.3% ± 0.5). The finding that antagonistic binding at 5-HT₃ receptors by a substance with no affinity to for α7 nAChRs provided no neuroprotection lends support to the hypothesis that tropisetron produces neuroprotection through a selective partial agonist action at α7 nAChRs and not through 5-HT₃ receptors.

![Figure 6. Ondansetron vs. Excitotoxicity](image)

*Figure 6. Ondansetron vs. Excitotoxicity*

Study showing the effect of the 5-HT3 antagonist, ondansetron, pretreatment. Ondansetron lacks the α7 nAChR agonist property of tropisetron and does not afford neuroprotection in RGCs. * represent significance from glutamate conditions, # represent significance from control (p < .05, n = 3).
**ELISA studies**

Recent studies have identified several intracellular signaling proteins that are involved in glutamate-induced excitotoxicity and neuroprotection by activation of α7 nAChRs. Asomugha et al. (2010) found that glutamate-induced excitotoxicity was associated with an increase in phosphorylation of p38 MAPK. Furthermore, pretreatment with ACh before glutamate eliminated the increase in p38 MAPK phosphorylation as well as increased phosphorylation of Akt. From these findings we hypothesized that if tropisetron protects RGCs through mechanisms similar to ACh, then pretreatment with tropisetron should also inhibit p38 MAPK phosphorylation and increase Akt phosphorylation.

To examine this hypothesis, cell lysates were obtained from adult pig RGCs collected from four different treatment conditions: untreated controls, 500 µM glutamate alone, 100 nM tropisetron alone, and tropisetron that was added 1 hour before glutamate. Figure 7 displays mean phosphorylation rates of p38 MAPK across treatment conditions. Each bar graph represents the mean levels of phosphorylated p38 MAPK in units/ml. The level of phosphorylated p38 MAPK in glutamate treated cells was significantly greater (15 units/ml ± 0.76) than in untreated controls (6.07 units/ml ± 0.99, p < .05). Cells treated with tropisetron, however, did not exhibit this increase in p38 MAPK phosphorylation (5.07 units/ml ± 0.99). When cells were treated with tropisetron prior to glutamate application, RGCs exhibited phosphorylated p38 MAPK levels near untreated control samples (7.3 units/ml ± 1.45). This suggests that glutamate increases p38 MAPK phosphorylation levels and that tropisetron inhibits this increase in p38 MAPK phosphorylation.
Figure 7. Phosphorylated p38 MAPK

ELISA study showing involvement of the p38 MAPK pathway in neuroprotection. Conditions treated with 500 uM glutamate (G) displayed a significant increase in phosphorylation of p38 MAPK over control conditions (C). Treatment with tropisetron alone (T) showed phosphorylation levels similar to control conditions. Pretreatment with tropisetron prior to a glutamate assault (T/G) prevented phosphorylation of p38 from rising to levels seen in excitotoxic conditions. * represent significance from glutamate conditions, # represent significance from control. (p < .05, n = 3)

Studies investigating Akt did not yield viable results when cells were treated with glutamate or when cells were treated with tropisetron. Therefore, these results have not been included. These findings suggest that tropisetron provides protection from glutamate by way of inhibition of the p38 MAPK pathway, however, it was undetermined if Akt phosphorylation plays a role in neuroprotection by tropisetron.

Receptor Internalization

Several recent studies have linked internalization of ion channels to neuroprotection (Akopian et al., 2006; Christofanelli, & Akopian, 2006; Mizuno et al.,
2010; Shen et al., 2010). Shen et al. (2010), found internalization of NMDA type glutamate receptors was linked to increased cell survival after treatment with the acetylcholinesterase inhibitor, donepezil. It was therefore hypothesized that if tropisetron protects cells in a similar way to donepezil, that cells treated with tropisetron may exhibit internalization of NMDARs. To determine if receptor internalization is involved in neuroprotection by tropisetron, immunocytochemistry studies were designed to label and image NMDARs within the cells with DAPI and on the cell surface with Rhodamine under different conditions. A primary antibody against the NMDAR1 subunit was used to label the NMDARs. The NMDAR1 subunit is the most commonly expressed subunit and is necessary for the formation of functional NMDARs throughout the body. The typical structure of all NMDARs is of a bilobed heterotetrameric ion channel composed of 2 NMDAR1 subunits and 2 other types of NMDAR2 subunits that are found in varying frequency throughout the nervous system. Selecting a primary antibody against NMDAR1 ensures that any NMDARs on the cultured cells will be bound with the primary antibody and able to be tagged with the secondary fluorescent antibodies.

Figure 8 displays images of control studies to demonstrate specificity of labeling. Fig. 8A is an image of a large adult pig RGC labeled with a .2% solution of primary monoclonal rabbit antibody against NMDAR1 for 1.5 hours at 37°C and a 1% solution of secondary anti-rabbit antibody conjugated to rhodamine overnight at 4°C under normal illumination. Fig 8B is the same cell under rhodamine illumination. Fluorescence indicates binding of the secondary antibody to receptors bound to primary antibody. Figure 8C demonstrates a control cell treated with rhodamine fluorescent secondary antibody without application of the primary antibody against NMDAR1. No
fluorescence indicates that fluorescent antibody was unable to bind to receptors that had not been previously labeled with the primary antibody against NMDAR1. Figure 8D is a cell labeled for 1.5 hours with a .2% solution of primary rabbit antibody against NMDAR1 at 37°C and a 1% solution of secondary anti-rabbit antibody conjugated to DAPI overnight at 4°C shown under normal illumination. Figure 8E shows the same cell under DAPI illumination. Fluorescence indicates binding of secondary antibody to receptors bound to primary antibody. Figure 8F is an image of another control cell treated with DAPI conjugated secondary antibody without application of the primary anti-NMDAR1 antibody shown under DAPI illumination. The lack of fluorescence indicates that DAPI conjugated anti-rabbit antibody did not bind to NMDAR1 without application of the primary anti NMDAR1 rabbit antibody.

Figure 8. Antibody Specificity

Antibody labeling control study. RGC labeled with a primary rabbit antibody for NMDAR1 and secondarily labeled with rhodamine anti-rabbit antibody under (A) normal illumination and (B) rhodamine illumination. In C, the primary antibody was omitted before application of the rhodamine labeled secondary antibody. (D) RGC labeled with a primary antibody for NMDAR1 and DAPI anti-rabbit antibody under normal illumination and (E) DAPI illumination. In F, the primary antibody was omitted prior to application of the DAPI labeled secondary antibody.
Figure 9 displays images of cells labeled with Rhodamine and DAPI under various treatment conditions. All cells were labeled with a .2% solution of primary monoclonal rabbit antibody against NMDAR1 for 1.5 hours at 37°C before any pharmacological treatment and before fixation and labeling with any secondary antibody. Figure 9A displays cells with no pharmacological treatment using tropisetron, fixed and treated with rhodamine-conjugated anti-rabbit antibody, then permeabilized and treated with DAPI conjugated antibody under rhodamine illumination. Labeled surface NMDAR1 receptors are illuminated showing bound receptors on the cell surface by this method. Figure 9B shows the same cells under DAPI illumination. No fluorescence indicates that the DAPI conjugated antibody was unable to bind to NMDAR1 as all surface receptors were already bound with rhodamine. Figure 9C is an overlay image composed of 9B and 9A displaying that DAPI conjugated antibody was unable to bind to receptors. In figure 9D cells were treated with 100 nM tropisetron for 1 hour after labeling NMDAR1s with .2% solution of primary monoclonal rabbit antibody for 1.5 hours at 37°C, fixed and treated with 1% rhodamine-conjugated antibody overnight at 4°C, then permeabilized and treated with 1% DAPI-conjugated antibody overnight at 4°C. Figure 9D is shown under rhodamine illumination displaying surface NMDAR1s labeled with rhodamine. Figure 9E shows the same cells under DAPI illumination. Internalized receptors as a result of tropisetron treatment are shown labeled with DAPI conjugated antibody. Figure 9F is an overlay showing surface receptors vs. internalized receptors. Notice the different populations of labeled receptors in the same cells. Figure 9G shows cells already labeled for 1.5 hours at 37°C with a .2% primary monoclonal rabbit antibody against NMDAR1s treated with MLA prior to tropisetron treatment, then
fixed and treated with 1% rhodamine-conjugated antibody overnight at 4°C, then permeabilized and treated with 1% DAPI-conjugated antibody. Under rhodamine illumination surface receptors fluoresce red with rhodamine labeling in figure 9G. Figure 9H shows the same cell population under DAPI illumination. There are no internalized receptors visible when MLA is used to block the nAChR action of tropisetron. Figure 9I is an overlay of 9G and 9H showing only surface receptors and no internalized receptors labeled. The data are consistent with the hypothesis that tropisetron protects RGCs from glutamate toxicity by internalizing NMDARs.

Figure 9. Receptor Internalization

Internalization study. Top row - RGCs with no pharmacological treatment under (A) rhodamine illumination, (B) DAPI illumination, and (C) overlay of both images. Middle row - RGCs treated with 100 nM tropisetron under (D) Rhodamine illumination, (E) DAPI illumination, and (F) overlay of both images. Bottom row - RGCs treated with MLA prior to tropisetron under (G) rhodamine illumination, (H) DAPI illumination, and (I) overlay of both images. Only cells treated with tropisetron displayed internalized receptors.
DISCUSSION

Glaucoma and Receptor Involvement

Considering the widespread and devastating effects of glaucoma, and the fact that current treatment options aimed mainly at reducing IOP have met only marginal success in delaying the effects of the disease, discovering effective therapeutic agents is of great importance from a public health standpoint. It has previously been shown that activation of nAChRs protects adult pig RGCs from glutamate-induced excitotoxicity (Wehrwein et al., 2004; Thompson et al., 2006, Asomugha et al., 2010), a key process in retinal degradation that occurs with glaucoma. However, attempts at raising concentrations of the endogenous agonist, ACh, to therapeutic levels in the retina have proven difficult to achieve physiologically. Anticholinesterase agents that have shown success in protecting neurons from amyloid-β induced neurodegeneration associated with Alzheimer’s disease by inhibiting degradation of ACh and increasing its presence at synapses (Buckingham et al., 2009; Hernandez et al., 2010). However this neuroprotective effect does not successfully translate to the retina. Therefore, discovering substances that can be directed at raising nAChR activity in the retina could provide a critical step in developing therapeutic agents for treating glaucoma.

Glutamate Receptors

Glutamate is the most abundant neurotransmitter of the vertebrate central nervous system, including the retina (Muller et al., 1992, Michaelis, 1998; Thoreson & Witkovsky, 1999). It is released from presynaptic vesicles onto glutamate receptors that are found on post-synaptic dendrites and cell bodies throughout the nervous system.
These receptors can be differentiated into two main types, metabotropic glutamate receptors (mGluRs) and ionotropic glutamate receptors (iGluRs), based on their physiological post-synaptic effect (Nakanishi, 1992). When glutamate activates mGluRs, the receptor binds to G-proteins that activate second messenger pathways that have a variety of intracellular results (Pin & Duvoisin, 1995; Conn & Pin, 1997). In contrast, when glutamate binds to iGluRs, nonspecific cation channels open allowing the passage of $\text{Na}^+$, $\text{K}^+$, and $\text{Ca}^{2+}$ (Michaelis, 1998). Both mGluRs and iGluRs are activated by glutamate, however because of subtle differences between the shapes of the binding sites in the two types, agonists specific for mGluRs are rarely able to bind to iGluRs and specific iGluR agonists are rarely able to bind to mGluRs (Thoreson & Witkovsky, 1999).

Eight different subtypes of mGluRs have been identified; mGluR1-mGluR8 (Pin & Duvoisin, 1995). These mGluRs are classified into three distinct groups (Group I – III) based on their affinities to varied specific agonists and their intracellular effect (Pin & Duvoisin, 1995; Conn & Pin, 1997). Activation of mGluRs leads to the initiation of a wide range of second messenger systems. The metabotropic nature of activation leads to a slow, but wide-ranging physiological response by the affected cell due to the signal amplification that occurs with second messenger systems. The ultimate physiological effect that occurs from mGluR activation depends on the type of receptor, cell, and messenger system involved. (Conn & Pin, 1997; Thoreson & Witkovsky, 1999).

Excitotoxicity has not been directly linked to mGluR activation, although release of $\text{Ca}^{2+}$ from intracellular stores could contribute to excess $\text{Ca}^{2+}$ load (Mattson, 2003).
Ionotropic glutamate receptors are ligand-gated ion channels, that, when activated by glutamate or another novel agonist, allow the passage of $\text{Na}^+$, $\text{K}^+$, or $\text{Ca}^{2+}$ ions. Three distinct types of iGluRs have been identified. They are classified based on their selective affinity to different agonists. N-methyl-D-aspartate (NMDA) is a selective agonist for NMDA receptors, kainic acid (KA) is a selective agonist for KA receptors, and $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) is a selective agonist for AMPA receptors (Michaelis, 1998). These three receptor subtypes are often referred to as being either NMDA or non-NMDA due to similarities between the AMPA and KA receptors (Michaelis, 1998).

Non-NMDA receptors are composed of various combinations of the known glutamate receptor subunits. AMPA receptors are composed of the subunits GluR1-4, and KA receptors are composed of combinations of the subunits GluR5-7 and KA1-2 (Michaelis, 1998). Different combinations of the subunits produce receptors with different properties. Generally, non-NMDA receptors exhibit lower levels of $\text{Ca}^{2+}$ permeability than NMDA receptors. When glutamate or the preferred agonist is applied to non-NMDA glutamate receptors, both KA and AMPA receptors allow a short inward $\text{Ca}^{2+}$ current that is quickly blocked (Lerma, 1997). The receptors remain desensitized for a short period, limiting the rate of $\text{Ca}^{2+}$ conductance through the activated receptors. The phasic, desensitizing response produced when glutamate or the preferred agonist is applied to non-NMDA GluRs suggests that they have a role in rapid excitatory synaptic transmission (Lerma, 1997; Thoreson & Witkovsky, 1999) and could also possibly provide a measure of protection from glutamate-induced excitotoxicity.
NMDA glutamate receptors are composed of different combinations of the subunits NMDAR-1 (occurring in forms a-h) and NMDAR-2 (occurring in forms A-D) (Michaelis, 1998). Generally, NMDA receptors differ from non-NMDA receptors in that they tend to exhibit higher Ca\textsuperscript{2+} conductance, and a more sustained, tonic response to glutamate (Michaelis, 1998). High Ca\textsuperscript{2+} permeability in response to glutamate is a major contributing characteristic to the NMDAR receptor’s role in glutamate-induced excitotoxicity. In the presence of high concentrations of glutamate Ca\textsuperscript{2+} influx occurs in excess. The excessive influx of Ca\textsuperscript{2+} acts as a second messenger that initiates apoptotic pathways that lead to the resulting cell death by glutamate-induced excitotoxicity (Quigley et al., 1995; Sucher et al., 1997; Lam et al., 1999; Brandt et al. 2010).

**Acetylcholine Receptors**

Acetylcholine is another neurotransmitter found widely throughout the nervous system. It acts on two distinct types of receptors that are defined from one another by specific affinity to different exogenous agonists. The muscarinic acetylcholine receptors, or mAChRs, which exhibit metabotropic effects, are defined by their selective affinity for the drug, muscarine (Caulfield & Birdsall, 1998). Nicotinic acetylcholine receptors, or nAChRs, are ionotrophic and are defined by their selective affinity for the drug, nicotine (Colquhoun et al., 2003).

Muscarinic AChRs are generally found in involuntary types of muscle cells, but are sometimes found in CNS cell bodies. They occur in five different subtypes, m\textsubscript{1-5} AChRs that are all expressed in various concentrations throughout many systems of the body (Caulfield & Birdsall, 1998). Although specific actions of each subtype of mAChR
are not fully understood, they generally exhibit a metabotropic response to activation by ACh or muscarine, utilizing second messenger pathways (Caulfield & Birdsall, 1998). However, a previous study by our lab determined that mAChRs have no significant effect on glutamate-induced excitotoxicity in studies involving adult pig RGCs (Wehrwein et al., 2004).

Nicotinic AChRs occur widely throughout the body. They are found in all autonomic ganglia, some CNS cell bodies and dendrites, and at the motor end plates of skeletal muscle fibers. There are 12 different subunits, nine α (α2-α10) subunits and three β (β2-β4) subunits that are expressed in various combinations to construct a wide variety of different nAChRs throughout the nervous system (Colquhoun et al., 2003). All of the receptors are ionotrophic, having direct effect on the cell membrane’s permeability to Ca$^{2+}$ and Na$^+$. A number of recent studies have shown that the activation of these receptors can also stimulate second messenger pathways that play a key roles in the protection of neural cells from glutamate-induced excitotoxicity (Kaneko et al., 1997; Dajas-Bailador et al., 2000; Wehrwein et al., 2004; Thompson et al., 2006; Wang et al., 2007; Asomugha et al., 2010). Using immunocytochemical techniques to label specific nAChR subunits, Thompson et al. (2006) found that neuroprotection of adult pig RGCs by nAChR activation is partially mediated through multiple nAChR subtypes that are distributed differently on large and small adult pig RGCs. In their study, it was shown that nAChRs composed of α4 and β2 subunits were mainly found on small RGCs, while α7 nAChRs were found primarily on large RGCs. They also found that application of partial ACh agonists for α4 nAChRs produced significant neuroprotection in cultured RGCs. However, protection did not reach the level found with ACh or nicotine, pointing
to involvement of multiple nAChR subtypes.

_Tropisetron_

Tropisetron, a known 5-HT₃ receptor antagonist, has been shown to exhibit a selectively potent partial agonist action at α7 nAChRs (Papke et al., 2005; Macor et al., 2001). In addition to its history of use as an anti-emetic agent, studies show that tropisetron is able to produce effects in the central nervous system when administered orally and intraperitoneally (Hashimoto et al., 2005). This supports the notion of its possible use as a therapeutic agent in the glaucomatous retina. Another recent study (Rahimian et al., 2011) found that tropisetron was effective at suppressing inflammation and preventing the degenerative effects associated with ischemic brain injury in a rat model of embolic stroke. Furthermore, the study was able to show that the protective effect occurs independently of the 5-HT₃ receptor antagonist action. When rats were injected with tropisetron 1 hour prior to inducing stroke conditions the size of brain infarct, neurological dysfunction and inflammatory response were all diminished when compared to untreated controls. This provides further evidence of tropisetron as a possible neuroprotective agent. However, the study did not investigate the role of nAChR activity in neuroprotection.

In the current study it was hypothesized that pre-treatment of RGCs with a therapeutic dose of tropisetron will protect RGCs from glutamate-induced excitotoxicity by activation of nAChRs. Results from pharmacology studies provide evidence in support of this hypothesis. A 500 µM dose of glutamate applied to isolated adult pig RGCs resulted in death of an average of 62% of cells in culture. Studies showed that
tropisetron was able to protect RGCs from the glutamate-induced excitotoxicity in a
dose-dependent manner when applied to cultures 1 hour prior to glutamate application,
with a maximal effect at a concentration of 100 nM. These results lend support to the
hypothesis by showing that tropisetron exhibits significant neuroprotection in adult pig
RGCs when applied 1 hour prior to inducing excitotoxic conditions.

Inhibition studies contributed further evidence that tropisetron exhibits
neuroprotection through activation of α7 nAChRs. When the α7 nAChR inhibitor, MLA,
was applied to RGCs 1 hour prior to tropisetron application, protection from glutamate
was significantly reduced. Studies investigating 5-HT₃ receptor activity showed that
application of the 5-HT₃ receptor agonist, SR 57227, before tropisetron to counteract the
effect of tropisetron’s 5-HT₃ antagonist action did not produce a significant decrease of
neuroprotection, nor did pretreatment with SR 57227 alone provide protection from
 glutamate. Further studies were performed to examine if tropisetron’s action as a 5-HT₃
receptor antagonist might plays role in neuroprotection. Ondansetron, another known 5-
HT₃ receptor antagonist that does not share the α7 nAChR agonist property of
tropisetron, was tested in dose-response studies as a neuroprotective agent. Pretreatment
with ondansetron provided no significant protection to isolated RGCs when applied for 1
hour prior to 500 µM glutamate.

An interesting trend found in this study was tropisetron’s tendency to provide a
greater degree of neuroprotection to large RGCs over small RGCs. In culture conditions
treated with tropisetron where neuroprotection was found, the cells that survived after 3
days in culture were primarily large RGCs; only a small number of small RGCs were
found surviving after 3 days in culture. This finding is in line with the notion that tropisetron’s effect selectively involves the α7 nAChR. Immunocytochemistry studies investigating localization of the α7, α4, and β2 nAChR subunits by Thompson et al. (2006) found that α7 nAChR subunits are found exclusively on large RGCs, and that α4 and β2 nAChR subunits are found exclusively co-localized on small RGCs. Furthermore, it was shown that neuroprotection by ACh in large RGCs occurs through α7 nAChRs and occurs in small RGCs through activation of α4β2 nAChRs. Further analysis of the current data quantifying the differential survival rates between large and small RGCs could provide evidence supporting this notion. Studies with other nAChR subtype inhibitors could further specify the receptor action of tropisetron.

*Intracellular Signaling Pathways*

Next we sought to determine by what mechanism tropisetron protects RGCs from glutamate-induced excitotoxicity. Several studies have suggested involvement of various intracellular signaling pathways in excitotoxicity and neuroprotection (Dineley, et al., 2001; Pearson et al., 2001; Manabe & Lipton, 2003; Zarubin & Han, 2005; Wang et al., 2007; Asomugha et al. 2010). Asomugha et al. (2010) investigated the involvement of p38 MAPK and Akt in glutamate-induced excitotoxicity. Using ELISA techniques they found that when adult pig RGCs are cultured with 500 μM glutamate for 12 hours there is 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. Application of glutamate alone did not have an effect on Akt phosphorylation. These findings
suggest that activation of the MAPKKK > MAPKK > p38 MAPK pathway is involved in regulating cell death in response to excessive glutamate activity.

Further ELISA studies by Asomugha et al. (2010) investigated involvement of intracellular signaling pathways in neuroprotection by ACh. When adult pig RGCs were cultured with ACh alone, RGCs survived at a rate comparable to control and showed an increase in phosphorylation of Akt. When cultured with glutamate alone, an average of 60% of RGCs survived and there was no observed increase in phosphorylated Akt. When ACh was applied before glutamate, RGCs survived at rates comparable to control and exhibited significantly increased levels of phosphorylated Akt and decreased levels of phosphorylated p38 MAP kinase compared to glutamate alone. When the PI3-K inhibitor, wortmannin, was applied before ACh and glutamate, Akt phosphorylation did not increase, and p38 MAPK phosphorylation occurred at rates comparable to glutamate application without ACh. These results suggest that not only does ACh exert its neuroprotective effect through activation of the PI3-K > Akt pathway, but also through inactivation of the p38 MAP kinase pathway, and that activation of the PI3-K > Akt pathway is involved in inhibiting the p38 MAPK pathway. To test the involvement of these pathways in the current study, ELISA studies were conducted to investigate phosphorylation rates of the target proteins.

Present results from ELISA studies were only able to suggest involvement of p38 MAPK in using tropisetron 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 from rising to levels
found in samples treated with glutamate alone. Phosphorylation levels for Akt were not
able to be determined. Experiments testing for phosphorylated Akt did not function
properly and yielded no viable results. Some experiments failed to produce a signal,
others produced too much signal and could not be interpreted. Previous studies done
using ACh exhibited an increase in phosphorylation of Akt. The present studies using
tropisetron did not show this trend. One possible explanation is that the Akt activation
seen in studies using ACh occurred through action at α4β2 nAChRs which, are found
only on small RGCs. ACh is non-specific AChR agonist and studies used lysates
prepared from large and small RGC cultures. Tropisetron is a specific agonist of the
α7nAChR and tends to protect large RGCs better than small RGCs since α7nAChRs are
found only on large RGCs and not on small RGCs. If Akt phosphorylation occurs in
response to α4β2 nAChRs then tropisetron would show little effect since it does not act at
α4β2 nAChRs. Our findings provide evidence that tropisetron provides neuroprotection
through inhibition of p38 MAPK, however involvement of the Akt pathway was unable
to be determined.

One interesting finding that came about through the control pharmacology studies
was that the 1nM and 100nM treatment conditions of ondansetron alone before glutamate
application showed significantly lower survival rates than excitotoxic control cells treated
with glutamate alone. Further literature review uncovered a study (Liu et al., 2011)
showing that ondansetron provides protection against liver injury in a rat model of hemorrhagic shock via stimulation of the p38 MAPK pathway. In the study, hemorrhagic shock was induced in rats by bleeding the rat through a catheter in the left femoral artery until a mean arterial blood pressure of 40 mmHg was achieved, then animals were gradually resuscitated. At 30 minutes before the end of resuscitation, animals were administered ondansetron, ondansetron and the p38 MAPK inhibitor, SB-203580, or an equal volume of vehicle. Animals treated with ondansetron showed significantly lower levels of liver proinflammatory markers when compared to animals that received the vehicle alone or ondansetron with SB-203580. This suggests that ondansetron has a protective effect on liver tissue through activation of a p38 MAPK dependent pathway. Furthermore, western blot assays showed increased levels of phosphorylated p38 MAPK in liver tissue homogenate from animals treated with ondansetron when compared to animals treated with the vehicle or ondansetron and SB-203580. This suggests that ondansetron increases activation p38 MAPK pathway in liver tissue, although the exact mechanism of p38 MAPK activation was not thoroughly explored.

It is possible that ondansetron treatment also increases p38 MAPK activity in cultured adult pig RGCs. This finding is interesting since our ELISA studies suggest that tropisetron protects adult pig RGCs by inhibiting the p38 MAPK pathway in cells treated with glutamate (Fig. 7). It is possible that, while the effect of ondansetron on increasing p38 MAPK activity has a protective effect on liver tissue, the increase in p38 MAPK activation by ondansetron combined with an excitotoxic concentration of glutamate could produce even higher levels of p38 MAPK activity involved in apoptotic pathways leading to increased cell death in adult pig RGCs than in conditions treated with glutamate alone.
Further studies assessing cell viability in RGC cultures treated with ondansetron and p38 MAPK inhibitors are needed to investigate this possibility.

The finding that increased p38 MAPK activity has a protective effect in the liver, yet is related to neurodegeneration in the retina is not surprising. Each intracellular signaling protein is involved in a number of signaling pathways and each protein can have many and varied outcomes. The outcome of activating a particular intracellular signaling protein depends on many factors, such as, mode of activation, voltage across the cell membrane, Ca$^{2+}$ concentration, etc. It is possible that the activation of p38 MAPK in liver tissue occurs without a massive Ca$^{2+}$ influx, or that when p38 MAPK activity is initiated in adult pig RGCs by NMDAR stimulation an apoptotic pathway is activated via a receptor associated protein. The mechanism by which ondansetron lead to increased p38 MAPK activity was not mentioned in the studies on liver tissue. What is interesting is that 5-HT$_3$ receptors are not known to occur in RGCs. Our control studies investigating 5-HT$_3$ receptor activity did not indicate that it played a significant role in neuroprotection and that tropisetron’s neuroprotection occurred independently of action at 5-HT$_3$ receptors. Further studies investigating the mechanism by which ondansetron increases p38 MAPK activity are needed to explain our results. It is possible that immunocytochemistry and binding studies with ondansetron in RGC cultures may be able to expose the localization of another receptor subtype through which ondansetron may act. It is also possible that ondansetron increases p38 MAPK activity independent of a known receptor linked mechanism. Studies investigating intracellular conditions that occur with these activities are needed to address these questions.
Calcium Involvement

Multiple studies have determined that Ca\textsuperscript{2+} is the key ion involved in excitotoxicity as well as neuroprotection (Sucher et al., 1996; Kihara et al., 2001; Brandt et al., 2010). Ca\textsuperscript{2+} has been shown to act as a second messenger linked to a number of different cellular responses. Excitotoxicity occurs after a prolonged influx of Ca\textsuperscript{2+} creates a Ca\textsuperscript{2+} overload and activates a cascade of calcium-activated proteases, or caspases that initiate apoptosis. Brandt et al. (2011) provided strong evidence that neuroprotection is also dependent on Ca\textsuperscript{2+} influx. In cell culture studies, neuroprotection by nicotine and ACh were affected in a dose-dependent manner when the extracellular concentration of Ca\textsuperscript{2+} was manipulated during induced excitotoxic conditions. Lower concentrations of extracellular Ca\textsuperscript{2+} led to lower degrees of neuroprotection. It was further shown that the physiological outcome did not depend on the avenue of Ca\textsuperscript{2+} entry. Neuroprotection could be induced by Ca\textsuperscript{2+} influx through voltage-gated Ca\textsuperscript{2+} channels via KCl depolarization. Even more interestingly, neuroprotection against a 500 µM glutamate treatment was also induced by Ca\textsuperscript{2+} entry through glutamate receptors via a low-dose pretreatment of glutamate. All of this evidence suggests that the amount of Ca\textsuperscript{2+} that enters the cell is what determines the fate of the cell. When stimulation of nAChRs occurs, the receptors characteristically rapidly desensitized allowing only a small amount of Ca\textsuperscript{2+} to enter the cell, initiating signaling pathways that promote cell survival and inhibit apoptosis. Therefore, it can be reasoned that a method of decreasing the inward Ca\textsuperscript{2+} current in the presence of excessive amounts of glutamate may be a plausible method of providing neuroprotection in excitotoxic conditions.
Receptor Internalization

Several recent studies have investigated a role of Ca\(^{2+}\) channel internalization in response to glutamate receptor activity in salamander RGCs (Schubert & Akopian, 2004; Akopian et al., 2006; Christofanelli & Akopian, 2006). It was found that disruption of the actin cytoskeleton by actin disrupters regulates the Ca\(^{2+}\) current through voltage-gated L-type Ca\(^{2+}\) channels (Schubert & Akopian, 2004) and ionotropic glutamate receptors (Akopian et al., 2006). In turn, it was also found that Ca\(^{2+}\) influx through voltage-gated L-type Ca\(^{2+}\) channels and activated ionotropic glutamate receptors both caused destabilization of actin filaments (Cristofanilli & Akopian, 2006). Further studies from the same lab found that treatment with actin destabilizing agents caused internalization of Ca\(_{v}1.3\) 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). It was later found that disruption of the actin cytoskeleton by glutamate receptor activation below the threshold of excitotoxicity also caused internalization of the Ca\(_{v}1.3\) L-type calcium channel (Mizuno et al., 2010). Further experiments also confirmed that internalization of Ca\(_{v}1.3\) L-type calcium channels protects RGCs from excitotoxicity suggesting a role of ligand-mediated internalization of Ca\(^{2+}\) channels in neuroprotection. Another study has 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\(^{2+}\) current, 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 using tropisetron as a neuroprotective agent.

In our receptor internalization studies, we showed that NMDA receptor internalization occurs in adult pig RGCs when neuroprotective conditions are induced by tropisetron. Our images showed that cells treated with tropisetron displayed internalized receptors bound to fluorescent antibody that was applied after fixed cells with surface receptors occupied were made permeable with Triton-X allowing access to the interior of the cell. When cells were not treated with tropisetron or when the specific α7 nAChR antagonist MLA was applied prior to tropisetron, cells did not display receptors bound to the fluorescent antibody inside the cells. The current study is only able to suggest that internalization of NMDARs is possibly involved in neuroprotection. Further quantitative studies are needed to confirm involvement of this process in neuroprotection. Cells displaying internalized receptors after tropisetron treatment could be quantified by setting a threshold of fluorescence for determining which cells display evidence of internalization (i.e. cells in which approximately 15% of the cell fluoresced with the internalized receptor antibody were considered to display evidence of internalization) and counting the number of cells that reach that threshold. These counts could then be compared to total cell counts to determine the percentage of cells that display evidence of internalization. Percentages from tropisetron treated cells would then be compared to conditions with no tropisetron treatment and with conditions inhibiting tropisetron’s action. Results from these studies would be expected to support the hypothesis that tropisetron protects cells from excitotoxicity via NMDAR internalization by showing an
increase in the amount of cells displaying evidence of internalized NMDARs after treatment with tropisetron. Another interesting angle to approach would be to apply these studies to ACh treated cells to determine if NMDAR internalization is involved in ACh neuroprotection. Since tropisetron acts through α7 nAChRs, the evidence shown in this study is mainly displayed in large RGCs. Since ACh also acts on α4β2 nAChRs this study could be used to examine if internalization also occurs in small RGCs.

It is interesting to note that Ca$^{2+}$ influx is required for both excitotoxicity and neuroprotection. It is thought that the amount of Ca$^{2+}$ entering the cell determines the cellular response with excessively large influx leading to cell death and lower levels of Ca$^{2+}$ entry leading to neuroprotection. What is not known is that if encouraging protection by nAChR activation protects cells by preventing excessive Ca$^{2+}$ influx through glutamate receptors or by stimulating protective mechanisms that encourages cell survival regardless of the amount of Ca$^{2+}$ that enters the cell. Ca$^{2+}$ imaging studies could help determine this by quantifying the amount of Ca$^{2+}$ entering the cell under different treatment conditions. With evidence suggesting involvement of NMDAR internalization in neuroprotection, it would be expected that treatment with tropisetron would lead to a reduction of Ca$^{2+}$ influx in response to glutamate treatment. Conducting these studies using ACh would also be helpful in determining if ACh provides protection through a reduction of Ca$^{2+}$ influx or if the effect occurs independently of Ca$^{2+}$ alteration. Electrophysiology studies could also be of interest to examine changes in Ca$^{2+}$ current during excitotoxic and neuroprotective conditions. These studies would be expected to show a large inward Ca$^{2+}$ current in response to glutamate application. Treatment with tropisetron would be expected to display a small increase in the inward Ca$^{2+}$ current. The
inward $\text{Ca}^{2+}$ current initiated by subsequent glutamate application would be expected to be much lower than with glutamate alone, supporting the idea that tropisetron protects cells by attenuating the inward $\text{Ca}^{2+}$ current.

Concluding Remarks

In conclusion, our 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 $\text{Ca}^{2+}$ influx through NMDARs. Furthermore, these results suggest the possibility that neuroprotection by tropisetron also involves attenuation of the inward $\text{Ca}^{2+}$ current through NMDARs by removal of NMDARs from the cell membrane via receptor internalization. These results occurred at physiologically relevant concentrations that can be achieved through conventional therapeutic measures. The findings of this study suggest that tropisetron could be an effective therapeutic agent for the treatment of glaucoma and other degenerative disorders of the central nervous system and warrants further exploration as a treatment option for such disorders.
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