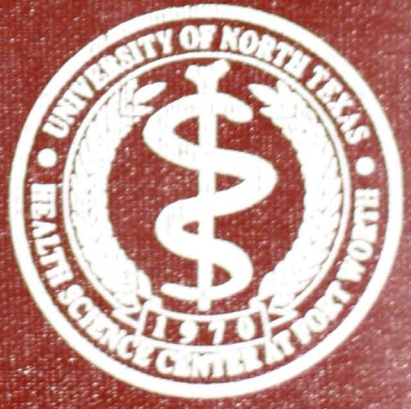
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Kumar, Domalapalli Maneesh, Role of Nonfeminizing Estrogen Analogues in Neuroprotection of Rat Retinal Ganglion Cells against Glutamate-Induced Cytotoxicity, Doctor of Philosophy (Cell Biology and Genetics), May, 2007, 210 pp., 3 tables, 23 figures, bibliography, 427 titles.

Retinal ganglion cell death has been determined to be the final common pathway in glaucoma. Continuous loss of retinal ganglion cells results in irreversible progressive visual field deterioration that culminates in blindness. No effective therapy is currently available to reverse retinal ganglion cell loss. Therefore, preventing the loss of retinal ganglion cells is a logical approach to maintaining vision in effected individuals. Of the methods of investigation, *in vivo* models of ganglion cell death provide a physiological system in which to study neuroprotective drugs and their effects, but these systems are inefficient for initial screening studies. We have addressed this by utilizing the RGC-5 clonal rat retinal ganglion cell line. Glutamate treatment of RGC-5 cells induces apoptotic death which can be attenuated by pretreatment with the anti-oxidants *N*-acetyl cysteine and thiourea, implicating oxidative stress as a major component of glutamate's cytotoxicity. Also antioxidants, estrogens have been demonstrated to be potent neuroprotectants in a variety of *in vitro* and *in vivo* models of neurodegeneration. Estrogens' antioxidant capacity has been attributed to the ability of the phenolic A ring to quench and resonance stabilize oxidative free radicals. It is also known that the estrogen A ring is responsible for binding of these hormones to estrogen receptors, producing feminizing phenotypes. The feminizing effects of estrogens narrow or preclude their use as neuroprotectants in males, and in females that may be predisposed to their deleterious

effects. To address these shortcomings we screened 13, non-feminizing, non-receptor binding estrogen analogues in our glutamate-induced RGC-5 model of oxidative stress-induced cell death. The most effective of these drugs was ZYC-3. ZYC-3 was synthesized by the addition of an adamantly group to the C2 position on the A ring of estrone. This modification produced a neuroprotective compound with potency and efficacy at least equal to the prototypical estrogen neuroprotectant, 17β-estradiol, but with no appreciable binding affinity for estrogens receptors α or β. Our preliminary findings suggest that ZYC-3 enhances glutathione synthesis and blocks mitochondrial apoptotic pathways. However, as a novel drug we are naive to its effects on cellular physiology and as to how it affords neuroprotection. Understanding how this drug regulates cellular destructive and protective mechanisms could lead to further innovations in drug design and in methods to prevent retinal ganglion cell degeneration. *In vivo* studies of this drug may then form the bridge to a better clinical approach to managing ocular disorders in which ganglion cell loss is the culprit for vision loss. Although promising, evidence supporting the application of estrogen analogues in models of ocular neurodegenerative diseases are nearly non-existent. *It is our objective to study the neuroprotective effects of ZYC-3 in glaucomatous models with the goal of maintaining retinal ganglion cell viability and preventing vision loss.*

ROLE OF NONFEMINIZING ESTROGEN ANALOGUES IN NEUROPROTECTION OF RAT RETINAL GANGLION CELLS AGAINST GLUTAMATE-INDUCED CYTOTOXICITY

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ROLE OF NONFEMINIZING ESTROGEN ANALOGUES IN NEUROPROTECTION OF RAT RETINAL GANGLION CELLS AGAINST GLUTAMATE-INDUCED CYTOTOXICITY

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

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DOCTOR OF PHILOSOPHY

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Domalapalli Maneesh Kumar, B.S.

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Please Note:

Appendix C has been retracted
from this dissertation.

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CHAPTER I

INTRODUCTION TO THE STUDY

Among the host of optic neuropathies in which retinal ganglion cell loss has been observed, glaucoma is the most prominent. One to two percent of Americans over the age of forty are affected by glaucoma; each year more than twelve thousand are blinded by the disease (Kahn et al., 1980; Tielsch et al., 1990). Its growing prevalence produces tremendous economic burdens, with over three million annual office visits in the U.S. alone (Klein et al., 1992). This single disease affects nearly 70 million people worldwide, and approximately ten percent suffer from bilateral blindness due to primary glaucoma (Quigley et al., 1996). Additionally, increased mortality has been observed among glaucoma patients, making glaucoma a significant source of morbidity as well as mortality (Egge et al., 1999).

Glaucoma Pathophysiology. Glaucoma is a family of optic neuropathies that result in progressive visual field deterioration, leading to complete vision loss due to retinal ganglion cell death. Retinal ganglion cell death and vision loss are most commonly associated with elevated intraocular pressure due to blockage of aqueous humor outflow. As aqueous humor is formed by the ciliary body, it passes anteriorly between the lens and the iris, where it travels through the trabecular meshwork and drains into the canal of

Schlem. This canal is located at the abutment of the cornea and sclera, and may function as a damn to aqueous humor outflow. Retinal ganglion cell death and glaucoma may also occur in patients with normal intraocular pressures, commonly known as normotensive glaucoma. While several etiologies of glaucoma exist, it is thought that the ultimate cause of vision loss is apoptosis of retinal ganglion cells (Quigley et al., 1995). Retinal ganglion cells are the output neurons that encode and transmit information from the eye to the brain that produce the perceived images in the visual cortex (Nickells, 1996). Elimination of retinal ganglion cells and their axons occurs by apoptosis, and because retinal ganglion cells are neurons, they cannot be regenerated once dead (Li et al., 1999; Levin et al., 1997). The pathologic changes observed in glaucomatous retinas on clinical examination are attributable to progressive loss of these cells, resulting in characteristic cupping of the optic disk at the optic nerve head (Quigley et al., 1995; Wygnanski et al., 1995; Neufeld et al., 2003; Danias et al., 2003). Experimentally a variety of pathologic situations may be modeled *in vitro* to study the loss of retinal ganglion cells; one such model is glutamate-induced apoptosis of the cells (Goldblum et al., 2002). Similarly, a number of *in vivo* models also exist. One of the best is the Morrison model, created by reduction of aqueous humor outflow by scarring the episcleral veins to elevate intraocular pressure.

Glutamate challenge of retinal ganglion cells. Glutamate normally functions as the major excitatory amino acid neurotransmitter in the retina, but at high concentrations it becomes neurotoxic. When glutamate is injected into the vitreous in *in vivo* studies or when cultured retinal ganglion cells are treated with glutamate *in vitro*, retinal ganglion cells

have been shown to undergo apoptosis (Lam et al., 1999; Lucas et al., 1957; Otori et al., 1998; Aoun et al., 2003; Pang et al., 1999). The apoptosis observed in such models has been attributed to the influx of calcium into cells and the generation of free radicals. Pathologic quantities of nitric oxides (NO), produced due to elevated extracellular glutamate concentration, facilitate the production of superoxide free radical anions by the mitochondria, triggering cell death (Haefliger et al., 1999; Bonfoco et al., 1995). Of interest in our model is the additional oxidative stress imposed through the inhibition of glutamate/cystine antiporters, which decreases the availability of intracellular cysteine, a necessary precursor in glutathione synthesis (Murphy et al., 1989; Schubert et al., 2001; Atlante et al., 2001; Bridges et al., 2001). In the absence of adequate intracellular cysteine, cells cannot produce adequate glutathione to cope with the resultant overwhelming oxidative stress that glutamate challenge creates.

Studies investigating glutamate concentrations in the vitreous humor of glaucoma patients and in various glaucomatous animal models have left the occurrence of neurotoxic retinal glutamate levels up to debate (Dreyer et al., 1996; Carter-Dawson et al., 2002). Nonetheless, increases in glutamine, glutathione, and glutamate/aspartate transporter levels have been demonstrated in experimental glaucomatous monkey retinas, indicating an elevation in extracellular glutamate and enhanced glutamate transport and metabolism (Carter-Dawson et al., 2002). It is also known that glutamate causes excitotoxicity via excess stimulation of its receptors, initiating a cascade of events that result in an overload of intracellular calcium and the release of reactive oxygen species. More importantly, elevated extracellular glutamate inhibits glutamate/cystine antiporters

(Schubert et al., 2001; Atlante et al., 2001; Li et al., 1998; Savolainen et al., 1998). These effects, along with resultant perturbations of mitochondrial membrane potential, direct the cells toward programmed death (Murphy et al., 1989; Pereira et al., 2000). The toxic effects of glutamate on mammalian retinal ganglion cells have been well documented and result in a glaucomatous phenotype both *in vivo* and *in vitro* (Bridges et al., 2001; Vorwerk et al., 1996).

A number of laboratories have reported that retinal ganglion cells are highly susceptible to glutamate toxicity *in vitro* and *in vivo* (Kawasaki et al., 2000 and 2002; Caprioli et al., 1996; Kido et al., 2000; Otori et al., 1998; Moncaster et al., 2002; Li et al., 1999; Moore et al., 2001). It is important to note that Ullian et al., have demonstrated that *in vitro* and *ex vivo* retinal ganglion cells are invulnerable to 1 h 500 μM glutamate elicited NMDA receptor excitotoxicity (Ullian et al., 2004). As we have already published succinyl concanavalin A (sConA) differentiated RGC-5 cells are susceptible to 50μM glutamate treatment (Krishnamoorthy et al., 2001). Furthermore, we have shown that our characterized RGC-5 cells are highly sensitive to 24 h 5 mM glutamate treatment (without sConA differentiation), which produces 50% or greater cell death. This cytotoxic effect was not attenuated or reversed by the NMDA receptor antagonist MK801, nor by the sodium dependent glutamate/aspartate transport inhibitor dl-TBOA (Aoun et al, 2003). Additionally, the antioxidants thiourea and NAC, respectively, minimized and reversed the typical cell death seen with glutamate treatment (Aoun et al, 2003). These findings indicate that glutamate's toxicity at higher concentrations may be independent of its transporters and receptors, suggesting late phase toxicity (Figure 1). As

mentioned above, glutamate induces tremendous oxidative stress that can contribute to mitochondrial dysfunction. Such dysfunctions have been observed in optic neuropathies and can promote retinal ganglion cell loss (Carelli, et al., 2002 and 2004). To address this, we examined the signaling mechanisms of glutamate-induced toxicity in parallel with ZYC-3 protection in our mitochondrial apoptosis pathway work. Taken together, excessive glutamate concentrations pose an overwhelming oxidative stress on retinal ganglion cells, which may lead to their death and is the basis for the present studies. Comprehensive discussions on the role of oxidative stress in glaucoma and glutamate-induced cytotoxicity of RGC-5 cells are included in Appendices A and B.

In vivo retinal ganglion cell death model. Although the debate over elevation of intravitreal glutamate concentration in glaucoma patients and *in vivo* glaucoma models continues, it is believed that oxidative stress plays an important role in retinal ganglion cell death. As opposed to most parts of the central nervous system, the retina is vulnerable to potentially high levels of oxidative stress due to light exposure in addition to the generation of lipid peroxides and endogenous pro-oxidant events (Mainster et al., 1987; Organisciak et al., 1998). Elevated intraocular pressure, with which glaucoma is most commonly associated, has been demonstrated to further increase oxidative stress (Moreno et al., 2004). The Morrison model of elevation of intraocular pressure in rats is an accepted paradigm for the study of glaucoma *in vivo* and a logical choice for the continuation of our studies *in vivo* (Kim et al., 2004).

Estrogen Neuroprotection. Estrogens have long been recognized as antioxidants in a variety of *in vitro* and *in vivo* studies, and evidence is mounting that implicates their antioxidant nature as part of their neuroprotective value (Gridley et al., 1998; Harms et al., 2001; C et al., 1997; Dykens et al., 2003; Prokai et al., 2003; Lui et al., 2002; Yang et al., 2000). Estrogens have specifically been demonstrated to be neuroprotective against glutamate-induced cytotoxicity (Zaulyanov et al., 1999; Green et al., 1998; Bhavnani et al., 2003). In addition to their role as neuroprotectants, estrogens play vital roles in normal human development, growth, maturation, and aging (Clark et al., 1996; Hyder et al., 1999; Bulun et al., 2000). Estrogens' effects are classically mediated by the binding of estrogen to its receptors, followed by binding of the steroid-receptor complex to estrogen response elements on various genes, modulating transcriptional events. Those functions are accomplished via classical steroid hormone receptors and receptor dependent signaling pathways (Azcoitia et al., 2002; Bjornstrom et al., 2002; Qui et al., 2003). This allows estrogens to modulate the expression of genes, many of which produce female phenotypes in both females and males (Findlay et al., 2001). Estrogens also have the propensity to exacerbate pathologic conditions such as female hormone sensitive malignancies and hypercoagulable states, further limiting their potential for widespread clinical application (Rosendaal et al., 2003; Anderson et al., 2003). Additional findings of the Women's Health Initiative study suggest that estrogen therapy may have negative effects on cognitive function and stroke incidence, also bring caution to the application of natural estrogens as neuroprotectants (Rapp et al., 2003; Shumaker et al., 2003 and 2004; Wassertheil-Smoller et al., 2003; Schneider 2004). Estrogens may afford significant

protection in neurodegenerative disesaes such as glaucoma, but their therapeutic applications stand to be limited by their vast array of physiologic and pathophysiologic effects. A comprehensive discussion on the role of estrogens in the retina, with an emphasis on glaucoma, is presented in Appendix C.

Estrogen analogues as neuroprotectants. The body of evidence supporting the use of estrogens as neuroprotectants is large and growing. However, it is becoming clear that neuroprotective responses may be elicited in neurons independent of the classical pathways of estrogen receptor mediated activation of gene expression (Green et al., 1998; Rupprecht et al., 1999; Behl et al., 2000). Non-receptor binding enantiomers, quinols, and synthetic analogues of estrogens have all demonstrated neuroprotection of central nervous tissues in various models of insult (Prokai et al., 2003; Xia et al., 2002; Yang et al., 2003; Green et al., 1998, 2000, and 2001; Lui et al., 2002). Many of the protective mechanisms of estrogens are believed to occur through fast acting intracellular signaling pathways too rapid to involve gene expression (Singer et al., 1999; Honda et al., 2000; Linford et al., 2000; Kuroki et al., 2001; Singh 2001; Cordey et al., 2003; Mize et al., 2003; Yu et al., 2004). Furthermore, the ability of estrogens to maintain mitochondrial membrane potential and enhance cellular responses to oxidative stress, in part via glutathione synthesis, appear to be intimately related to their ability to prevent apoptosis (Green et al., 1998; Honda et al., 2001; Bertrand et al., 2002; Borras et al., 2003; Chen et al., 2003; Chiueh et al., 2003; Nilsen et al., 2003; Lu et al., 2004).

The studies mentioned above were conducted in central nervous tissue from the brain parenchyma, but none have addressed the neural retinal ganglion cell layer of the eye. Therefore, there is compelling evidence that estrogen analogues may confer protection to retinal ganglion cells independent of estrogen receptors by activating anti-apoptotic pathways, enhancing their capability to cope with oxidative stress, and maintaining mitochondrial function in the face of a potentially lethal insult (a discussion on the role of non-feminizing estrogen analogues as neuroprotectants in optic neuropathies is presented in Appendix D). Each of these well documented observations lead to the creation of our specific aims.

Specific Aims

Maintenance of cellular redox status, mitochondrial function, and control of apoptotic pathways are all key to saving neurons in neurodegenerative diseases. Synthetic estrogen analogues, which lack classic hormonal properties, are promising alternatives to natural estrogens for neuroprotection against a host of insults and pathologic states. Such drugs have tremendous potential to protect against potentially lethal oxidative stresses. The study of designer estrogens allows us to better understand their pharmaco-dynamics and therapeutic potential. Such non-feminizing estrogen analogues stand to provide therapeutic applications in both males and females, including but not limited to ocular disorders and neuroprotection. We tested the hypothesis that non-feminizing estrogen analogues can protect rat retinal ganglion cells against glutamate-induced cytotoxicity.

The following specific aims were proposed to test the hypothesis:

Specific Aim 1: Determine the neuroprotective efficacy of estrogen analogues against glutamate-induced cytotoxicity of RGC-5 cells
RGC-5 cells were pretreated with estrogen analogues followed by 5mM glutamate challenge for 24 hours. Cell viability was determined using neutral red dye uptake assay. Maximum efficacy of each compound was assessed.

Specific Aim 2: Examine the capability of estrogen analogue ZYC-3 to effect pathways critical to combating oxidative stress in RGC-5 cells
RGC-5 cells pretreated with 1μM ZYC-3 followed by 5mM glutamate insult for 24 hours were assayed for reduced glutathione levels. Additionally, enzyme mediated steps key to glutathione synthesis and cycling were examined to determine if ZYC-3 can enhance the ability of RGC-5 cells to combat oxidative stress.

Specific Aim 3: Assess the ability of estrogen analogue ZYC-3 to affect mitochondrial function and signal transduction pathways in RGC-5 cells.
The mitochondrial membrane potential of RGC-5 cells, pretreated with 1μM ZYC-3 followed by 5mM glutamate insult, was examined. Furthermore, we determined if ZYC-3 can enhance neuronal survival via signal transduction.

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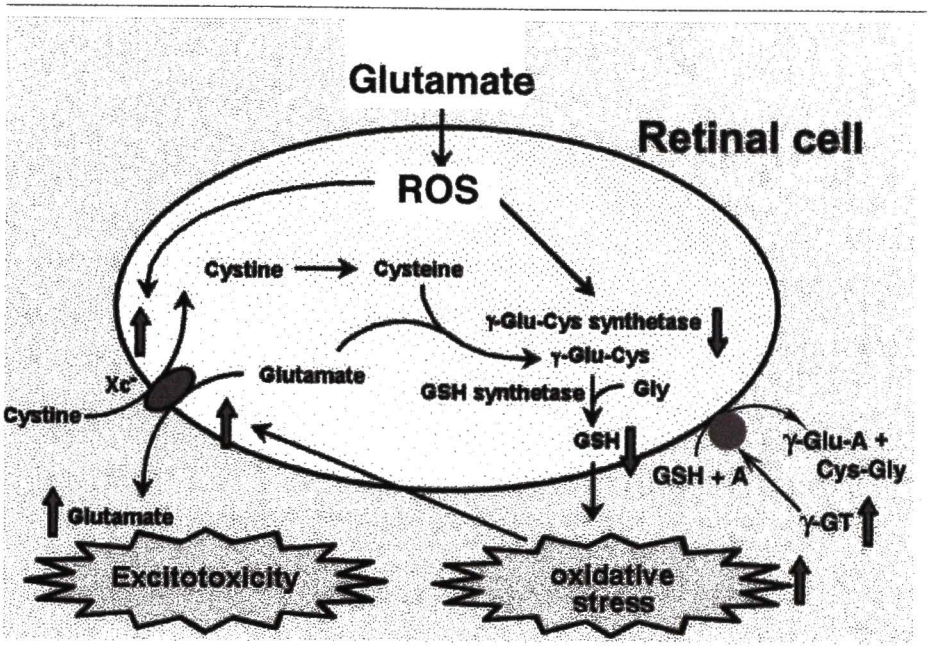
Figure Legends

Figure 1: Glutamate induced generation of excitotoxicity and oxidative stress, independent of glutamate receptors (adapted from Bridges et al., 2004). Elevated extracellular levels of glutamate inhibit or reverse glutamate /cystine antiporters, depleting intracellular cysteine concentrations. When intracellular cysteine is depleted, glutathione, the essential endogenous antioxidant, cannot be produced. As indicated γ-glutamylcysteine synthetase (glutamate cysteine ligase), glutathione synthetase, and γ-glutamyl transpeptidase are all essential components in the synthesis and cycling of glutathione.

Figures

Figure 1



PREFACE TO CHAPTER II

Here we examine the efficacy of three estrogen analogues and 17β-estradiol, in an *in vitro* model of glaucoma. Glutamate-induced cytotoxicity of RGC-5 rat retinal ganglion cells was used as a model for the study of neuroprotection in glaucoma. Additionally, we demonstrate that these drugs may be highly effective direct antioxidants.

CHAPTER II

Role of Nonfeminizing Estrogen Analogues in Neuroprotection of Rat Retinal Ganglion Cells against Glutamate-Induced Cytotoxicity

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Abstract-----Glaucoma is a family of eye disorders whose ultimate cause of vision loss is apoptosis of retinal ganglion cells. While several etiologies of glaucoma exist, oxidative stress is thought to be a key mechanism by which ganglion cells die. From this perspecive, the work presented here was designed to examine the efficacy of 17β-estradiol and three synthetic estrogen analogues (ZYC-1, ZYC-3, ZYC-10) as retinal ganglion cell neuroprotectants. Compounds ZYC-1 and its enantiomer ZYC-10, containing an additional double bond in the steroid C-ring of 17β-estradiol, had similar (ZYC-1) or modestly reduced (ZYC-10) affinity for estrogen receptors as compared to the parent estrogen. In the case of ZYC-3, the addition of an adamantyl group to the C2 position of the A ring of estrone abolished its binding to the estrogen receptors. RGC-5 cells (an established clonal rat retinal ganglion cell line) and rat retinas were shown to predominantly express estrogen receptor- α (ER-α) with minimal detectable levels of estrogen receptor-β (ER-β). The affinity of the synthetic compounds for estrogen receptors was as follows: ZYC-3 < ZYC-10 < ZYC-1. An *in vitro* model of glutamate induced RGC-5 cell death was used. Glutamate treatment resulted in 50-60% RGC-5 cell death with respect to control untreated cells. 17β-estradiol and the three estrogen analogues (0.5 to 1.0μM) protected the RGC-5 cells against glutamate cytotoxicity. The efficacy of neuroprotection by the estrogen analogues was as follows: ZYC-3 > ZYC-1 > ZYC-10. EC_{50} values for inhibition of TBAR levels were as follows: ZYC-3 > ZYC-10 > ZYC-1. Furthermore, these compounds worked independent of estrogen receptors, as inclusion of 100 nM ICI 182,780 did not reverse their neuroprotective properties against

glutamate insult. These compounds appear to affect neuroprotection via pathways independent of the classical estrogen receptors. The data support the hypothesis that estrogen analogues may be useful in the treatment of neurodegenerative diseases, particularly in neuroprotection of retinal ganglion cells in ocular pathologies such as glaucoma.

Introduction

Estrogens are a family of cholesterol-derived steroid hormones that have been demonstrated to be neuroprotective against a variety of insults in both *in vitro* and *in vivo* models of neurodegenerative diseases. As an insidiously progressive neurodegenerative disorder, glaucoma has the capacity to rob effected individuals of their vision. Nearly 70 million people are affected worldwide, with approximately ten percent suffering from bilateral blindness due to primary glaucoma [1]. Additionally, increased mortality has been observed among glaucoma patients, making glaucoma a significant source of morbidity and as well as mortality [2].

The pathologic changes observed in glaucomatous retinas are attributable to apoptosis of retinal ganglion cells within the optic nerve head, resulting in characteristic cupping of the optic disk [3-6]. Experimentally a variety of pathologic situations may be modeled to study the loss of retinal ganglion cells; one such model is a glutamate cytotoxicity induced apoptosis of these cells [7].

Glutamate normally functions as the major excitatory amino acid neurotransmitter in the retina, but at high concentrations it becomes neurotoxic. When glutamate is injected into the vitreous in *in vivo* studies or when cultured retinal ganglion cells are treated with glutamate *in vitro*, the retinal ganglion cells have been shown to undergo apoptosis [8-12]. The apoptosis observed in such models has been attributed to the influx of calcium into cells and the generation of free radicals. Pathologic quantities of nitric oxides (NO), produced due to elevated extracellular glutamate concentration, facilitate the production of superoxide free radical anions by the mitochondria triggering cell death

[13, 14]. Additional oxidative stress may be imposed through the inhibition of glutamate/cystine antiporters, which decreases the availability of intracellular cysteine, a necessary precursor in glutathione synthesis [15-18]. Taken together, excessive glutamate concentrations pose an overwhelming oxidative stress on retinal ganglion cells, which may lead to their death and is the basis for the present studies.

Estrogens have long been recognized as antioxidants in a variety of *in vitro* and *in vivo* studies, and evidence is mounting that implicates their antioxidant nature as part of their neuroprotective value [19-25]. Furthermore, estrogens have been demonstrated to be neuroprotective against glutamate cytotoxicity [26-28]. In addition to their role as neuroprotectants estrogens play vital roles in normal human development, growth, and maturation [29-31]. Those tasks are accomplished via classical steroid hormone receptors and innumerable signaling pathways [32-34]. This allows estrogens to modulate the expression of genes, many of which produce female phenotypes in both females and males [35]. Estrogens also have the propensity to exacerbate pathologic conditions such as female hormone sensitive malignancies and hypercoagulable states, further limiting their potential for widespread clinical application [36, 37]. Although estrogens may afford significant protection in neurodegenerative states such as glaucoma, their therapeutic applications stand to be limited by their vast array of physiologic and pathophysiologic effects.

Synthetic estrogen analogues, which lack classic hormonal properties may be promising alternatives to natural estrogens for neuroprotection against a host of insults and pathologic states. Such non-feminizing estrogen analogues could find widespread

therapeutic indications in both males and females, with a number of potential applications beyond ocular disorders and neuroprotection.

The novel estrogen analogues utilized here are structural variants of natural estrogens, retaining their neuroprotective function with reduced affinity for the classical estrogen receptors α and β [38]. The compounds ZYC-1 and ZYC-10 are synthetic variants of 17β-estradiol, and ZYC-3 is a synthetic variant of estrone. ZYC-1 contains an additional double bond in the C-ring of 17β-estradiol that is conjugated to the aromatic A-ring. This structural change enhances the stability of the C3 phenoxy radical when it is formed by loss of the hydrogen atom from the hydroxyl group. ZYC-10 is the enantiomer, or non-superimposeable mirror image, of ZYC-1. The physical properties of ZYC-1 and ZYC-10 are identical but their mirror image shapes confer different affinities for binding to estrogen receptors. ZYC-3 contains a 1-adamantyl group at the C2 position of estrone. This bulky electron donating substituent decreases the affinity of the compound for estrogen receptors and enhances the stability of the phenoxy radical (Figure 1). As a result, such compounds may provide enhanced *in vitro* neuroprotection at similar doses to natural estrogens, without utilizing estrogens classical mechanisms of action [22, 24].

In the present study we investigated the effects of estrogen and a set of synthetic estrogen analogues on an established retinal ganglion cell line (RGC-5), in response to oxidative stress induced by glutamate treatment. This was used as an acute *in vitro* model to determine the possible mechanisms of retinal ganglion cell death in experimental

glaucoma. Both estrogen and its analogues utilized here protected RGC-5 cells against toxic doses of glutamate, independent of classical estrogen receptors.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone (Logan, UT). Trypsin, Penicillin, Streptomycin, Neutral Red dye, and HEPES were purchased from Sigma-Adrich (St Louis, MO). L-glutamic acid was purchased from Fisher Scientific (Fair Lawn, NJ). The estrogen receptor antagonist, ICI 182,780 (ICI), was obtained from Tocris (Ballwin, MO) and 17β-estradiol from Steraloids (New Port, Rhode Island). ZYC-1 was synthesized from estrone by first introducing the C-ring double bond according to a literature procedure [39]. The 17-ketone group was then reduced to the 17β-OH group using sodium borohydride in ethanol to yield ZYC-1, which had the expected physical and spectroscopic properties [40]. ZYC-10 was prepared by total synthesis and had physical properties identical to those of ZYC-1 but an opposite optical rotation [41]. ZYC-3 was prepared as describe previously [42]. Antibodies against estrogen receptors α and β were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Estrogen and analogue receptor binding assays were performed using a HitHunter™ Enzyme Fragment Complementation Estrogen Chemiluminescence Assay (Fremont, CA) kit.

Culture of Retinal Ganglion Cell Line RGC-5

RGC-5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100μg/ml streptomycin (Sigma-Aldrich) in a humidified atmosphere of 95% room air and 5% CO_2 at 37°C as described

[43]. These cells have a doubling time of approximately 20 h and were passaged by trypsinization every two to four days.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Southern blot analysis for Expression of Estrogen Receptors α and β mRNA by RGC-5

Total RNA from RGC-5 cells was extracted using the RNAzol B reagent (Tel-Test Inc., Friendswood, TX) and subjected to cDNA synthesis using AMV reverse transcriptase [43]. The PCR primers for estrogen receptors α and β, and β-actin were designed from published sequences using Oligo Software (National Biosciences, Inc., Plymouth, MN) and Primer 3 (MIT, Cambridge, MA) programs downloaded from the world wide web (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/results_from_primer3) ERβ sense primer: GAAGCTGGCTGACAAGGAAC; ERβ antisense primer: AACGAGGTCTGGAGCAAAGA; ERβ internal oligo: CTCAGCCTGTTGGACCAAGT; ERα sense primer: ACCATTGACAAGAACCGGAG ERα antisense primer: CCTGAAGCACCCATTTCATT; ERα internal oligo: TACGAAGTGGGCATGATGAA). All the test samples were amplified simultaneously with a particular primer pair as per the annealing temperature of the individual set of primers. The master mix utilized contained all of the components in the PCR reaction, except the target cDNA; in the case of the control water was used. Although the RT-PCR product gave the expected product size, the authenticity of RT-PCR products was established by Southern blot hybridization using an internal hybridization oligonucleotide.

Immunofluorescent Staining of Rat Retinal Ganglion Cells (RGC-5) and Rat Retinas for Estrogen Receptors α and β

RGC-5 cells were seeded onto 12 mm glass coverslips and fixed with 4% para-formaldehyde and used in parallel with retina sections to perform immunocytochemistry for ERα and ERβ. 5 μm thick sections of 4% para-formaldehyde fixed and paraffin-imbedded rat retinas were mounted on poly-L-Lysine coated glass slides and dried over night on a slide warmer at 37-45°C. The paraffin sections were deparaffinized in xylene, hydrated in series of different ethanol dilution solutions, and rinsed in PBS, pH 7.4 for 10 min at each step. To unmask antigens the sections were immersed in a 0.1% TritonX-100 solution for 30 min and rinsed in PBS, pH 7.4 at least three times for 5 min each. To bind free aldehydes the sections were immersed in a 0.05M glycine for 15 min. To suppress non specific binding of IgG, the sections were incubated in a blocking solution of 5% BSA and 10% normal serum of the secondary antibody of the host animal in PBS, pH 7.4 for 15 min, followed by the primary antibody incubation of ERα or ERβ (Santa Cruz Biotechnologies) diluted in dilution buffer (1% BSA and 1% normal serum of 2° antibody host animal in PBS, pH 7.4) to 1 μg/ml final concentration. On control slides, the 1° antibody was substituted by dilution buffer or goat IgG in dilution buffer at the same concentration as the primary antibody. All slides were kept in a moist chamber at 4°C over night. The next day all slides were rinsed four times in PBS pH 7.4 for 5 min and incubated with the Alexa red 594 (Molecular Probes) attached secondary antibody 1:200 in dilution buffer (without normal serum added) for 1 h at room temperature. All slides

were rinsed in PBS, pH 7.4 followed by, nuclear acid staining DAPI (Molecular Probes, Inc.) diluted to the manufacturer's recommendation for 5-10 min, then rinsed two times in PBS, pH 7.4 and three times in distilled water for 5 min at each step. The sections were placed on cover slips with aqueous mounting media and viewed on a Nikon fluorescent microscope (Microphot FXA). Fluorescent images were collected digitally on a Nikon Microphot FXA microscope (Nikon, Melville, NY) set up for epi-fluorescence and using a 40X (NA 0.70) planapochromatic lens. Excitation wavelengths were selected by a computer-controlled Ludl filter wheel (Ludl Electronic Products Ltd., Hawthorne, NY) in front of the mercury vapor lamp source. A single emission filter was used, which allows the passage of DAPI and Alexa Red wavelengths, depending on the excitation filter selected. This allows multiple labels to be captured and the images overlayed without any spatial shifting of the image data. Images were captured with a Photometrics SenSys cooled CCD camera (Roper Scientific, Tuscon, AZ) as 10 bit, 1024 pixel X 1024 pixel, grayscale images. The camera and microscope automation were controlled with an Apple G4 Power PC computer (Apple Computer, Cupertino, CA), OS 9.0.4, running Scanalytic's IP Lab Spectrum software (Scanalytics, Fairfax, VA). Images were deconvoluted using Vaytek's Microtome plug-in (Vaytek, Fairfield, IA) for IP Lab Spectrum. Deconvoluted images were merged in IP Lab Spectrum to determine relative label distribution and to visualize co-localization of labels. Images were then saved as TIFF files and prepared for display and printing in Adobe Photoshop 7 (Adobe, San Jose, CA).

Estrogen Receptor Binding Assay

Competition binding assays were performed using a commercially available kit, HitHunter™ EFC Estrogen Chemiluminescence Assay kit from Discoverx (Fremont, CA). This assay uses an enzyme fragment complementation (EFC) method. In brief, competing ligands at final concentrations ranging from 10 pM to 10 μM were incubated with 5 nM recombinant estrogen receptor α (ERα) or 10 nM estrogen receptor β (ERβ) (Panvera, Madison, WI) and 17β-estradiol-conjugated enzyme donor for 1.5 h. The enzyme acceptor was then added for another 1.5 h after which chemiluminescence substrate was added for another 1 h. Relative luminescence units (RLU) were determined using a Biotek FL600 plate reader. Sigmoidal standard curves were created using a 4-paramater log curve with constant top and used to determine IC_{50} values for estrogen and analogue receptor binding (GraphPad Prism, version 3.02 for Windows, GraphPad Software, San Diego, CA).

Glutamate Cytotoxicity and Effects of 17β-estradiol and Estrogen Analogues on Retinal Ganglion Cells

RGC-5 cells were seeded at 10,000 cells/well in 24 well plates. After 16-24 h, cells were subjected to various concentrations of 17β-estradiol or estrogen analogues (ZYC-1, ZYC-3, or ZYC-10); those experiments with estrogen receptor antagonists were incubated with ICI for 2 hours prior to estrogen or analogue treatment. Once treated, the cells were

incubated for 6-18 h at 37°C in a humidified chamber incubator. Following this incubation, the cells were treated with 5 mM L-glutamic acid. The drugs were present during the glutamate treatment of the cells. Non-drug and glutamate treated cells were included as controls. Cell viability was determined using neutral red dye uptake assay 24 hours after glutamate treatment. To conduct the neutral red dye assay, the cells were washed of growth medium with HEPES buffer (125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM D-glucose, 10 mM HEPES, pH 7.2). Neutral red dye was added to a final concentration of 0.033% in HEPES buffer and incubated for 1-2 h at room temperature. Following neutral red dye uptake by living cells, the wells were gently washed with 2-4 volumes of a HEPES buffer to wash away excess dye. Cells were then allowed to air dry for 20 min, and then treated with ice-cold solubilization buffer (1% acetic acid/50% ethanol; 300-500 μl) to extract the dye taken up by living cells. Twenty minutes later, 100 μl aliquots were transferred to wells of flat-bottomed 96 well plates and optical densities of samples were read at 570 nm. The viability of the RGC-5 cells was calculated as percent survival of control RGC-5 cells and plotted as bar graphs.

Thiobarbituric Acid Reactive Substances (TBARS) Assay

To evaluate the role of 17β-estradiol and our synthetic analogues as antioxidant neuroprotectants, lipid peroxidation was determined using an adapted TBARS assay [44, 45]. Malondialdehyde is an end product of lipid peroxidation; it is derived from the

breakdown of polyunsaturated fatty acids (PUFAs) and related esters. Rat brain homogenates were subjected to iron chloride (50 μM) for 15 minutes. Compounds were added to assess their ability to inhibit this stimulated lipid peroxidation event. EC_{50} values for each of the examined compounds were obtained; values represent the concentration of estratriene needed to reduce lipid peroxidation by 50%.

Statistical Analysis

Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. $P<0.05$ was considered significant for all experiments. The values are reported as the mean±SEM.

Results

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Southern blot analysis for Expression of Estrogen Receptors α and β mRNA by RGC-5 Cells

RT-PCR analysis results demonstrated significant ERα mRNA expression in the RGC-5 cells as compared with the ERβ, whose mRNA expression was minimal (Figure 2). Authenticity of the RT-PCR product was assured by PCR-Southern blot analysis using a middle primer as the probe (data not shown).

Immunofluorescent Detection of Estrogen Receptors in RGC-5 Cells and the Rat Retina

To further confirm the results of Figure 2, immunodetection of the classical estrogen receptors, ERα and ERβ was performed in RGC-5 cells using isoform specific antibodies against estrogen receptors. The results demonstrated the localization of ERα within both the soma and axonal processes of RGC-5 cells (Figure 3A, arrows in panel 2). On the other hand, the presence of ERβ was almost undetectable in RGC-5 cells (Figure 3A, panel 4). These results further confirm that RGC-5 cells predominantly express ERα, not β.

To compare the results of ERα expression in RGC-5 cells and retina, localization of the classical estrogen receptors in rat retinas was carried out by immunohistochemistry. The results showed the presence of ERα with a paucity of ERβ (Figure 3B). Intense fluorescence for ERα was observed throughout the retinal thickness including the photoreceptor cells. The outer nuclear and outer plexiform layers also

exhibited ERα. ERα was also observed in the inner nuclear and inner plexiform layers. At the inner most portion of the retina the density of ERα appears to increase from the soma of the retinal ganglion cells to the nerve fiber layer (Figure 3B, arrows in panel 2).

ERβ was barely detectable in the photoreceptor, outer limiting, and outer nuclear layers. The outer plexiform, inner nuclear, and inner plexiform and retinal ganglion cell layers demonstrated minimal fluorescence for ERβ (Figure 3B, arrowheads in panel 4).

Estrogen Receptor Binding

Competition binding experiments revealed that 17β-estradiol bound to ERα and ERβ with EC_{50} values of 3.04 nM and 4.51 nM, respectively. Reductions made to the C ring of the steroid structure (ZYC1) did not drastically change the affinity of the estratetraenes for the estrogen receptors. ZYC-1 receptor binding EC_{50} values were 3.98 nM for ERα and 4.06 nM for ERβ. ZYC-10, the enantiomer of ZYC-1, has four fold reduced affinity for ERα (EC_{50} = 16.32 nM) and two fold reduced affinity for ERβ (EC_{50} = 8.14 nM). The addition of an adamantyl group on the A ring of estrone (ZYC-3) effectively abolished binding to the estrogen receptors. ZYC-3 receptor binding EC_{50} values were >10000 nM for ERα and >10000 nM for ERβ (Table 1).

Effects of 17β-estradiol, ICI 182,780, and Glutamate on RGC-5 Cell Viability

Glutamate treatment of RGC-5 cells resulted in approximately 65% loss of cell viability (Figure 4A). Pretreatment of RGC-5 cells with 17β-estradiol, followed by glutamate

challenge, resulted in a significant dose-dependent maintenance of cell viability as compared to RGC-5 cells challenged with glutamate alone (Figure 4A). A protection of 50% of control at a dose of 0.2 μM 17β-estradiol and 86% of control at 2.0 μM 17β-estradiol was observed (Figure 4A).

To determine if estrogen receptors are involved in neuroprotection of RGC-5 cells against glutamate cytotoxicity by 17β-estradiol, preincubation with ICI 182,780 at 100 nM, a concentration that is >200 times the IC_{50} of ICI for both estrogen receptors, minimally attenuated the neuroprotective effects of estrogen against glutamate cytotoxicity (Figure 4B). As shown in Figure 4B, the glutamate treatment resulted in 50% cell loss, which was reversed by inclusion of 1 μM 17β-estradiol even with the blocking of the estrogen receptors by ICI 182,780 (Figure 4B). These results indicated that 17β-estradiol was an effective neuroprotectant against glutamate cytotoxicity and this may be rendered via estrogen receptor independent mechanisms.

Effects of Estrogen Analogues (ZYC-1, ZYC-3, and ZYC-10), ICI, and Glutamate on RGC-5 Cell Viability

Treatment of RGC-5 cells with various ZYC compounds alone did not result in a significant change in the cell survival as compared to untreated RGC-5 cells (Figures 5,6, and 7). Pre-incubation of RGC-5 cells with ZYC-1 at concentrations of 0.3 to 1 μM, followed by 5 mM glutamate challenge, resulted in preservation of cell viability over glutamate treated cells alone. Maximum cell viability of approximately 70% of control was observed at 0.5 μM dose of ZYC-1 as compared to 5 mM glutamate treated cells,

which resulted in cell viability of approximately 35% of control RGC-5 cells (Figure 5A). When 100 nM of ICI 182,780 was included to block estrogen receptors prior to pre-incubation with 1 μM ZYC-1, no significant attenuation of cell viability was observed (Figure 5B).

Pre-incubation of RGC-5 cells with ZYC-3 at concentrations of 0.3 to 1 μM, followed by 5 mM glutamate challenge, resulted in maintenance of cell viability over glutamate treatment alone. Maximum cell viability of nearly 100-125% of control was observed at 0.3 μM-1 μM of ZYC-3 (Figure 6A). Inclusion of ICI 182,780 at 100 nM prior to pre-incubation with 1 μM ZYC-3, produced no significant attenuation of cell viability (Figure 6B).

Pre-incubation of RGC-5 cells with ZYC-10 at concentrations of 0.3 to 1 μM, followed by 5 mM glutamate challenge, resulted in enhanced cell survival as compared with the glutamate treated cells, only at the highest dose of ZYC-10 tested. Maximum cell viability, of nearly double that of glutamate challenged samples, was observed at a 1.0 μM dose of ZYC-10 (Figure 7A). When ICI at 100 nM was included prior to treatment with 1 μM ZYC-10 and glutamate, a small and insignificant attenuation of cell viability was observed. ZYC-10 protected the cells against glutamate cytotoxicity to approximately 75% of the cells not treated with the ICI compound (Figure 7B).

Effects of 17β-estradiol and ZYC-3 on RGC-5 Cell Viability During Glutamate Exposure: Morphological Assessment

RGC-5 cells were plated in a 24 well tissue culture dish and preincubated with

either 17β-estradiol or ZYC-3 for 6 h before treating with 5 mM glutamate along with untreated control cells. Cells were observed microscopically for morphological changes such as pyknosis, confluency, and growth pattern. Photomicrographs were taken 24 h post glutamate insult. Glutamate cytotoxicity resulted in a profound cell loss with rounding up of the cells (arrows in Figure 8B). Preincubation of RGC-5 cells with either 17β-estradiol or ZYC-3 reversed the cytotoxic effects of glutamate (Figure 8C and D respectively) and the cells appeared to be morphologically similar to untreated control RGC-5 cells (Figure 8A).

Estratriene and estratetraene inhibition of lipid peroxidation, TBARs

The potency of the compounds for protection of polyunsaturated fatty acids against iron chloride induced lipid peroxidation was examined for malonyldialdehyde, by TBAR assays. 17β-estradiol, the prototype neuroprotectant and parent compound to ZYC-1, exhibited an IC_{50} of 19.83 μM. ZYC-1 produced an IC_{50} of 4.92 μM, representing a four-fold increase in potency as compared to 17β-estradiol (Table 1). Similarly, ZYC10 was more potent than 17β-estradiol in the TBAR assay with an IC_{50} of 3.13 μM. Of the estrogen analogues examined, ZYC-3 was the most potent with an IC_{50} of 1.13 μM. This represents a twenty-fold increase in potency as compared to the prototype 17β-estradiol, and a nearly three fold greater potency than the next most potent compound, ZYC-10 (Table 1).

Discussion

Immunocytochemistry demonstrated the presence and relative abundance of ERα, with a paucity of ERβ, in the clonal rat retinal ganglion cell line, RGC-5. RT-PCR performed on RGC-5 mRNA for the presence of ERα and ER-β corroborated these findings. These *in vitro* findings were further supported by immunohistochemical analysis of rat retinas. ERα was detectable in all layers of the retina including the ganglion cell layer, but ERβ levels were barely detectable. The binding affinity of ZYC-1, ZYC-3 and ZYC-10 was examined, with the greatest reduction of binding affinity resulting from the addition of an adamantyl group to the A ring of the estrogen back bone (ZYC-3). We have also shown that 17β-estradiol and three synthetic estrogen analogues protected retinal ganglion cells, RGC-5 cells, against glutamate-induced cytotoxicity. This protection was independent of estrogen receptors, since ICI 182,780, a compound that antagonizes classical estrogen receptors, minimally inhibited the protective effects of the estrogen and analogues utilized here. The most effective neuroprotectant of this group was ZYC-3, which also had the least affinity for the estrogen receptors α and β. TBAR assay, used to assess compound antioxidant potency, determined that each of the analogues were more potent than the natural estrogen 17β-estradiol. The compound ZYC-3, which has the least affinity for estrogen receptors and is the most effective neuroprotectant, was also the most potent inhibitor of the lipid peroxidation product malondialdehyde.

Studies investigating glutamate concentrations in the vitreous humor of glaucoma patients and in various glaucomatous animal models, have left the occurrence of

neurotoxic retinal glutamate levels up to a debate [46, 47]. Nonetheless, Carter-Dawson's group has observed increases in glutamine, glutathione, and glutamate/aspartate transporter levels in experimental glaucomatous monkey retinas, indicating an elevation in extracellular glutamate and enhanced glutamate transport and metabolism. Furthermore, it is known that glutamate causes excitotoxicity via excess stimulation of its receptors and initiates a cascade of events that result in an overload of intracellular calcium, the release of reactive oxygen species, and the inhibition of glutamate/cystine antiporters [16, 17, 48, 49]. These effects, along with perturbations of mitochondrial membrane potential, direct the cells toward programmed death [15, 50]. The toxic effects of glutamate on mammalian retinal ganglion cells have been well documented and result in a glaucomatous phenotype both *in vivo* and *in vitro* [18, 51].

A number of laboratories have reported that retinal ganglion cells are highly susceptible to glutamate toxicity *in vitro* and *in vivo* [52-59]. It is important to note that Ullian et al., have recently demonstrated that *in vitro* and *ex vivo* retinal ganglion cells are invulnerable to 1 h 500 μM glutamate elicited NMDA receptor excitotoxicity [60]. We have shown that our characterized clonal RGC-5 cells are highly sensitive to 24 h 5 mM glutamate treatment, which produces 50% or greater cell death. This cytotoxic effect was not attenuated or reversed by the NMDA receptor antagonist MK801, nor by the sodium dependent glutamate/aspartate transport inhibitor dl-TBOA [11]. Furthermore, the antioxidants thiourea and NAC, respectively minimized and reversed the typical cell death seen with glutamate treatment [11]. These findings indicate that glutamate's toxicity at higher concentrations may be independent of its transporters and receptors. In

the present studies, 5 mM L-glutamic acid was used to create an oxidative stress to RGC-5 cells in order to study the neuroprotective effects of ZYC estrogen analogues.

Estrogens' effects are classically mediated by the binding of estrogen to its receptors, followed by binding of the steroid-receptor complex to estrogen response elements on various genes that modulate transcriptional events. However, it is becoming clear that responses may be elicited in neurons independent of the classical pathways of estrogen receptor mediated activation of gene transcription [27, 61, 62].
Presented here is evidence supporting the observation that the neuroprotective effects of estrogens, against glutamate cytotoxicity, do not require ER-dependent gene transcription. First and foremost, estrogen receptor antagonists do not attenuate the protective effects of estrogens in models of neurotoxicity [63]. Second, non-receptor binding estrogen analogues have shown neuroprotective efficacy as potent as 17β-estradiol [64-66]. Finally, 17β-estradiol has been shown to activate the mitogen activated protein kinase signal transduction pathway within minutes of 17β-estradiol treatment, far too rapidly to involve the expression of genes [67]. Paramount to these neuroprotective effects is estrogen's structural basis for free radical scavenging that can prevent oxidative neuronal cell death [23, 68, 69]. As has been described, the key components to their function as free radical scavengers are the phenolic A ring and quinol-based cyclic antioxidant mechanisms [22, 23, 70]. The compounds utilized in the studies presented here take advantage of such estrogen receptor independent mechanisms of action, which may be predicted based upon the modifications made (Figure 1).

Estrogen analogue ZYC-1contains a double bond in the C ring not found in its parent, 17β-estradiol. This modification was introduced in order to enhance free radical stability by resonance stabilization. Additionally, appropriate modifications in the structure made near the phenolic A ring may stabilize phenoxy radicals, allowing the 3-hydoxyl group of the A ring to donate its hydrogen atom to free radicals more readily. As predicted based on its structure, ZYC-1's affinity for ERα or ERβ was nearly equivalent to that of 17β-estradiol. ZYC-1 demonstrated significant, yet incomplete protection of RGC-5 cells against glutamate insult. Maximum protection was less than that observed with 17β-estradiol. However, in those studies utilizing ICI the protective effects of ZYC-1 were comparable to 17β-estradiol. Furthermore, ZYC-1 was four-fold more potent at inhibiting the production of malonyldialdehyde (MDA). This result provides evidence for the use of extended conjugation, a means to enhance 17β-estradiol antioxidant capacity. Although ZYC-1 did not show enhanced cell viability against glutamate insult when compared to 17β-estradiol, these observations support their role as antioxidant neuroprotectants independent of estrogen receptors.

Compound ZYC-10 is the enantiomer of ZYC-1. Because enantiomers have identical physical and chemical properties, its inherent antioxidative capacity is identical to that of ZYC-1. However, its mirror shape is expected to further reduce the compound's affinity for estrogen receptors, which are highly stereospecific for ligand binding. This expectation was supported by the estrogen receptor binding assay data. ZYC-10 had less than one fifth the affinity for ERa and nearly half the affinity for ERb as compared to its natural estrogen parent. ZYC-10 also demonstrated significant yet incomplete

neuroprotection against glutamate challenge. Maximum protection was less than that observed with 17β-estradiol and ZYC-1. Surprisingly, in those studies utilizing ICI the protective effects of ZYC-10 were less than those observed with 17β-estradiol as well as ZYC-1. The fact that ZYC-1 and ZYC-10 did not have identical neuroprotective actions may be explained by differences in the metabolism or distribution of the enantiomers in the cell. The TBARs assay revealed that ZYC-10 was over six times more potent at inhibiting the MDA formation with respect to 17β-estradiol. Although ZYC-10 did not show enhanced cell viability against glutamate insult when compared to 17β-estradiol or ZYC-1, these observations support its role as an antioxidant neuroprotectant with appreciable independence of estrogen receptors.

Compound ZYC-3 contains the bulkiest modification of the synthetic estrogen analogues used here. An electron-donating adamantly moiety was introduced to the C-2 position on the A ring of estrone. The addition of this bulky electron-donating group was predicted to decrease binding of the steroid to estrogen receptors as well as increase the ability of the compound to scavenge free radicals. As predicted, binding of ZYC-3 to the classical estrogen receptors was unappreciable. Maximum protection was equal to or greater than that observed with prototypical 17β-estradiol, and significantly better than that of compounds ZYC-1 and ZYC-10. Similar results were observed in those studies utilizing ICI. Furthermore, ZYC-3 was the most potent compound at inhibiting the formation of the lipid peroxidation product MDA. It was twenty times more potent than 17β-estradiol and roughly four times more potent than either of the other two estrogen analogues. The enhanced cell viability observed in ZYC-3 pretreated samples against

glutamate insult supports its structural basis as an effective antioxidant neuroprotectant, independent of estrogen receptors. Of the estrogens tested ZYC-3 was the most effective against glutamate insult.

The affininty of each of the ZYC compounds for ERα and ERβ was as follows: ZYC-3 < ZYC-10 < ZYC-1. Each of the ZYC compounds protected against glutamate cytotoxicity as described above. Their efficacy against 5 mM glutamate challenge, based on values from the percentage cell viability curves, was as follows: ZYC-3 > ZYC-1 > ZYC-10. Neuroprotection by the ZYC compounds was nearly equal to that of the estrogen analogue with the estrogen receptor antagonist at the ICI 182,780 dose examined. Efficacy at inhibiting lipid peroxidation was as follows: ZYC-3 > ZYC-10 > ZYC-1. Regardless of its concentration, ZYC-3 demonstrated greater protection against glutamate cytotoxicity when compared to ZYC-1 and ZYC-10.

Of the compounds examined, ZYC-3 had the least affinity for classical estrogen receptors, the greatest neuroprotective effect, and the highest potency against lipid peroxidation. Although differences were observed in these categories between ZYC-1 and ZYC-10, they were very small in terms of the greatly exaggerated values of ZYC-3.

Taken together, in the present studies we have shown that 17β-estradiol and three novel estrogen analogues, each with unique modifications to native estrogen structures, are effective in protecting RGC-5 cells against glutamate cytotoxicity. Estrogen receptor antagonist ICI 182,780 minimally attenuated the neuroprotective effects of estrogen and the synthetic analogues. Of the non-feminizing estrogen analogues examined, ZYC-3 was the most effective. Thus these findings provide support for continued investigation for the

synthesis and use of synthetic estrogens as potential neuroprotectants in a host of neurodegenerative diseases, including glaucoma.

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Figure Legends:

Figure 1. Structure of Various Estrogen Analogues

Estrogen analogue ZYC-1contains a double bond in the C ring not found in 17β-estradiol. ZYC-3 contains an electron donating adamantly moiety at the C-2 position on the A ring of estrone. Compound ZYC-10 is the enantiomer (non-superimposeable mirror image) of ZYC-1.

Figure 2. RT-PCR Analysis for mRNA Expression of ERα and ERβ by RGC-5 Cells

Total RNA was isolated from of RGC-5 cells and subjected to RT-PCR analysis using ERα and ERβ specific primers as described in the methods. The results showed that RGC-5 cells express predominantly ERα.

Figure 3. Immunohistological Detection of ERα and ERβ in RGC-5 Cells and Rat Retinas

To further confirm the RT-PCR results, the protein for ERα and ERβ protein was detected by immunocytochemistry using specific antibodies for ERα and ERβ by RGC-5 cells and rat retinas. The protein levels of ERα were predominant for RGC-5 cells (A, arrows in panel 2) as well as retinas (B, arrows in panel 2) as compared with the ERβ in RGC-5 cells (A, 4) and retina (B, arrowheads in panel 4), where it was detected minimally. In Figures A and B: panels 1 and 3 represent the differential interface

contrast (DIC) pictures respectively for RGC-5 and retinas. Red fluorescence represents either ERα or ERβ and blue dye represents the nuclear stain DAPI. ERα was present in neurites of the RGC-5 cells with a little labeling in the nucleus (A, arrows in panel 2). In the retina, ERα (B, arrows in panel 2) was expressed throughout the retina including photoreceptor cell layer (PRC), outer nuclear layer (ONL), outer plexiform layer, inner nuclear layer (INL), and nerve fiber layer (NFL) (including retinal ganglion cells). ERβ was detected in the outer plexiform (B, arrowheads in panel 4) and retinal ganglion cell layers alone (B, arrowhead in panel 4).

Figure 4. Effects of 17β-estradiol on RGC-5 viability during glutamate exposure. RGC-5 were pretreated with 17β-estradiol for 6 h after which the cells were treated with glutamate (5 mM) without the removal of 17-β-estradiol from the treated wells. Glutamate treatment alone resulted in 60-65% loss of RGC-5 cells and pre-incubation with 17β-estradiol resulted in neuroprotection of RGC-5 cells in a dose dependent manner (A). To distinguish if 17β-estradiol exerts its neuroprotective effects via estrogen receptors, RGC-5 cells were treated with ICI 182,780 (100 nM) for 2 h to block the estrogen receptors, followed by 6 h with 17β-estradiol (1 μM) before treating with glutamate (B). Similar to A, glutamate treatment resulted in 50% cell loss, which was reversed by 17β-estradiol treatment. ICI 182,780 did not block the neuroprotective effects of 17β-estradiol, suggesting that 17β-estradiol, a natural estrogen possesses anti-oxidative properties. The data is represented as mean±SEM. * denotes $p<0.001$ vs untreated control cells and ** denotes $p<0.01$ vs glutamate treated cells (A) and **

denotes $p<0.001$ as compared with the glutamate treated cells (B) and * denotes $p<0.001$ as compared to control untreated cells (B).

Figure 5. Effects of ZYC-1 on RGC-5 viability during glutamate exposure. RGC-5 were pretreated with ZYC-1 for 6 h after which the cells were treated with glutamate (5 mM) without the removal of ZYC-1 from the treated wells. Glutamate treatment alone resulted in 65% loss of RGC-5 cells and pre-incubation with ZYC-1 resulted in neuroprotection of RGC-5 cells with 0.3 to 1 μM concentration. ZYC-1 (1 μM) treatment alone to RGC-5 cells did not affect the viability of the cells as compared with the untreated control (A). The maximum neuroprotection was observed with 0.5 μM as compared with the 0.3 μM and 1 μM ZYC-1 treatment (A). To distinguish if ZYC-1 exerts its neuroprotective effects via estrogen receptors, RGC-5 cells were treated with ICI 182,780 (100 nM) for 2 h to block the estrogen receptors, followed by 6 h with ZYC-1 (1 μM) before treating with glutamate (B). Glutamate treatment resulted in 50% cell loss, which was reversed by ZYC-1 treatment. ICI 182,780 did not block the neuroprotective effects of ZYC-1, suggesting that ZYC-1 exerts its effects via anti-oxidative properties. The data is represented as mean±SEM. * denotes $p<0.001$ vs untreated control cells and ** denotes $p<0.001$ vs glutamate treated cells (A) and ** denotes $p<0.05$ as compared with the glutamate treated cells (B) and * denotes $p<0.001$ as compared to control untreated cells (B).

Figure 6. Effects of ZYC-3 on RGC-5 viability during glutamate exposure.

RGC-5 were pretreated with ZYC-3 for 6 h after which the cells were treated with glutamate (5 mM) without the removal of ZYC-3 from the treated wells. Glutamate treatment alone resulted in 30% loss of RGC-5 cells and pre-incubation with ZYC-3 resulted in neuroprotection of RGC-5 cells in a dose dependent manner. ZYC-3 (1 μM) treatment alone to RGC-5 cells did not affect the viability of the cells as compared with the untreated control (A). The maximum neuroprotection was observed with 0.5 μM as compared with the 0.3 μM and 1 μM ZYC-3 treatment (A). To distinguish if ZYC-3 exerts its neuroprotective effects via estrogen receptors, RGC-5 cells were treated with ICI 182,780 (100 nM) for 2 h to block the estrogen receptors, followed by 6 h with ZYC-3 (1 μM) before treating with glutamate (B). Glutamate treatment resulted in 50% cell loss, which was reversed by ZYC-3 treatment. ICI 182,780 did not block the neuroprotective effects of ZYC-3, suggesting that ZYC-1 exerts its effects via anti-oxidative properties. The data is represented as mean±SEM.

* denotes $p<0.001$ vs untreated control cells and ** denotes $p<0.001$ vs glutamate treated cells (A) and ** denotes $p<0.01$ as compared with the glutamate treated cells (B) and * denotes $p<0.001$ as compared to control untreated cells (B).

Figure 7. Effects of ZYC-10 on RGC-5 viability during glutamate exposure.

RGC-5 were pretreated with ZYC-10 for 6 h after which the cells were treated with glutamate (5 mM) without the removal of ZYC-10 from the treated wells. Glutamate treatment alone resulted in 30% loss of RGC-5 cells and pre-incubation with ZYC-10

resulted in neuroprotection of RGC-5 cells with 1 μM concentration. ZYC-10 (1 μM) treatment alone to RGC-5 cells did not affect the viability of the cells as compared with the untreated control (A). To distinguish if ZYC-10 exerts its neuroprotective effects via estrogen receptors, RGC-5 cells were treated with ICI 182,780 (100 nM) for 2 h to block the estrogen receptors, followed by 6 h with ZYC-10 (1 μM) before treating with glutamate (B). Glutamate treatment resulted in 50% cell loss, which was reversed by ZYC-10 treatment. ICI 182,780 did not block the neuroprotective effects of ZYC-10, suggesting that ZYC-10 exerts its effects via anti-oxidative properties. The data is represented as mean±SEM. * denotes $p<0.001$ vs untreated control cells and ** denotes $p<0.001$ vs glutamate treated cells (A) and ** denotes $p<0.001$ as compared with the glutamate treated cells (B) and * denotes $p<0.001$ as compared to control untreated cells (B).

Figure 8. Effects of 17β-estradiol and ZYC-3 on RGC-5 Cell Viability During Glutamate Exposure.

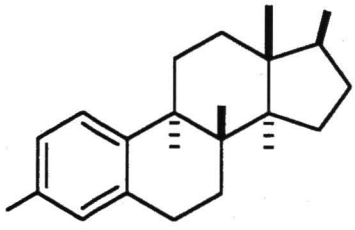
RGC-5 cells were preincubated with either 17β-estradiol or ZYC-3 for 6 hrs before treating with 5 mM glutamate along with untreated control cells. Cells were observed microscopically for morphological changes, and photomicrographed 24 h post glutamate insult. Glutamate cytotoxicity resulted in a profound cell loss with rounding up of the cells (B). Preincubation of RGC-5 cells with either 17β-estradiol or ZYC-3 reversed the cytotoxic effects of glutamate (C and D respectively) and the cells appeared to be morphologically similar to untreated control RGC-5 cells (A).

Table 1. EC_{50} values for estrogen receptor binding and IC_{50} values for inhibition of TBAR formation of estrogen and three estrogen analogues.

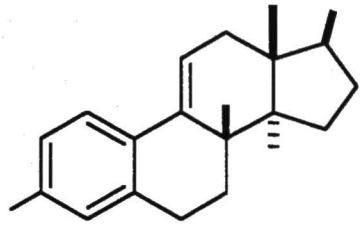
The estrogen receptor binding data show that the synthetic estrogen analogues have dramatically reduced affinity for estrogen receptors as predicted by their structural modifications: ZYC-3 < ZYC-10 < ZYC-1. Furthermore, these compounds were at least four-fold more potent than 17β-estradiol at preventing the formation of lipid peroxidation products, in rat brain homogenates, as assessed by the TBARS assay.

Figure 1

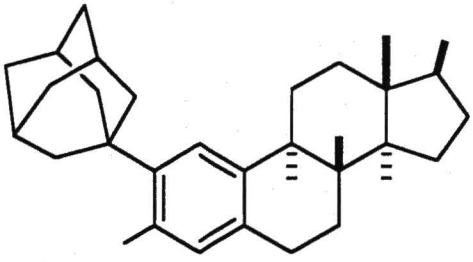
Stuctures of 17β-estradiol, ZYC-1, ZYC-3, and ZYC-10



17β-estradiol



ZYC-1



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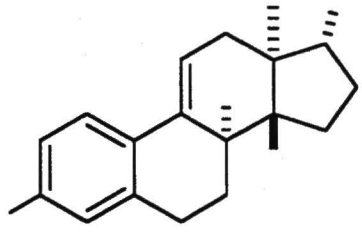


ZYC-10

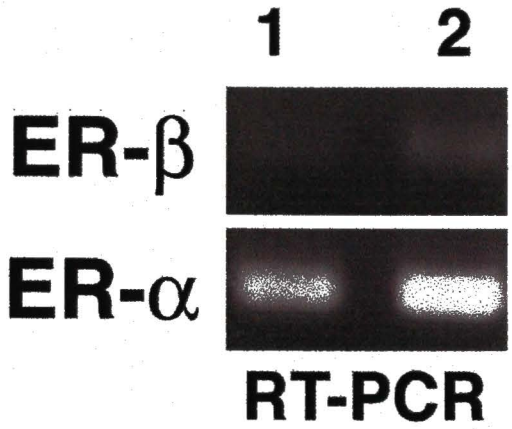
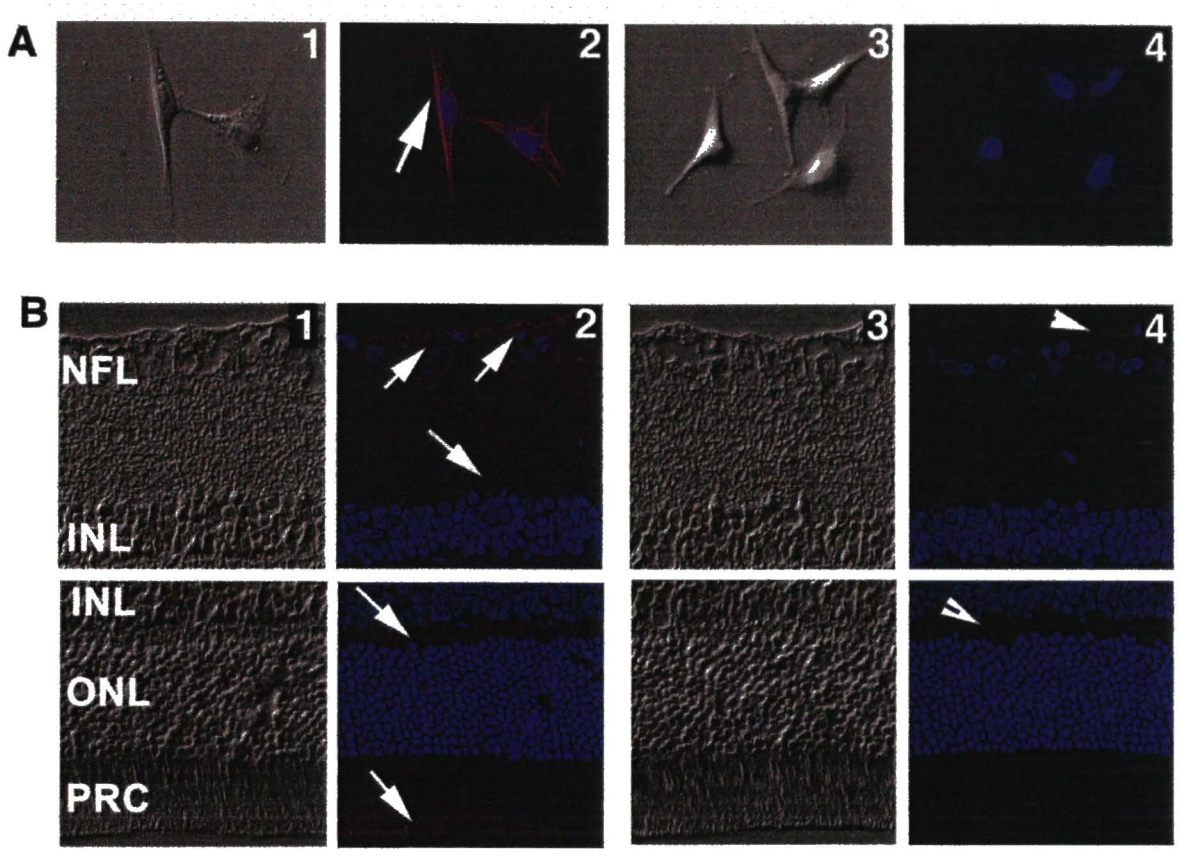
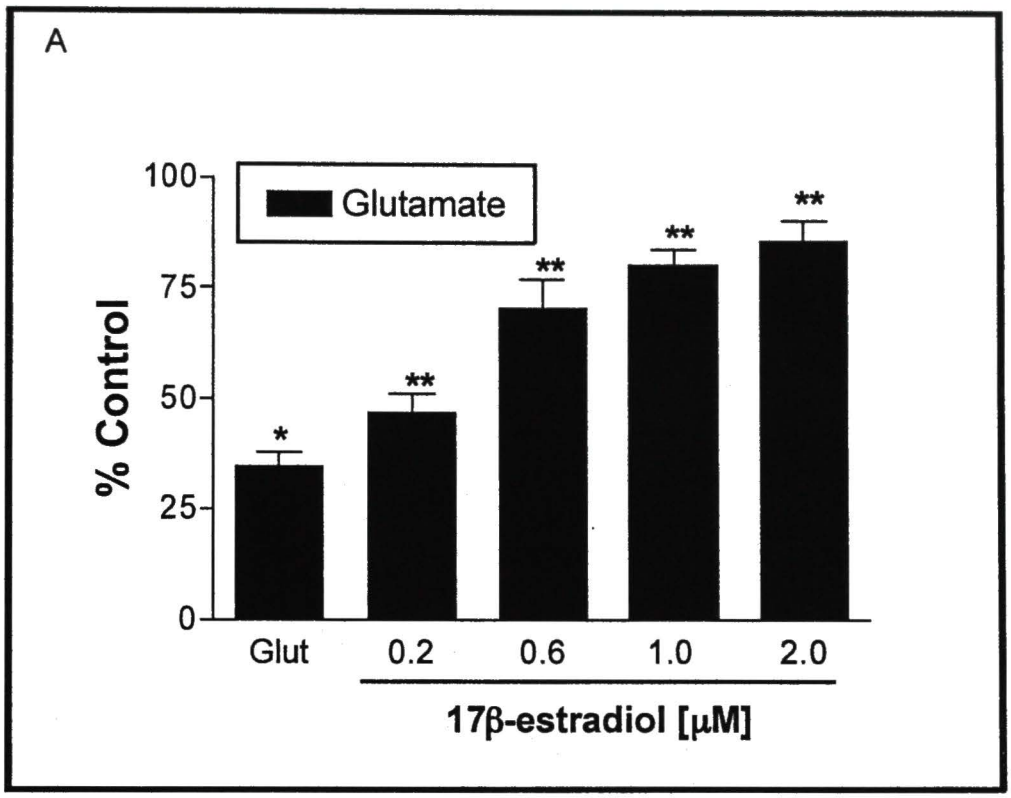


Figure 2

Figure 3




A
Glutamate
% Control
100
75
50
25
0
*
**
**
**
**
Glut
0.2
0.6
1.0
2.0
17β-estradiol [μM]

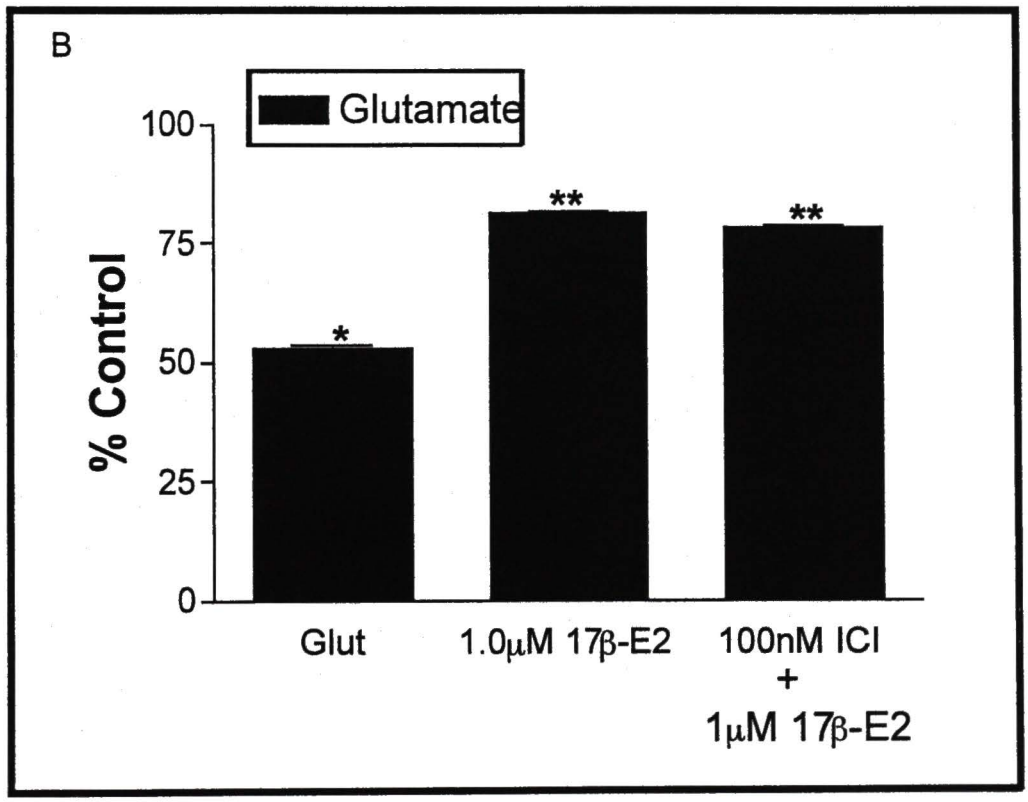
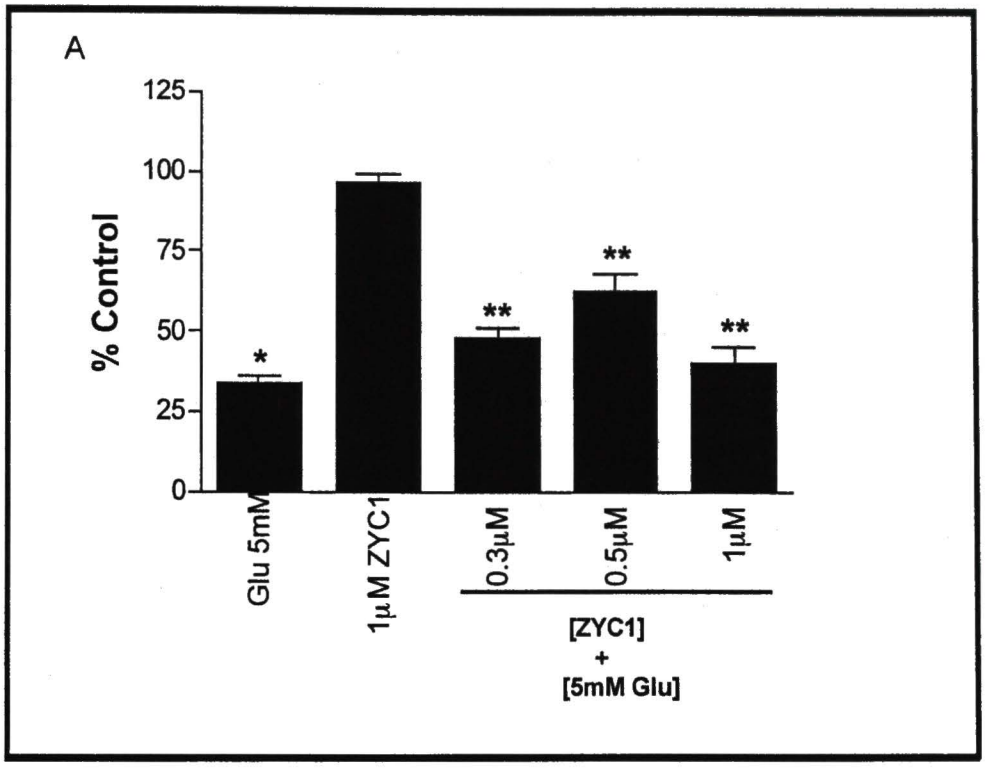

B
Glutamate
% Control
100
75
50
25
0
*
**
**
Glut
1.0μM 17β-E2
100nM ICI
+
1μM 17β-E2

Figure 4


A
125
100
75
50
25
0
% Control
*
**
**
**
Glu 5mM
1μM ZYC1
0.3μM
0.5μM
1μM
[ZYC1]
+
[5mM Glu]

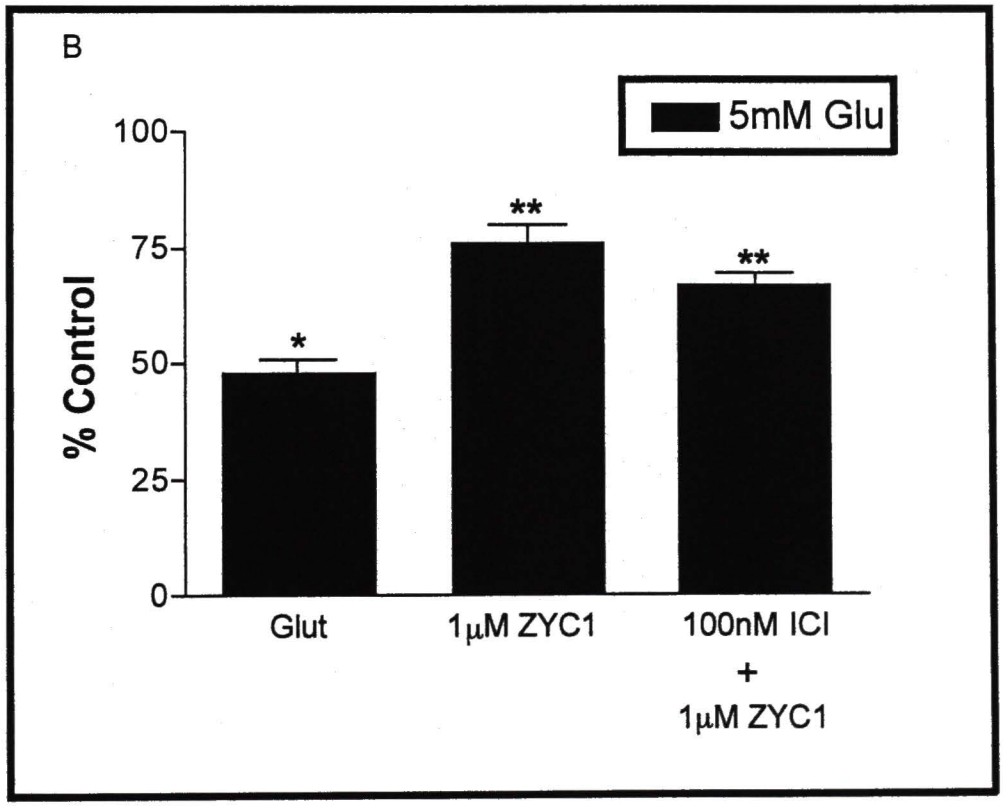
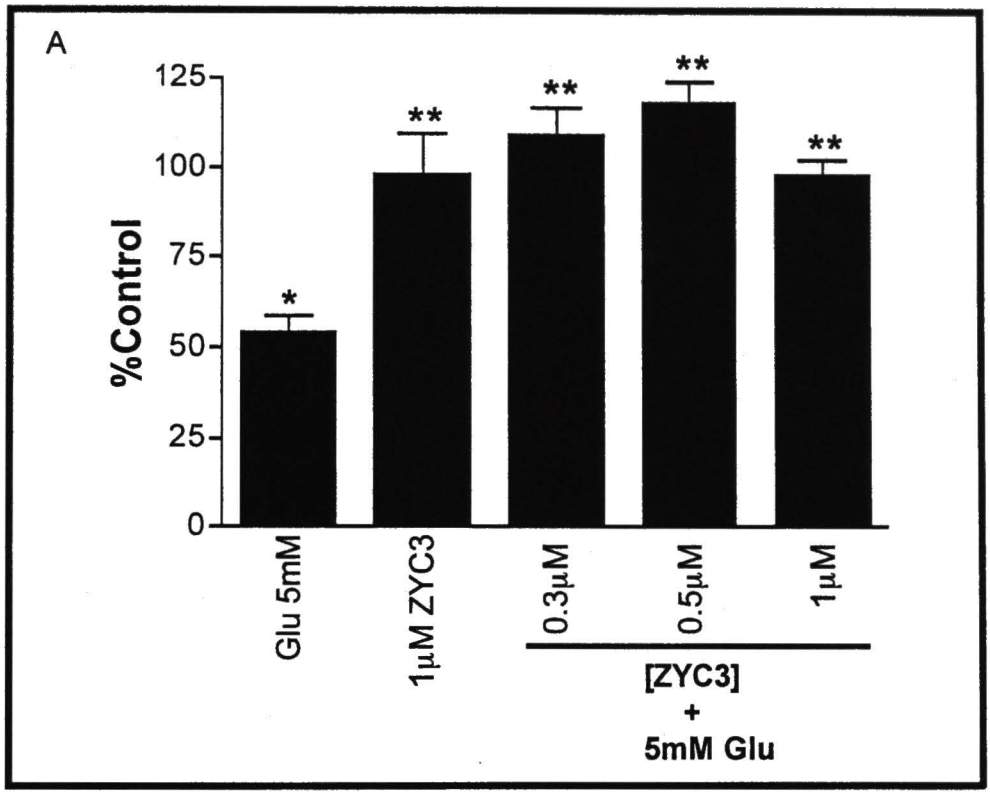

B
5mM Glu
100
75
50
25
0
% Control
*
**
**
Glut
1μM ZYC1
100nM ICI
+
1μM ZYC1

Figure 5


A
125
100
75
50
25
0
%Control
*
**
**
**
**
Glu 5mM
1μM ZYC3
0.3μM
0.5μM
1μM
[ZYC3]
+
5mM Glu

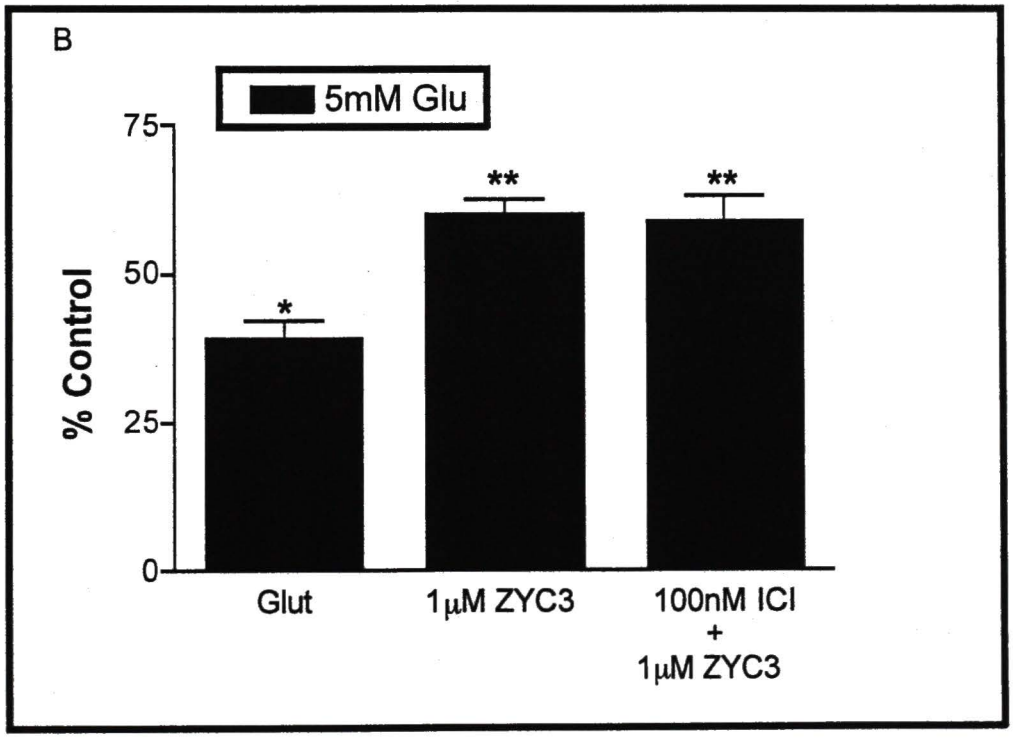
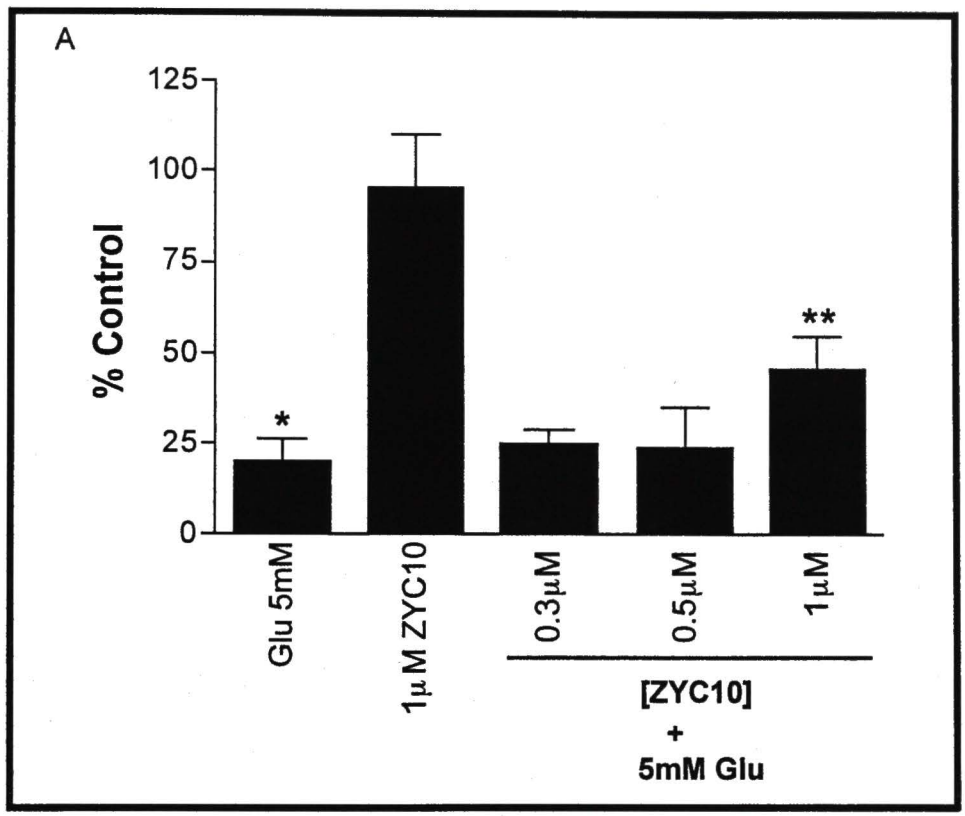

B
5mM Glu
75
50
25
0
% Control
*
**
**
Glut
1μM ZYC3
100nM ICI
+
1μM ZYC3

Figure6


A
125
100
75
50
25
0
% Control
*
**
Glu 5mM
1μM ZYC10
0.3μM
0.5μM
1μM
[ZYC10]
+
5mM Glu

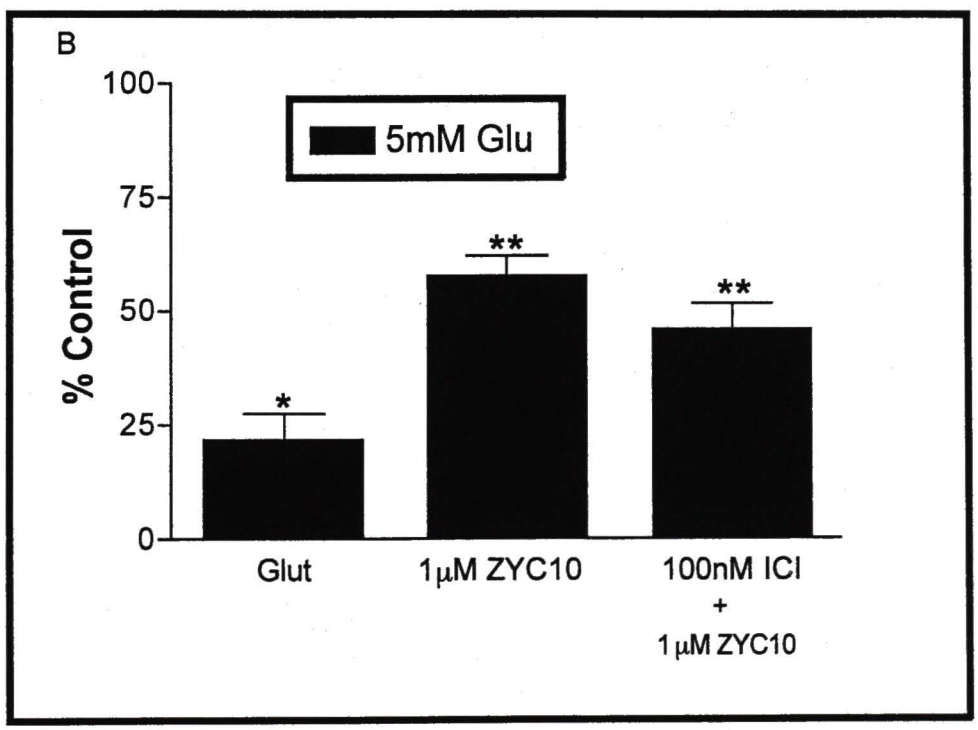
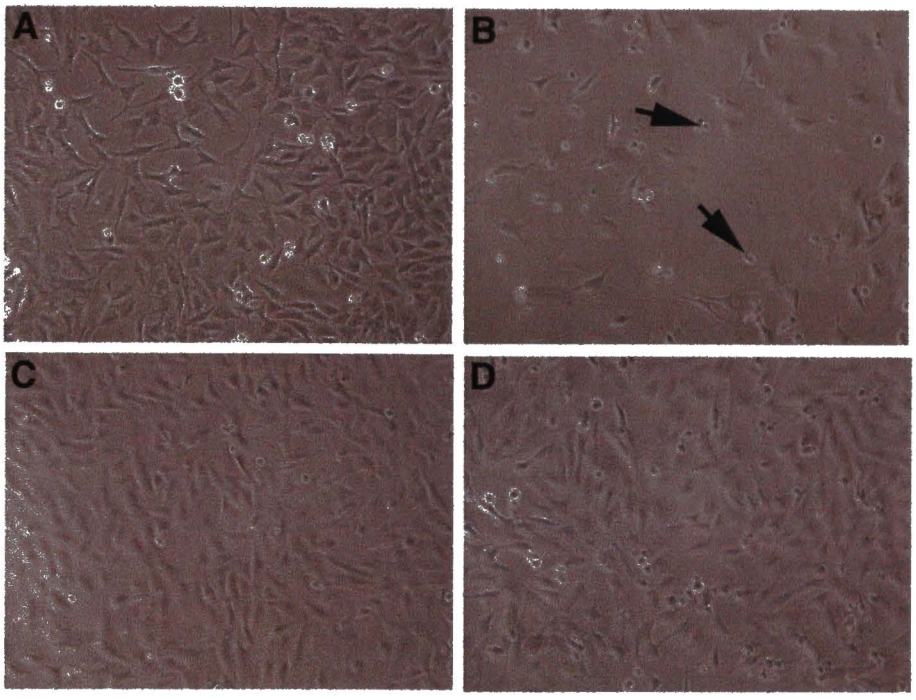

B
100
75
50
25
0
% Control
5mM Glu
*
**
**
Glut
1μM ZYC10
100nM ICI
+
1μM ZYC10

Figure 7

Figure 8



Compound	ERα binding (nM)	ERβ binding (nM)	TBARS (μM)
17β-Estradiol	3.04	4.512	19.83
ZYC-1	3.98	4.06	4.92
ZYC-3	>10000	>10000	1.13
ZYC-10	16.3	8.14	3.13

Table 1

PREFACE TO CHAPTER III

The neuroprotective efficacy of non-feminizing estrogen analogues in a glutamate-induced in vitro model of glaucomatous retinal ganglion cell death was established by the previous chapter. Here we illustrate the neuroprotective mechanisms of the synthetic estrogen analogue ZYC-3.

CHAPTER III

Neuroprotective mechanisms of the non-feminizing estrogen analogue ZYC-3 against glutamate-induced cytotoxicity of RGC-5 cells

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In preparation for publication

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Abstract----- Glutamate exposure depletes glutathione (GSH) and depolarizes mitochondrial membranes, leading to neuronal injury and death. Here we examined the neuroprotective mechanisms of the novel estrogen analogue ZYC-3, in an *in vitro* model of glaucomatous rat retinal ganglion cell (RGC-5) death against glutamate cytotoxicity. We demonstrated that cystine uptake via glutamate/cystine antiporters, rate limiting glutamate cysteine ligase activity, and intracellular GSH content were significantly reduced with glutamate challenge, but protected with ZYC-3 pretreatment. Furthermore, GSH utilization by GSH peroxidase and cycling by GSH reductase were inhibited with glutamate treatment, but promoted with ZYC-3 pretreatment. These findings were corroborated by activation of antioxidant response elements with glutamate treatment that was attenuated by ZYC-3. In the face of glutamate challenge, ZYC-3 enhanced GSH pathway antioxidant defenses and protected mitochondrial membrane potential ($\Delta\Psi_m$). These are the first reports of the effects of ZYC-3 treatment on the GSH antioxidant system and $\Delta\Psi_m$ in glutamate insulted RGC-5 rat retinal ganglion cells. The data support the hypothesis that ZYC-3 may function as a neuroprotective antioxidant and mitochondrial stabilizer.

Introduction

Neuroprotective strategies for the treatment of glaucoma have been a diverse and controversial subject of investigation since this approach was first addressed more than a decade ago (Schwartz et al., 1996). Disease modifying strategies have included immune modulation by vaccination (Ben Simon et al., 2006; Baudouin et al, 2006; Bakalash et al., 2005; Bakalash et al, 2003), cell surface receptor mediators (Ward et al., 2007; Das et al., 2006; Ji et al., 2004; Wheeler et al., 2001), and axonal regeneration and gene therapy (Renwick et al., 2006; Watanabe et al., 2006). In recent years compelling evidence supporting the use of estrogens and estrogen analogues as neuroprotectants has been reported (reviewed by Hoffman et al., 2006; reviewed by Simpkins et al., 2005; Simpkins et al., 2004; Rau et al., 2003; reviewed by Merchanthaler et al., 2003). These findings are relevant in spite of the initial conclusions from the Women's Health Initiative Study indicating that conjugated equine estrogens and estrogen plus medroxyprogesterone acetate (a synthetic progestin) sharply increase the risk of ischemic stroke in generally healthy postmenopausal women (Hendrix et al., 2006; Wassertheil-Smoller S, et al., 2003). Critical reexamination of these reports plus additional data indicate that low doses of 17β-estradiol may in fact protect against ischemia and reperfusion injury (Macrea et al., 2006; reviewed by Wise et al., 2005). Nonetheless, the suggestion that natural estrogens may have detrimental effects opens the door for investigation and development of synthetic estrogen analogues as highly effective neuroprotectants without prothrombotic and feminizing sequelae.

The mechanism of brain parenchymal neuronal death in ischemic stroke is an overwhelming oxidative burden lethal to insulted cells. In the eye, two dominant hypotheses have been proposed to explain glaucomatous retinal ganglion cell (RGC) loss in humans. The vascular hypothesis is based on ischemia-induced production of free radicals, due to compromised blood flow in retinal vessels, with subsequent oxidative damage to RGC axons (Flammer et al, 1994). The mechanical pressure hypothesis is based on elevation of intraocular pressure (IOP) inhibiting retrograde neurotrophin support of RGCs (Quigley et al., 1999). Interestingly, the final common pathway of RGC death in both theories is oxidative stress, similar to that in ischemic stroke.

Therefore a reasonable approach to neuroprotection in glaucoma would include the consideration of estrogens as neuroprotectants. However, in spite of their demonstrated efficacy in a variety of *in vitro* and *in vivo* models of neurodegenerative diseases, the application of estrogens and their synthetic analogues in ophthalmic disorders of neurodegeneration remains an enigmatic approach.

We have previously shown the role of 17β-estradiol and three synthetic estrogen analogues (ZYC-1, ZYC-3, ZYC-10) in neuroprotection of RGC-5 rat retinal ganglion cells in an *in vitro* model of glutamate-induced cytotoxicity This model utilizes the overwhelming oxidative burden that results from glutamate challenge, as evidenced by an increase in lipid peroxidation end products in our study (Kumar et al., 2005). Pretreatment with the estrogen analogue ZYC-3 yielded complete protection of insulted RGC-5 cells, comparable to that of 17β-estradiol pretreated samples. Furthermore, lipid peroxidation in glutamate challenged cells pretreated with ZYC-3 was comparable to that

of control cells and only 0.5% that of 17β-estradiol pretreated samples. The estrogen receptor antagonist ICI 182,780 minimally attenuated the neuroprotective effects of ZYC-3. Additionally, ZYC-3 had no appreciable affinity for estrogen receptors α and β based on the results of competition binding assays (Kumar et al, 2005). We have shown such compounds could possiblybe used as neuroprotectants, independent of estrogen receptor mediated mechanisms, while potentially avoiding the harmful effects associated with conjugated equine and human estrogens. How does ZYC-3, a synthetic variant of estrone modified with the addition of an adamantyl group to the C2 position of the A ring, afford neuroprotection without the estrogen receptor mediated effects of its parent drug?

To better understand the neuroprotective mechanisms of ZYC-3 against glutamate induced cytotoxicity, RGC-5 rat retinal ganglion cells were challenged with L-glutamic acid as a model of RGC death in glaucoma. Although the role and the presence of elevated intravitreal glutamate in glaucoma remains controversial, this model continues to serve as a reproducible high-throughput means of studying RGC biology *in vitro*. In the present study we have elucidated some of the basic mechanisms of RGC-5 death induced by glutamate and neuroprotection with ZYC-3. This body of data demonstrates that ZYC-3 mobilizes critical steps for the maintenance of intracellular GSH, which can then be utilized by GSH dependent antioxidant systems. Even more impressive is that this receptor-independent drug protects mitochondrial membrane potential.

Methods

Culture of Retinal Ganglion Cell Line RGC-5

RGC-5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich) in a humidified atmosphere of 95% room air and 5% CO_2 at 37°C as described (Krishnamoorthy et al., 2001). These cells have a doubling time of approximately 20 h and were passaged by trypsinization every 2-4 days.

Preparation of Cell Lysates for Immunoblot Analysis

RGC-5 cells were plated on 100 mm tissue culture treated dishes, and the following day (12-16 hours), ZYC-3 was administered to a final concentration of 1μM. After a 6 h incubation period with ZYC-3, glutamate was administered to a final concentration of 5 mM and the treated cells incubated for an additional 24 h. The following experimental groups were included for all studies: untreated cells, DMSO (drug vehicle) treated cells, ZYC-3 treated cells, glutamate treated cells, DMSO pretreated glutamate challenged cells, and ZYC-3 pretreated glutamate challenged cells. This treatment protocol was followed for all experiments. The cells were then collected and the lysates prepared. Briefly, the cells were collected, pelleted, and washed three times in equal volumes of PBS. They were then resuspended in 200 μl of lysis buffer (50mM Tris, 150mM NaCl, 10% glycerol, 1mM EGTA, 1mM Na-orthovanadate, 5μM $ZnCl_2$, 100mM NaF, 10μg/ml aprotinin, 1μg/ml leupeptin, 1mM PMSF, 0.4 μg/ml okadaic acid, 1% Triton X-100), sonicated, and centrifuged. The protein concentrations of the resultant supernatants (no

pellet was visible) were determined by the Bradford-Lowry method. The samples were stored at –80°C until used for immunoblot analysis.

Immunoblot Analysis

After separation by 10% SDS-PAGE at 20 mA, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) in a BioRad (Hercules, CA) trans-Blot electrophoresis apparatus at 150 mA for 12-16 h using Towbin's buffer (25 mM tris, pH 8.3, 192 mM glycine, and 20% methanol). The membranes containing immobilized proteins were blocked with 3% bovine serum albumin (BSA) in assay buffer (10mM Tris [pH 7.5], 150 mM NaCl, and 0.2% TWEEN-20). Antibodies that react with rat proteins of interest were prepared in 3% BSA in assay buffer and added to the transblots for overnight (12-16 h) incubation at 4°C. After washes, the membranes were incubated with a goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody as the secondary antibody in 3% BSA in assay buffer for 2 h at room temperature. To verify equal loading of protein in each lane, the blots were reprobed with an antibody against ß-actin. Immunoreactive bands were visualized by a standard enhanced chemiluminescence solution procedure (Amersham Biosciences-Buckinghamshire, England). The primary antibody against the transporter subunit of glutamate/cysteine antiporters (1:1000), was generously provided by Dr.Sylvia Smith (Department of Cellular Biology and Anatomy, Medical College of Georgia). The primary antibodies against the catalytic (1:20,000) and modulatory (1:20,000) subunits of glutamate cysteine ligase (GCL) were generously provided by Dr. T.J. Kavanagh

(Department of Environmental Health, Center for Ecogenetics and Environmental Health, University of Washington, Seattle, WA). The primary antibodies against glutathione reductase (1:2,000) and glutathione peroxidase (1:2,000) were purchased from GeneTex (San Antonio, TX). The primary antibody against Bcl-2 (4C11) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a dilution of 1:1,000. The signal transduction antibody Pathscan® Multiplex Western Cocktails I and II were purchased from Cell Signaling Technology (Beverly, MA) and used at the recommended dilution of 1:200.

Glutamate/Cysteine Antiporter Cysteine Uptake

RGC-5 cells were seeded at 10,000 cells/well in 24 well plates. After 14-18 h, the cells were pretreated with ZYC3 and DMSO (vehicle control) and incubated for 6 h at 37°C in a humidified chamber incubator. Following this incubation the cells were treated with 5 mM glutamate. The drug and vehicle were present during the glutamate treatment of the cells. Non-drug, non-vehicle, drug alone, vehicle alone, and glutamate treated cells were included as controls.

Cell viability was determined using neutral red dye uptake assay 24 h after glutamate treatment. The cells were washed of growth medium with HEPES buffer (125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM D-glucose, 10 mM Hepes, pH 7.2). Neutral red dye was added to a final concentration of 0.033% in HEPES buffer and incubated for 1-2 h at room temperature. Following neutral red dye uptake by living cells, the wells were gently washed with 2-4

vol HEPES buffer to remove excess dye. Cells were then allowed to air dry for 20 min, and then treated with ice-cold solubilization buffer (1% acetic acid/50% ethanol; 300-500 μl) to extract the dye taken up by living cells. 20 min later, 100 μl aliquots were transferred to wells of flat-bottomed 96 well plates and optical densities were read at 570 nm. The viability of the RGC-5 cells was expressed as percentage control RGC-5 cell survival.

Cystine uptake via glutamate/cystine antiporters was determined using a ^{35}S-cystine uptake assay. RGC-5 cells were seeded at 10,000 cells/well in 24 well plates. After 14-18 h, the cells were pretreated with ZYC3 or DMSO (vehicle control) and incubated for 6 h at 37°C in a humidified chamber incubator. Next, the cells were treated with 5 mM glutamate in the presence of drug or vehicle. ^{35}S-cysteine uptake was determined 24 h after glutamate treatment. To conduct the uptake assay the cells were washed of growth media with 37°C isotope-free assay buffer (25 mM HEPES/Tris, 140 mM N-methyl-D-glucamine chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, pH 7.5). 250µl ^{35}S-cysteine uptake buffer (5 ml assay buffer as above, 250 µl of 500 µM Cystine, 50 µl 5000 µCi ^{35}S-cysteine – 25 µM cysteine were) applied to each well and incubated for 15 min at 37°C in a humidified chamber incubator. After the wells were washed with 4°C isotope-free assay buffer, 500µl solubilization buffer (1% SDS in 0.2 N NaOH) was added to each well and incubated at room temperature with shaking for 15 min. The solubilized cells combined with 5 ml of ScinitiVerse scintillation fluid from (Fisher Scientific, Pittsburgh, PA) and counts were read in a Tri-Carb 2100 TR scintillation counter.

Preparation of Cell Lysates for Enzyme Activity Assays and Glutathione Measurement

Cells were plated on 100 mm tissue culture treated dishes, and after 12-16 hours, ZYC-3 was administered to the cultures at a final concentration of 1 μM. After 6 h incubation with ZYC-3, glutamate was administered to a final concentration of 5 mM and the treated cells incubated for an additional 24 h. The drug and vehicle were present during the glutamate treatment of the cells. Following incubation, cells were collected in assay specific buffer, sonicated if required, then stored at –80°C.

Measurement of Glutathione

Cell lysates prepared in PBS were used for measurement of reduced (GSH) and total (GSH + GSSG) glutathione concentrations by high-pressure liquid chromatography (Shimadzu), with fluorescence detection (Hitachi, Tokyo, Japan), as previously described (Medved et al., 2003). Glutathione disulfide (GSSG) concentrations were calculated by subtraction of GSH from total glutathione concentration.

Glutathione Peroxidase and Glutathione Reductase Activities

Cell lysates were prepared in assay specific buffer and enzyme activities were determined with the use of commercially available kits (Cayman Chemical Company, Ann Arbor, MI) . Glutathione reductase activity was determined from the rate of oxidation of NADPH to NADP+ in the presence of GSSG, monitored at 340 nm. Glutathione peroxidase activity was determined by coupling the rate of GSH oxidation to the rate of

NADPH oxidation in the presence of cumene hydroperoxide (5mg/ml) and excess (0.67 U/ml) glutathione reductase.

Transient transfection and Analysis of Antioxidant Response Element Luciferase Reporter Gene Activity.

Cells were seeded at a density of 2 x 10^5 per well in 24-well plates and grown in the culture medium as described (Kumar et al., 2005). After incubation overnight, the cells were transiently transfected with antioxidant response element-luciferase reporter plasmids (pare-luciferase)(5-CTCAGCCTTCCAAATCGCAGTCACAGTGACTCAGCAGAATC-3) that were created (Moehlenkamp, et al., 1999) using pGL3 Luciferase Reporter Vectors (Promega, Madison, MI). The plasmid pGL3, encoding firefly luciferase, was used as a positive control for transfection and luciferase activity. Transfections were done using FuGene 6 reagent (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instructions. Following transfection, the culture medium was replaced and the cells were pretreated with ZYC3 and DMSO (vehicle control) and incubated for 6 hours at 37°C in a humidified chamber incubator. Next, the cells were challenged with 5 mM glutamate. The drug and vehicle were present during the glutamate exposure.. pGL3 transfection positive control was induced with 50 μM *t*-BHQ (in a solution giving a final concentration of 0.1% v/v DMSO). Cells were exposed to treatments for 24 h before being collected, and the firefly luciferase activities in their lysates were measured using a

luminometer following addition of Luciferase Assay Reagent II (Promega). The relative luciferase activity was calculated by normalizing firefly luciferase activity to cellular protein concentrations.

JC-1 Mitochondrial Staining in Live RGC-5 Cells: Confocal Microscopy

Cells were seeded in 35-mm glass-bottomed dishes (World Precision Instruments, Inc., Sarasota, FL) with DMEM supplemented with 10% FBS and treated with glutamate and ZYC-3 as described (Charles et al., 2005). The mitochondrial membrane potential in healthy cells causes JC-1 to form red fluorescent "J aggregates" that exhibit a broad excitation spectrum and an emission maximum at 590 nm.However, JC-1 exists as a monomer at low mitochondrial membrane potential, as seen in apoptotic cells, and appears green. Thus, the emission of this cyanin dye can be used as a sensitive measure of mitochondrial membrane potential (Molecular Probes, Eugene, OR) (Martin et al., 2004). JC-1 stock solution (Molecular Probes) was prepared at 4 mg/ml in dimethylsulfoxide (DMSO). The stock JC-1 solution was added drop-wise, while vortexing, to a final concentration of 10 μg/mL, then passed through a 0.2-μM syringe filter (GeneMate, Kaysville, UT). An appropriate volume was added to the RGC-5 culture dishes and incubated for 15 min at 37°C. After incubation, the staining solutions were decanted, each dish was washed three times with Ringer's buffer (in mM: 130 NaCl, 5 KCl, 2 $CaCl_2.2H_2O$, 1 $MgSO_4$, 8 NaOH, 1 NaH_2PO_4, 5.5 D-glucose, 37°C, pH 7.4) at, and an appropriate volume of Ringer's buffer was added to each dish. Live cell images were then acquired with JC-1 mitochondrial staining, using the Argon laser (488 nm/568 nm) on a

confocal microscope (model LSM410; Carl Zeiss Meditec, Dublin, CA). The JC-1 was excited at 488 nm and nonconjugated light emissions were collected at 530 nm (green) and conjugated at 590 nm (red). To quantitate the effects of glutamate and ZYC-3 on the mitochondrial membrane potential of RGC-5 cells, red to green fluorescence image ratios of individual cells for JC-1 were then calculated on computer (MetaMorph ver. 6.1; Universal Imaging Corp., Downingtown, PA).

Statistical Analysis and Enzyme Activity Normalization

Statistical significance was determined by one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. $P<0.05$ was considered significant for all experiments. The values are reported as the mean±SEM. Because the experimental design produces a heterogeneous sample of viable, dying, and dead cells, activities of the enzymes and dependent products were normalized to protein concentration/cell viability to assess effective enzyme activity in the surviving cell population. This method was used to determine the effects of glutamate and ZYC-3 treatment on the systems being studied while accounting for glutamate-induced cytotoxicity.

Results

Glutamate/Cysteine Antiporter (x_c^-) Cystine Uptake

We examined x_c^- mediated ^{35}S-cystine uptake in glutamate treated RGC-5 cells to determine if glutamate/cystine antiporter function was perturbed in glutamate cytotoxicity and if ZYC-3 protected cystine uptake. Glutamate treatment resulted in a 60% decrease in cystine uptake. ZYC-3 treatment prior to glutamate insult significantly increased cystine uptake by 180%, to 80% that of uninsulted control cells (Figure 1A).

Immunoblot Analysis of Glutamate/Cystine Antiporter

The marked enhancement of cystine uptake in neuroprotected RGC-5 cells raised the possibility that the cellular content of x_c^- protein may have been enhanced with ZYC-3 pretreatment. Immunoblot analysis revealed that x_c^- contents in Control, DMSO (vehicle control), and ZYC-3 (drug control) samples were comparable. Treatment of RGC-5 cells with glutamate lowered β-actin-normalized x_c^- band density by nearly 20% versus control. Pretreatment with ZYC-3 prevented the glutamate-induced reduction in x_c^-. Pretreatment with the ZYC-3 vehicle DMSO exacerbated glutamate induced reduction in x_c^- content (Figure: 1B, 1C).

Immunoblot Analysis of Glutamate Cysteine Ligase

Immunoblot analysis demonstrated that content of both the catalytic (GCLC) and modifier (GCLM) subunits of glutamate cystine ligase were comparable in control and DMSO (vehicle control) cells (Figure 2). ZYC-3 treatment increased GCLC by 30% over control cells, but did not alter GCLM content. Glutamate exposure did not alter GCLC and GCLM protein band densities. In contrast, in ZYC-3 pretreated cells, glutamate challenge sharply increased contents of both subunits, to roughly double the respective contents in control cells (Figure 2).

Glutathione Content

Glutamate challenge resulted in a striking 93% reduction in GSH content versus control cells. ZYC-3 pretreament limited glutamate-induced GSH depletion to 72%, thereby increasing GSH content fourfold versus glutamate challenged, non-ZYC-3 pretreated cells (Figure 3). The vehicle DMSO did not alter glutamate depletion of GSH. Oxidized glutathione (GSSG) was below the detection limit in these cells.

Glutathione Peroxidase

Glutamate exposure lowered glutathione peroxidase (GPx) activity by 87% versus untreated control cells. In the absence of glutamate, ZYC-3 treatment increased GPx activity 2.5-fold (Figure 4A). Glutamate exposure sharply enhanced ZYC-3 activation of GPx, increasing the enzyme's activity 8-fold versus control. Pretreatment of RGC-5s with ZYC-3 vehicle DMSO did not appreciably alter GPx activity in the absence or presence of glutamate (Figure 4A).

Immunoblot analysis demonstrated that GPx protein band densities in Control, DMSO (vehicle control), and ZYC-3 (drug control) samples were similar. Glutamate challenge decreased GPx protein content by 80%. Pretreatment with ZYC-3 attenuated glutamate-induced GPx depletion (Figure 4B, 4C).

Glutathione Reductase

Neither ZYC-3 nor DMSO vehicle altered glutathione reductase (GR) activity in glutamate-free cells (Figure 5A). Remarkably, glutamate exposure alone abolished GR activity. ZYC-3 pretreatment completely protected GR from glutamate inactivation, and even increased GR activity to 70% above control values. DMSO alone had a modest GR-protective effect against glutamate exposure (Figure 5A).

Glutathione reductase contents (Figure 5B, 5C) roughly paralleled the changes in enzyme activity. Thus, contents in DMSO (vehicle control) and ZYC-3 (drug control) cells were similar to control values. Glutamate treatment sharply lowered GR content by 80%. DMSO pretreatment did not protect GR protein content from glutamate. In contrast, ZYC-3 nearly prevented GR depletion during subsequent glutamate challenge (Figure 5B, 5C).

Antioxidant Response Element Activation

Activation of antioxidant response elements (ARE) was determined as a measure of response to oxidative stress 24 hours post 5mM glutamate challenge. No statistically significant differences in ARE activity among control, DMSO (vehicle control), and ZYC-3 pretreated (drug control) cells were detected, although a strong trend toward

increased ARE activity was seen in the DMSO-pretreated cells. Glutamate challenge activated ARE 2.4-fold versus control. Interestingly, ZYC-3 pretreatment prevented glutamate-induced activation of ARE (Figure 6).

Mitochondrial Membrane Potential and Cell Morphology

Mitochondrial membrane potential ($\Delta\Psi_m$) was assessed to determine the ability of ZYC-3 to avert $\Delta\Psi_m$ depolarization in the face of glutamate challenge. Confocal image analysis of mitochondria in control (Figure 7A), DMSO (not shown), and ZYC-3 (not shown) treated cells showed no significant difference in JC-1 uptake (Figure 7A). Glutamate challenge caused a 62% reduction in JC-1 uptake (compare Figure 7A and 7B). $\Delta\Psi_m$ depolarization was attenuated with ZYC-3 pretreatment, with JC-1 uptake equivalent to 80% of control cells and double that of glutamate-insulted cells (Figure 7C and 7D). Morphologically, insulted soma appeared rounded, with simplification and loss of processes and neurites, and exhibited pyknotic chromatin. Control and ZYC-3 pretreated cells maintained multiple processes and neurites typically seen in healthy RGC-5 cells, and lacked apoptotic nuclear appearance.

Discussion

Neuroprotection remains a promising approach to saving vision in glaucoma patients. Although glaucoma is the second leading cause of vision loss in the United States, no single neuroprotective strategy is ubiquitously accepted. In this study, RGC-5 cells were subjected to 5mM L-glutamtic acid for 24 hours to study cellular responses to overwhelming oxidative stress (Kumar et al., 2005). In addition, the potentially cytoprotective effects of pretreatment with the synthetic estrogen analogue were studied. Pro-oxidant mechanisms of glutamate-induced RGC-5 death, and antioxidative neuroprotection by the non-feminizing estrogen analogue ZYC-3 are demonstrated. Glutamate exposure attenuated cystine uptake, severely depleted GSH reserves, and inactivated and depleted the crucial antioxidant enzymes GSH peroxidase and GSH reductase, leading to activation of the antioxidant response element, mitochondrial depolarization, and loss of cell and nuclear morphology. ZYC-3 pretreatment at least partially if not completely prevented all of these detrimental effects, and even caused an overshoot of GSH peroxidase and GSH reductase activities to levels well above the glutamate-free control cells. These are the first reports of the neuroprotective effects of ZYC-3 against glutamate –induced disruption of the glutathione antioxidant defense system and $\Delta\Psi_m$.

A less obvious explanation of ZYC-3's neuroprotective effects lies in the direct free radical scavenging structure of estrogens (Reviewed by Prokai et al, 2007). The process is thought to cause an interruption of free-radical chain reactions such as lipid

peroxidation (Prokai et al., 2006). However, such chain terminating events generate a radical product whose fate must be determined for a complete understanding of ZYC-3's antioxidant action. Phenoxyl radicals, such as those produced by quenching of free radicals by ZYC-3 or natural human estrogens, can be regenerated through glutathione-dependent free radical reductase (McCay et al., 1989). Therefore a continuous cycle that scavenges free radicals can be maintained by the antioxidant action of ZYC-3. The interdependence of estrogens on GSH is highlighted by their documented synergistic efficacy in *in vitro* models of neurodegeneration (Green et al., 1998; Gridley et al., 1998). Early observations supported a model in which glia take up cystine via x_c^- for production of GSH that is subsequently released to supply neurons (Reviewed by McBean, 2002). Whereas glia may be important for providing GSH to neurons, recent studies suggest that hippocampal and cortical neurons, and retinal ganglion cells in culture are able to efficiently transport cystine and maintain adequate GSH levels (Allen et al., 2001; Danbolt et al., 2001; Shanker et al., 2001; Dun et al., 2006).

Although glutamate-cystine antiporters were once thought to be expressed only in Mueller cells, current data clearly show that both primary retinal ganglion cells and RGC-5 cells utilize these antiporters to maintain homeostatic uptake of these amino acids critical to GSH production (Kato et al., 1993; Pow et al., 2001; Dun et al., 2006). Accordingly, elevated extracellular concentrations of glutamate have been hypothesized to inhibit and reverse function of the x_c^- system in RGCs, so that glutamate enters cells in exchange for cytosolic cysteine Perturbation of the glutamate/cystine antiporter system (x_c^-) may then deplete intracellular cysteine that is critical for the synthesis of glutathione

and maintenance of intracellular redox status (Dun et al., 2006; Allen et al., 2001; Danbolt et al., 2001; Shanker et al., 2001). Glutamate challenge of RGC-5 cells resulted in significant inhibition of x_c^--mediated cystine uptake that culminated in more than 30% cell death. However, depletion of cysteine was attenuated by pretreatment with ZYC-3, providing complete neuroprotection. Curiously, the steady state x_c protein content in cells pretreated with ZYC-3 was significantly greater than in glutamate treated samples, implicating an estrogen receptor-independent pathway of gene expression or protection of x_c- protein from degradation in glutamate cytotoxicity. Given its lipophilicity as an estrone derivative, ZYC-3 may be concentrating in the lipid rich cell membrane and functioning as a localized antioxidant, protecting membrane bound x_c^- (Prokai et al., 2006; Simpkins et al., 2005).

Although maintenance of intracellular cysteine is crucial for GSH synthesis and protection against lethal oxidative burdens, this process is rate limited by glutamate cysteine ligase (GCL) activity. Similar to the x_c^- system, current observations indicate that retinal GCL is expressed in Muller cells and is responsive to oxidative stresses via an antioxidant response element. GCL mRNA levels have also been correlated with the degree of GSH depletion (Agardh et al., 2006; Lu et al., 1999). In light of ZYC-3 enhanced cystine uptake and x_c^- content, complete protection against glutamate challenge, and the presence of an antioxidant response element in the GCL gene, we examined protein levels of the GCL enzyme (reviewed by Dickinson et al., 2004; Montano et al., 2004; Levonen et al., 2004). The oxidative burden induced by glutamate treatment resulted in no appreciable reduction in GCL levels, but GCL protein content was

enhanced with ZYC-3 pretreatment as demonstrated by Western blot analysis. Although GCL and GSH content were directly correlated, pretreatment with ZYC-3 increased GSH content by four-fold in glutamate challenged RGC-5 cells, yet the increased GSH content was at most 30% of that found in control RGC-5 cells. Although this increase in available GSH was modest, it was associated with complete neuroprotection against glutamate-induced cytotoxicity.

The participation of endogenous and mimetic peroxidases has been suggested to be protective under conditions of oxidative stress in glaucomatous models (Kortuem et al., 2000; Castagne et al., 2000). Due to presence of an antioxidant response element in the GPx gene, we examined content and activity of GSH-mediated antioxidant defenses in our experimental paradigm. Increased GPx protein content and activity paralleled ZYC-3 induced cytoprotection agianst glutamate-induced toxicity. This phenomenon may be an exploitation of the unique characteristics of RGC resistance and susceptibility to injury that has been prevented with minimal improvements in GSH available for use by GPx. A similar sequence of events has been described in an elevated intraocular pressure (IOP) model of glaucoma. At six weeks of IOP elevation GSH content was appreciably decreased; conversely, GPx activity was increased at 10 weeks of IOP elevation, whilst superoxide dismutase (SOD) and catalase (CAT) activities fell as early as three weeks after IOP elevation (Moreno et al., 2004). Incidentally, SOD and CAT activities were undetectable in RGC-5 cells. ZYC-3 treatment appeared to improve the utilization of available GSH and was accompanied by a greater than 50% increase in GR content and GR-mediated reduction of oxidized glutathione disulfide. The presence,

activity, and role of GR in glaucomatous retinas have not been established yet. Increased GPx activity is suggestive of enhanced utilization of available GSH by GPx followed by rapid redox cycling of glutathione by GR; ZYC-3 appears to enhance this redox biochemistry (Moreno et al., 2004; Kumar et al., 2005). As described above these steps are crucial to the maintenance of GSH for utilization by glutathione dependant free-radical reductase.

Glutmate-cysteine ligase and GPx are key glutathione pathway enzymes necessary for maintenance of intracellular redox status in healthy cells. In addition to GCL and GPx, SOD and CAT are under the regulation of antioxidant response elements and are critical components in combating the oxidative burden that develops in glaucoma (reviewed by Lyakhovich et al., 2006; Yildrim et al., 2005; Ferreira et al., 2004; Geiger et al., 2002). Our data clearly demonstrated that glutamate challenge of RGC-5 cells induces an overwhelming oxidative burden, produceing sustained activation of AREs that persist at least 24 hours after glutamate treatment. Activation of AREs was completely inhibited by ZYC-3 pretreatment, but accompanied by increased levels and activity of x_c^-, GCL, GPx, and GR. It should be noted that natural estrogens are known to upregulate the expression of antioxidant enzymes, but the present studies demonstrated for the first time with a synthetic non-feminizing estrogen analogue has similar effects (Borras et al., 2005). Working in concert with the direct antioxidant effects of ZYC-3, the induction of these genes and activity of these enzymes effectively inhibited the glutamate-induced cytotoxicity of RGC-5 cells and activation of AREs.

It is now generally accepted that RGC apoptosis is critically dependent on

oxidative redox state and is accompanied by mitochondrial depolarization (Tezel 2006; Izzoti et al., 2006; Ohia et al., 2005; Reviewed by Kumar et al., 2007; Carelli et al., 2004; Tatton et al., 2001; Abu-Amero et al., 2006). The severe depolarization of $\Delta\Psi_m$ we observed with glutamate challenge was prevented with ZYC-3 pretreatment. Mechanistic models indicate that estrogens intercalate into cell membranes where they block lipid peroxidation reactions, and are in turn recycled via glutathione. Such a mechanism would be particularly germane in mitochondria where function is directly dependent on the impermeability of the inner membrane, as is the case with highly energy dependant RGCs, and where GSH must be maintained at high concentrations (reviewed by Simpkins et al., 2005). Furthermore, signal transduction pathways that are evolved by estrogens are also important regulators of mitochondrial function and may mediate the indirect effects of estrogens on mitochondria. For example, estrogens can activate the Akt pathway (Singh 2001; Honda 2000&2001; Zang 2001) which can in turn phosphorylate the pro-apoptotic protein Bad. Phosphorylation inactivates Bad and prevents Bax-induced release of cytochrome c from mitochondria. Furthermore, estrogens have been shown to affect the concentration and localization of anti-apoptotic proteins that exert their effects through the maintenance of $\Delta\Psi_m$ in the face of cytotoxic insults (References from Singh et al., 2006). These effects are presumed to be estrogen receptor dependent and independent, but the precise mechanisms are still unknown.

These studies address glaucoma as a disorder of RGC cell body damage, but it has also been hypothesized that glaucomatous vision loss is a consequence of RGC axon damage. These two theories can be reconciled by a shared pathway of neuronal loss

caused by ischemia-induced oxidative burdens (Cioffi et al., 2004 & 2005; Schlamp et al., 2006). It is still debatable whether RGC loss begins as a result of axonopathy or primary somaopathy. Nevertheless, the real phenotype of the disease is the apoptotic loss of retinal ganglion cells. The experimental observations presented here demonstrate the efficacy of the synthetic estrogen analogue ZYC-3 against glutamate-induced cytotoxicity and key mechanisms of action within the glutathione pathway of redox modulation. Further, we demonstrated that ZYC-3 protects mitochondrial membrane potential. Taken together these data provide evidence that non-feminizing estrogen analogues can be effective neuroprotectants in glaucoma and that they bolster the endogenous antioxidant defenses of retinal ganglion cells.

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Figure Legends

Figure 1. Effect of ZYC-3 treatment on cystine uptake via glutamate/cystine antiporters (x_c^-) and immunoblot analysis of x_c^- content in glutamate-challenged RGC-5 cells.

^{35}S-cysteine uptake (panel A) was assessed after 24 h glutamate exposure, or a comparable glutamate-free incubation. The ZYC-3 groups were pretreated with 1μM ZYC-3 for 6 h before addition of 5mM glutamate. ^{35}S-Cystine uptake is expressed as counts per minute/(mg/ml protein/% cell viability). Values are means ± SEM. * $p<0.05$ versus untreated control cells; ** $p<0.05$ versus glutamate-treated cells. Immunoblots (panel A) were performed to assess the steady-state x_c^- protein content following 24-hour glutamate challenge in each RGC-5 treatment group. Panel C: densitometric quantitation of immunoblot band density.

Figure 2. Effect of glutamate challenge +/- ZYC-3 pretreatment on glutamate cystine ligase (GCL) content

To determine if GCL content may be affected by glutamate challenge and cytoprotection with ZYC-3, GCL catalytic (GCLC) and modulatory (GCLM) subunits were detected at steady state (panel A). RGC-5 cells were pretreated with 1μM ZYC-3 for 6 h followed by 5mM glutamate for 24h without the removal of ZYC-3 (A). Densitometric quantitation of band densities of GCLC (panel B) and GCLM (panel C) are presented.

Figure 3. GSH content in RGC-5 cells pretreated with ZYC-3 followed by glutamate challenge.

GSH content was measured in the same groups as in figures 1 and 2 and normalized to protein concentration (mg/ml) and percent cell viability. Values are means ± SEM. *$p<0.05$ versus untreated control cells; ** $p<0.05$ versus glutamate-challenged cells.

Figure 4. Effects of glutamate exposure and ZYC-3 pretreatment on glutathione peroxidase content and activity in glutamate insulted RGC-5 cells.

Glutathione peroxidase activity (panel A) is normalized to protein concentration (mg/ml) and percent cell viability. *$p<0.05$ versus untreated control cells. Immunoblot (panels B, C) demonstrated that 24-hour glutamate suppressed GPx protein content normalized to β-actin content. This effect was partially reversed by ZYC-3 pretreatment but not by its vehicle DMSO. Panel B: representative immunoblot; panel C: densitometric quantitation of band density.

Figure 5. Effects of glutamate exposure and ZYC-3 pretreatment on glutathione reductase activity in RGC-5 cells.

Glutathione reductase (GR) (panel A) is normalized to protein concentration (mg/ml) and percent cell viability. *$p<0.05$ versus untreated control cells; ** $p<0.05$ versus glutamate

challenged cells. Panel B: representative immunoblot of GR; Panel C: densitometric quantitation of band density.

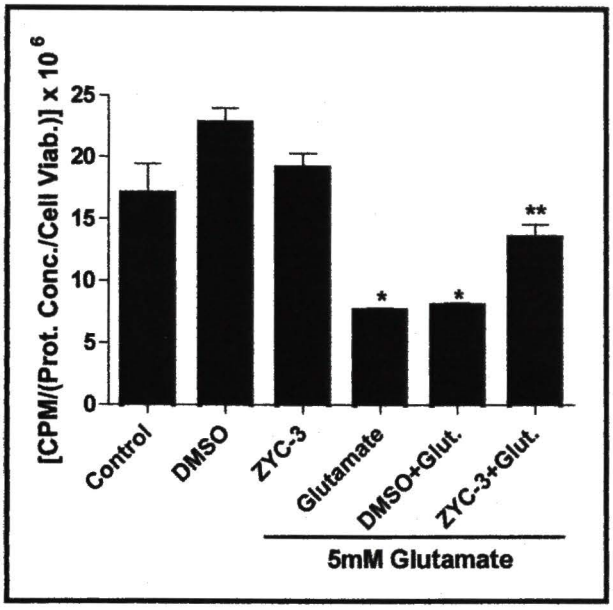
Figure 6. Activity of antioxidant response elements.

Global antioxidant response element activation was assessed by gene transfection using an ARE expression vector as described in the methods. Values are means ± SEM from three experiments per group. * $p<0.05$ versus untreated control cells; ** $p<0.05$ versus glutamate treated cells.

Figure 7. Glutamate –induced mitochondrial membrane potential depolarization and protection with ZYC-3.

Panes A-C: mitochondrial membrane potential was determined with JC-1 dye as described in methods. Aggregation of JC-1 mitochondria was visualized using confocal microscopy, and red:green fluorescence image analysis was used to determine the ratio of healthy:depolarized mitochondria. Panel D: Red/Green fluorescence ratios (means ± SEM from 3 experiments) are shown for control, glutamate-challenged, and ZYC-3 pretreated glutamate-challenged cells. * $p<0.05$ versus untreated control cells ; ** $p<0.05$ versus glutamate treated cells.

Figures



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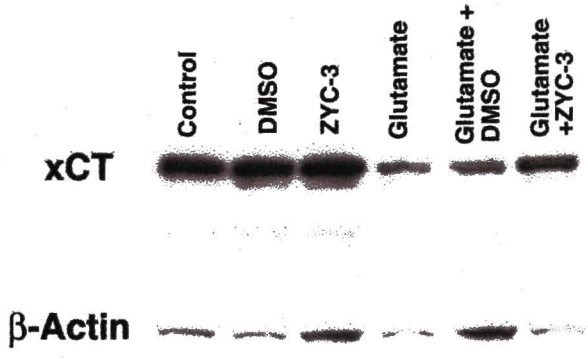

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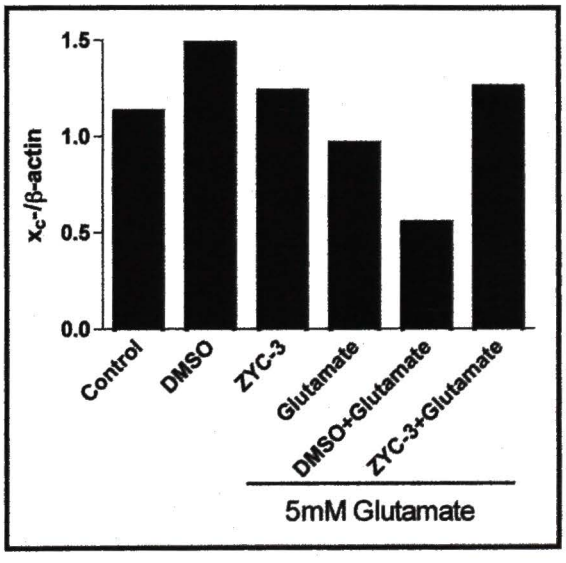

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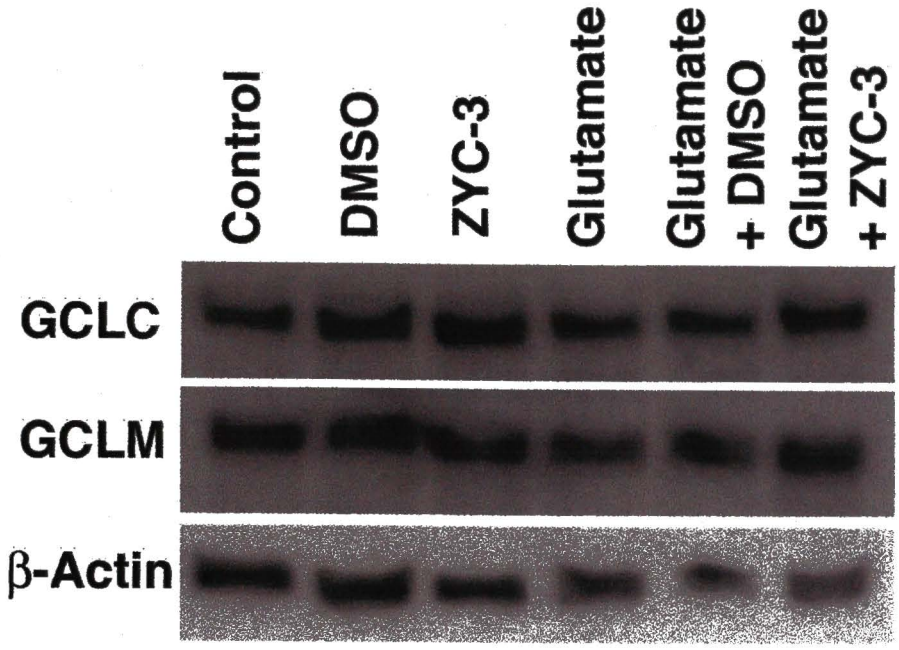

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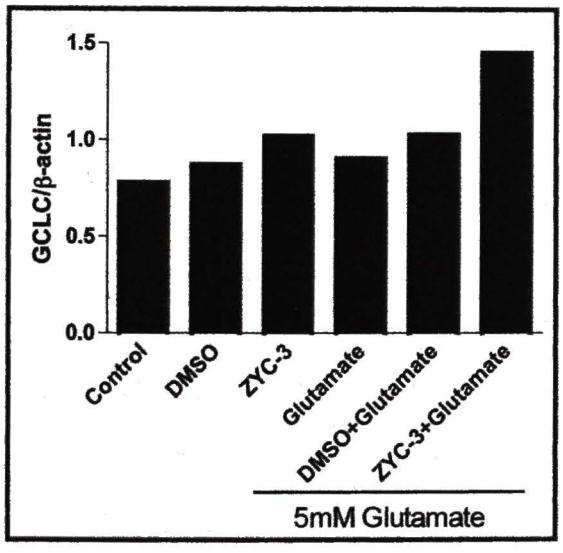

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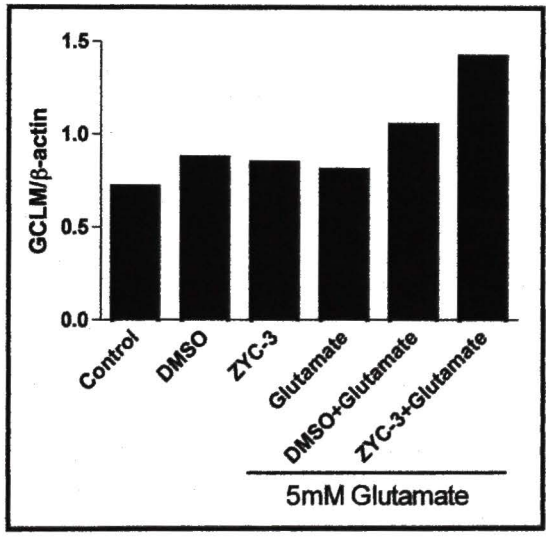

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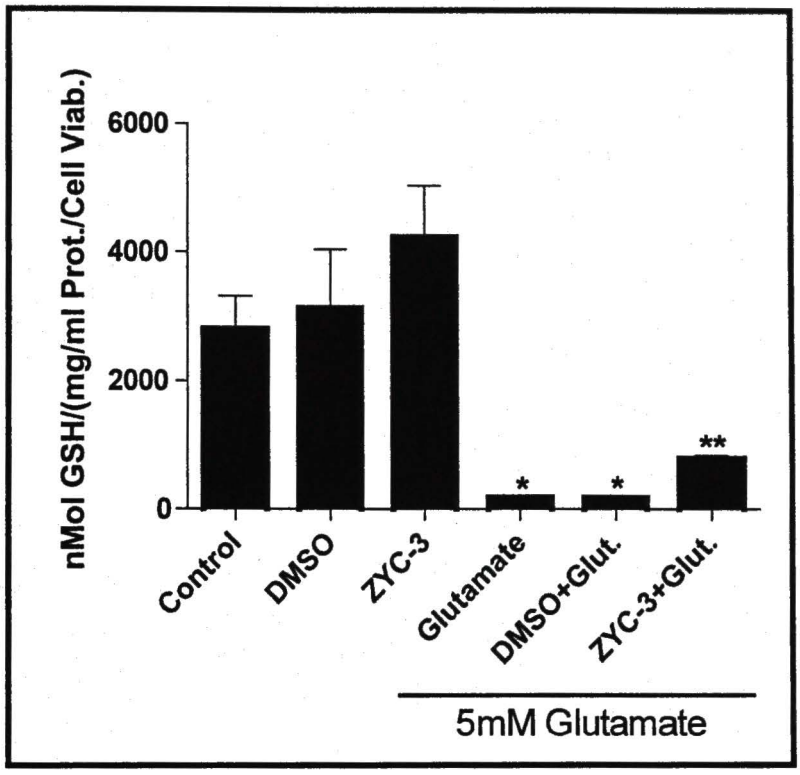
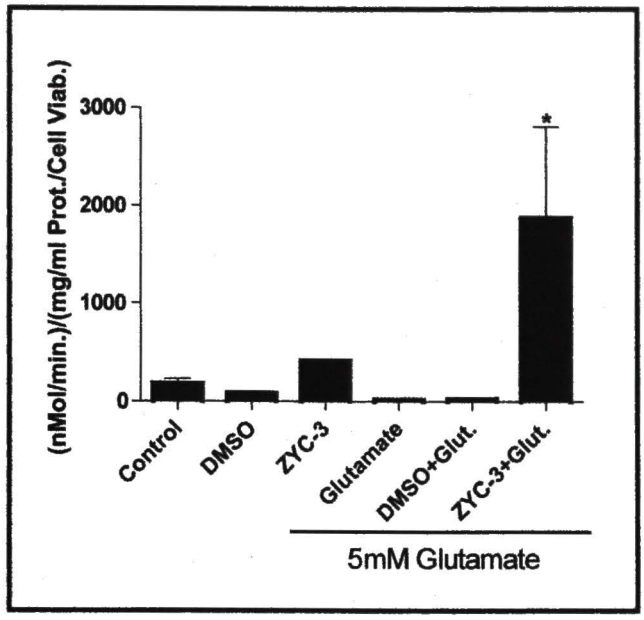

nMol GSH/(mg/ml Prot./Cell Viab.)
6000
4000
2000
0
*
*
**
Control
DMSO
ZYC-3
Glutamate
DMSO+Glut.
ZYC-3+Glut.
5mM Glutamate

Figure 3



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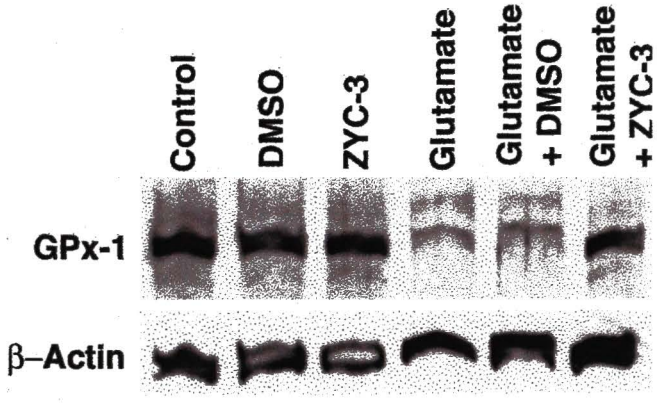

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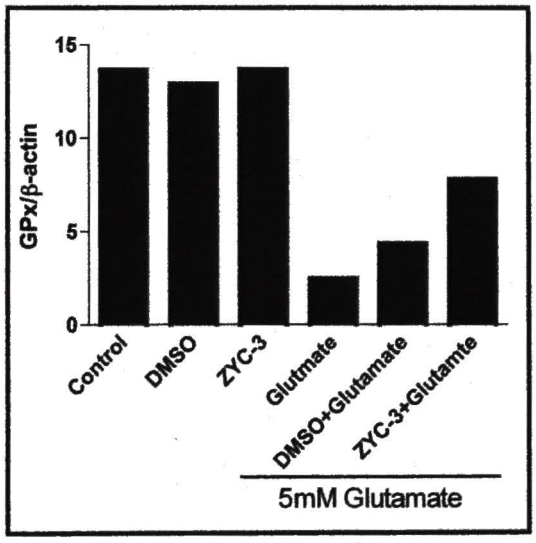

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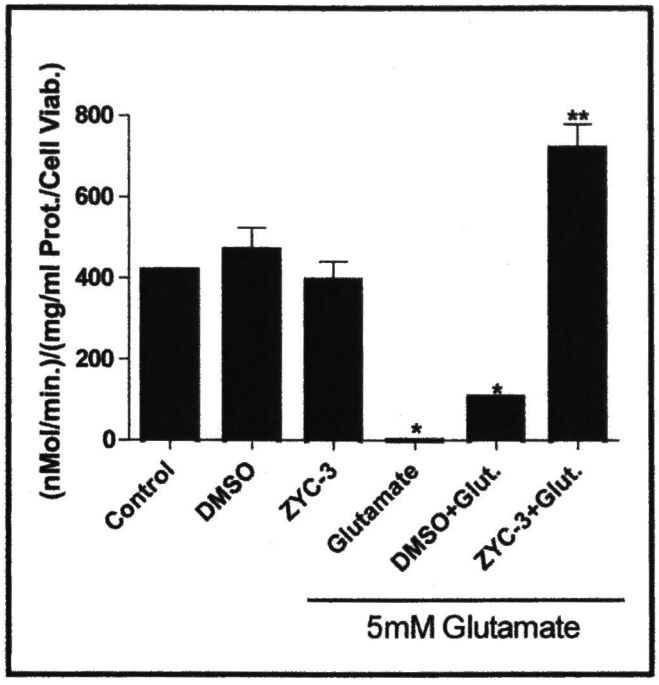

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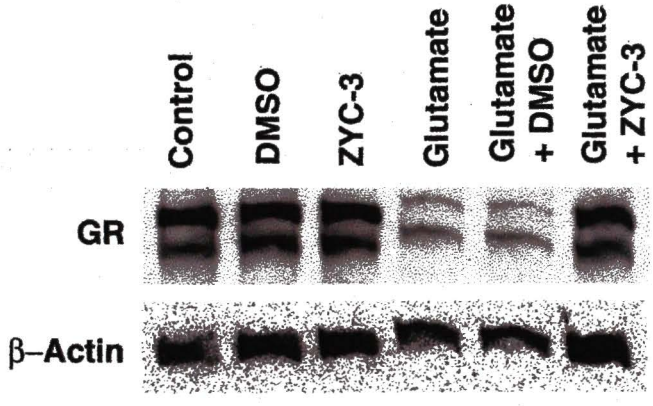

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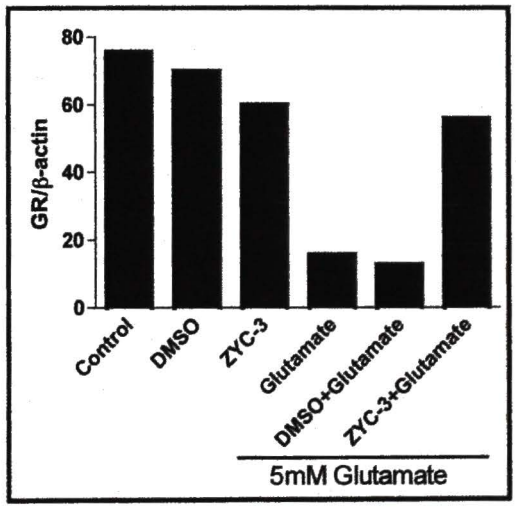

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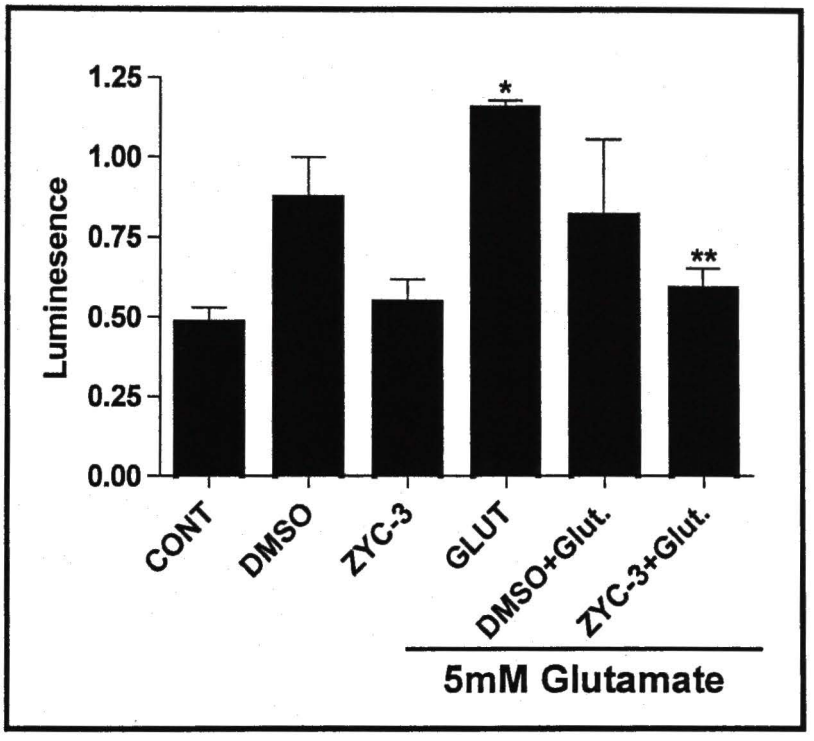
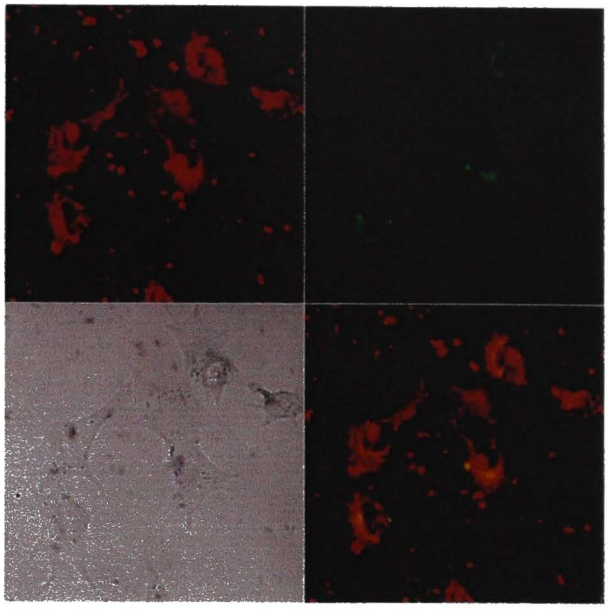

1.25
1.00
0.75
0.50
0.25
0.00
Luminesence
*
**
CONT
DMSO
ZYC-3
GLUT
DMSO+Glut.
ZYC-3+Glut.
5mM Glutamate

Figure 6



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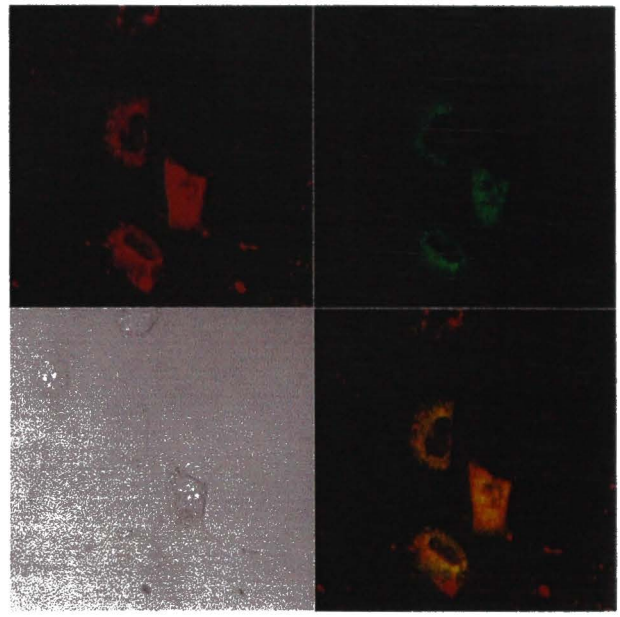

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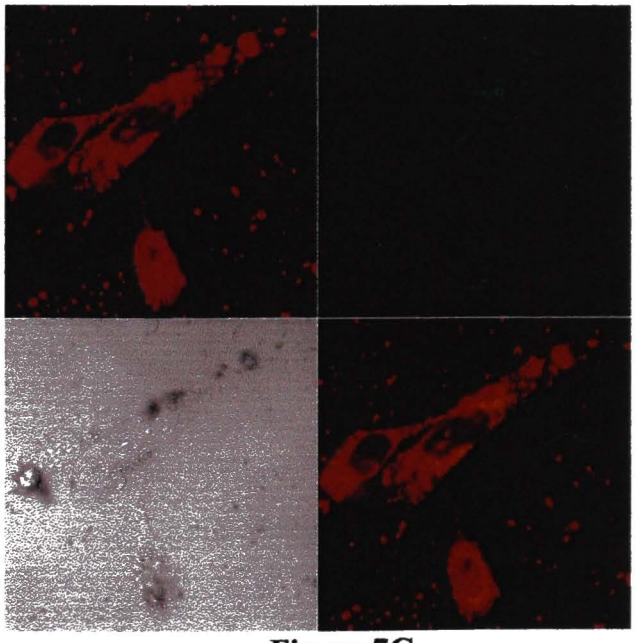

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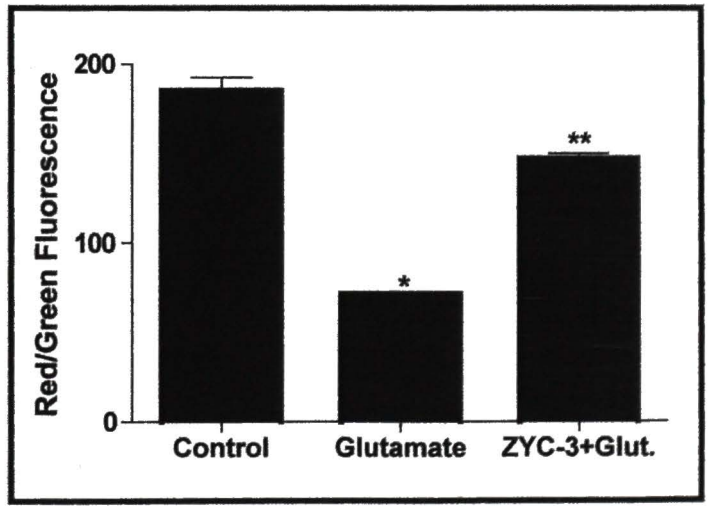

Figure 7D

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

Loss of retinal ganglion cell soma and axons are the cause of vision loss in glaucoma. Several etiologies of glaucoma have been proposed, but oxidative stress is thought to be a common underlying mechanism by which ganglion cells die. Estrogens are effective antioxidants and neuroprotectants in a wide variety of *in vitro* and *in vivo* models of neurodegenerative disease, but this has been shadowed by the deleterious outcomes of hormone therapy (HT) from the Women's Health Initiative study. These outcomes present a hurdle to application of HT and natural estrogens as neuroprotectants, paving the way for the consideration of non-feminizing (receptor independent) estrogen analogues as alternatives. From this perspective, we examined the efficacy of more than a dozen estrogens and synthetic non-feminizing estrogen-like compounds to determine if they may be neuroprotective. Glutamate-induced RGC-5 rat retinal ganglion cell cytotoxicity was used as an *in vitro* model of glaucomatous retinal ganglion cell death for these studies.

The synthetic estrogen analogues (ZYC-1, ZYC-3, ZYC-10) and 17β-estradiol (used as the prototype) were determined to be effective RGC-5 neuroprotectants. Compounds ZYC-1 and its enantiomer ZYC-10, containing an additional double bond in the steroid C-ring of 17β-estradiol, had similar (ZYC-1) or modestly reduced (ZYC-10)

affinity for estrogen receptors as compared to the parent estrogen. The addition of an adamantyl group to the C2 position of the A ring of estrone in ZYC-3 abolished its binding to estrogen receptors. RGC-5 cells and rat retinas were shown to predominantly express estrogen receptor-α (ER-α) with minimal detectable levels of estrogen receptor-β (ER-β). The affinity of these compounds for estrogen receptors was as follows: ZYC-3 < ZYC-10 < ZYC-1. Glutamate treatment resulted in 50-60% RGC-5 cell death with respect to untreated cells. 17β-estradiol and the three estrogen analogues (0.5 to 1.0μM) protected the RGC-5 cells against glutamate-induced cytotoxicity. The efficacy of neuroprotection by the estrogen analogues was as follows: ZYC-3 > ZYC-1 > ZYC-10. EC_{50} values for inhibition of TBAR levels were as follows: ZYC-3 > ZYC-10 > ZYC-1. Furthermore, these compounds worked independent of estrogen receptors, as inclusion of 100 nM ICI 182,780 did not inhibit their neuroprotective properties against glutamate insult. These compounds provide effect neuroprotection via mechanisms independent of the classical estrogen receptors. The data support the hypothesis that estrogen analogues may be useful in the treatment of neurodegenerative diseases, particularly in neuroprotection of retinal ganglion cells in ocular pathologies such as glaucoma.

We then examined the neuroprotective mechanisms of the novel estrogen analogue ZYC-3, again using glutamate challenge of RGC-5 cells as an *in vitro* model of glaucomatous retinal ganglion cell death. In glaucoma, elevated extracellular glutamate concentrations have long been presumed to induce an overwhelming oxidative stress by inhibiting cystine uptake that is critical for glutathione (GSH) dependent antioxidant

defense mechanisms. We demonstrated that cystine uptake via glutamate/cystine antiporters, rate limiting glutamate cysteine ligase activity, and intracellular GSH content were significantly reduced with glutamate challenge, but was enhanced with ZYC-3 pretreatment. Furthermore, GSH utilization by GSH peroxidase and cycling by GSH reductase were inhibited with glutamate challenge, but promoted with ZYC-3 pretreatment. These findings were supported by activation of antioxidant response elements with glutamate treatment, but reduced by antioxidant and neuroprotectant ZYC-3. In the face of glutamate challenge, ZYC-3 also enhanced GSH pathway antioxidant defenses and protected mitochondrial membrane potential ($\Delta\Psi_m$). In addition, ZYC-3 pretreatment upregulated activation of the Akt, Erk, p90, p53, and S6 prosurvival pathways, and downregulated the activation of the p38 pro-apoptotic pathway. The data support the hypothesis that ZYC-3 may function as an antioxidant and mitochondrial stabilizer, promoting cell survival.

The approaches we employed allowed us to elucidate the efficacy and mechanisms of neuroptrotection of non-feminizing estrogen analogues in an *in vitro* model of glaucoma. Data produced from *in vitro* neuroprotection experiments are handicapped by a lack of metabolic and drug distribution evidence. Establishing high throughput *in vivo* models of neurodegenerative disease, akin to our *in vitro* model of glaucoma would facilitate. To advance estrogen-mediated vision protection for glaucoma, two critical objectives should be achieved: 1) novel neuroprotective compounds of greater efficacy must be created, 2) the pharmacokinetics and pharmacodynamics of these novel neuroprotective compounds should be investigated *in vivo* models of glaucoma.

Medicinal, Computational, and Synthetic chemistry, in collaboration with pharmacology and cell biology, could produce highly evolved medications. We determined that cholesterol backbones containing phenols/quinols directly and effectively attenuated lethal oxidative burdens. It would be possible to design compounds without significantly different molecular weight or strucutres from native cholesterols that yield fold-level differences in efficacy. Together these approaches have the opportunity to irradicate neurodegenerative diseases.

We showed for the first time that non-feminizing estrogen analogues protect RGC-5 retinal ganglion cells against a glutamate-induced cytotoxic insult. Furthermore, we demonstrated that the estrogen analogue ZYC-3 protects intracellular glutathione content and glutathione dependent antioxidant defense mechanisms to effectively stabilize mitochondrial membrane potential in insulted RGC-5 cells. No other drug has been shown to be as effective a neuroprotectant in an *in vitro* model of glaucoma. Glaucoma is currently treated by reduction of intraocular pressure (IOP), but maintenance of IOP within the accepted reference range does not prevent the progression of visual field deterioration in all patients. Concomittant administration of an IOP lowering drug with a neuroprotectant, such as ZYC-3, has the potential to save vision in patients who are not optimally treated. This approach extended to other neurodegernative diseases has the potential to irradicate them.

APPENDIX A

Oxidative Stress In Glaucoma: A Burden of Evidence

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Introduction

Glaucoma is an insidiously progressive optic neuropathy which affects nearly 90 million people worldwide, and is the leading cause of irreversible blindness (Tomarev, 2001; Wang et al., 2001; Golderg, 2000). It is a neurodegenerative disorder of aging and occurs with increasing prevalence after the age of 40 (Quigley, 1998; Alward et al., 1998). Fundoscopic examination of the glaucomatous retina reveals characteristic excavation of the optic nerve head with concomitant visual field defects (Van Buskirk et al., 1992). Although most cases of glaucoma are associated with prolonged latent periods of elevated intraocular pressure, known as primary open angle glaucoma-POAG, approximately one third of glaucoma patients present with normal intraocular pressure (IOP). Furthermore, a significant number of cases progress despite clinically measurable reductions of IOP (Flammer et al., 2002). In spite of this paradox of pressure, the final common pathway of vision loss remains the apoptotic loss of retinal ganglion cells (RGC) with subsequent degeneration of the optic nerve head (ONH) (Quigley et al., 1995). Such observations have perpetuated research to determine the etiology of and risk factors for developing glaucoma.

In addition to elevated IOP (Krakau, 1981; Bonomi et al., 2001), retinal ischemia (Findl et al., 2000; Butt et al., 1997), and nutritional status (Veach et al., 2004), oxidative stress has been proposed as an etiologic factor in the pathophysiology of glaucomatous RGC death (Ferreira et al., 2004; Izzotti et al., 2003; Levin et al., 1996; Neufeld et al.,

1999). As central nervous system neurons, RGCs are especially susceptible to oxidative stress because of their tremendous oxygen consumption and high proportion of polyunsaturated fatty acids. But unlike cortical neurons, the RGCs are vulnerable to potentially high levels of oxidative stress as a result of direct light exposure (Mainster et al., 1987; Organisciak et al., 1998). Oxidative stresses are induced through the formation of multiple reactive oxygen species (ROS) including superoxide (O_2-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (·OH) that can initiate and propagate free radicals. The resultant free radicals can be terminated by antioxidant defense systems including the enzymes glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), and nonenzymatic components such as glutathione (reduced glutathione-GSH, oxidized glutathione-GSSG), thioredoxin, and antioxidant vitamins (Erden et al., 1984; Richer et al., 1998). The net oxidative burden between these opposing pro and antioxidant systems is the oxidative stress that damages lipids, protein, and DNA culminating in cell death (Valencia, 2002).

Despite the apparent role of oxidative stress in the glaucomatous death of retinal ganglion cells, developing neurotrophin-dependent RGCs require ROS as part of the signaling process for cell death, analogous to that seen in neurotrophin-dependent neurons (Estevez et al., 1998; Greenlund et al., 1995). Therefore, here we examine studies that attempt to provide evidence for and elucidate the role of oxidative stress in glaucoma.

Human Studies

There are two predominant schools of thought that may be used to explain the formation of oxidative free radicals that can result in RGC death. The vascular theory is based on ischemia-induced production of free radicals, due to compromised blood flow in retinal vessels, with subsequent oxidative damage to RGC axons (Flammer et al, 1994). The mechanical pressure theory is based on elevation of IOP inhibiting retrograde neurtrophin support of RGCs (Quigley et al., 1999). These theories are reconciled because the final common pathway of RGC death in both schools of thought is oxidative stress.

The propinquity of the anterior and posterior chambers of the eye makes changes in one critical to the biophysical state of the other. Reduced aqueous humor outflow or increased aqueous humor production results in additional mechanical force, and is the basis for the elevation of intraocular pressure that culminates in RGC loss. A recent study examined the antioxidant status of the aqueous humor of POAG patients. Aqueous humor was taken at the time of surgery from glaucoma patients and control cataract patients with no history of, or current glaucoma. The level of water-soluble antioxidants (glutathione, ascorbate, tyrosine) in the aqueous humor of glaucoma patients was found to be approximately half of that determined in the control group. Catalase (CAT) activity was no different in the patient and control groups, but superoxide dismutase activity (SOD) activity was nearly 60% greater in the glaucomatous group as compared to control

patients. Glutathione Peroxidase (GPx) activity was nearly three times greater in the aqueous humor of glaucoma patients with respect to controls (Ferreira et al., 2004). The enzymatic results demonstrate that an exaggerated oxidative burden in glaucoma patients induces the activity of mechanisms designed to combat oxidative stress.

As discussed above, POAG is the predominant form of glaucoma and can be attributed to reductions in outflow of aqueous humor via the trabecular meshwork in the anterior chamber of the eye. Izzotti et al. (2003) were able to demonstrate an oxidative stress dependent accumulation of trabecular meshwork region DNA damage in POAG patients. These findings correlated with IOP elevations and with visual field loss (Izzotti et al, 2003). The work of Sacca et al. (2005) confirmed that oxidative DNA damage is exaggerated in the trabecular meshwork of POAG patients. In this study oxidative DNA damage of human trabecular meshwork cells (HTM) was five-fold higher in glaucoma patients as compared to controls. Furthermore, oxidative damage of HTM DNA did not show statistically significant correlations with the age of the patients or the duration of disease. Visual field testing demonstrated increasing defects with elevated IOP peaks as well as with increasing HTM oxidative DNA damage (Sacca et al., 2005).

GSH is critical to combating oxidative stress, and changes in its levels may indicate variances in oxidative burden (Pompella et al., 2003). Glutathione levels may also be influenced by age and gender. It has been shown that more than half of healthy elderly patients have low levels of GSH and reduced capability to cycle GSSG to GSH

(Matsubara et al., 1991; Lang et al., 1992; Erden-Inal et al., 2002). Furthermore, it is known that men have higher levels of GSH than women. In spite of these age and gender based differences, Ghergel et al. (2005) recently demonstrated that glaucoma patients have lower serum GSH and total glutathoine (t-GSH) levels as compared to age-matched controls. One explanation offered by the authors is that glaucoma patients may have lower circulating levels of cysteine, the major determinant in t-GSH synthesis. Curiously though, GSSG levels were similar in the control and patient groups. It was suggested that this may have been due to defective redox cycling of GSSG to GSH, implying that this mechanism plays a role in reduced antioxidant defenses of glaucoma patients (Gherghel et al., 2005). It has also been suggested that low levels of circulating GSH, enhancing oxidative stress, may also reduce the availability of nitric oxide (NO). Low levels of NO may in turn inhibit relaxation of the ciliary muscle and trabecular meshwork resulting in elevation of IOP, perpetuating the cycle of elevated IOP and resulting in a higher rate of oxidative reactions (Wiederholt et al., 1994). These findings implicate reduced systemic levels of GSH in glaucoma patients, weakened antioxidant defenses, and the elevation of oxidative stress in glaucoma patients.

In another study of serum markers of oxidative stress, the activities of myeloperoxidase (MPO, an antioxidant defense enzyme), catalase (CAT), and plasma levels of malondialdehyde (MDA) were assessed. MDA is a lipid peroxidation end product that is generally accepted as a maker of oxidative stress. There was no evidence

of an association between POAG and alterations of systemic antioxidant activities of MPO and CAT. These results support those of Ferreira's work on antioxidant enzyme activities in the aqueous humor of POAG patients. However, a statistically significant relationship was found between plasma MDA levels and POAG. MDA levels were determined to be more than two-fold greater in the serum of POAG patients as compared to healthy controls. Patients with chronic comorbidities such as diabetes mellitus, systemic hypertension, inflammatory arthritis, and heart failure, which may also have pathophysiologic mechanisms dependent upon oxidative stress were excluded. This study exemplifies the role of oxidative stress in the pathogenesis of RGC death and glaucomatous vision loss, but also supports the potential use of plasma MDA as a serum marker of oxidative stress in POAG patients (Yildirim et al, 2005).

Each of the studies presented in this section were designed with different approaches to examine the potential role of oxidative stress in glaucoma patients. The work of the Gherghel et al. (2005) and Yildirim et al. (2005) examined systemic marker of oxidative stress independently, yet both groups demonstrated that an increased oxidative burden may be assessed through enzymatic and non-enzymatic indicators in serum. The work of Sacca et al. (2005) and Izotti et al. (2003) demonstrated that oxidative stress in the anterior chamber of the eye may be overwhelming enough that the trabecular meshwork cells, the critical gateway to the draining of aqueous humor and regulation of IOP, can directly damage the DNA of these cells and potentially propagate

the pathophysiologic mechanisms of glaucoma. The work of Ferreria et al. (2004) illustrates that oxidative stress in the eye is significant enough to elicit compensatory activation of antioxidant defense enzymes. The mechanisms of RGC death have been debated based on the growing body of data from *in vitro* and *in vivo* studies, but the current evidence from human studies suggests that oxidative stress may be the impetus for the final common pathway of RGC death and progressive vision loss in glaucoma. These studies demonstrate that oxidative burdens are increased outside of the retina and that oxidative stress in the retina may be an ocular manifestation of systemic disease.

Animal Studies

Experimental approaches designed to elucidate the role of oxidative stress in studies utilizing human subjects may be limited by ethical concerns, as well as by our ability to study the pathogenesis of glaucoma within the bounds of ethically acceptable methods. Animal based studies circumvent many limitations and allow investigators to further control potentially confounding variables in the study of oxidative stress in glaucoma. They also facilitate our ability to understand cellular events influenced by elevated IOP, neurotrophin deprivation, and other potential risk factors for glaucoma. Such studies provide further compelling evidence of the role of oxidative stress in the pathogenesis of RGC death and vision loss in glaucoma.

Utilizing intracameral injections of 1% hyaluronic acid (HA) to elevate the IOP in rats, Moreno et al. (2004) studied retinal oxidative stress induced by high IOP (Benozzi et al, 2002). Chronic elevation of IOP in this model demonstrated significant loss of RGCs and electroretinogram activity (Moreno et al., 2002). To determine the potential for oxidative stress and the response of antioxidant defenses, the enzymatic activities of SOD, GPx, and CAT were assessed along with GSH, thiobarbituric acid-reactive substances (TBARS), and melatonin levels from retinal homoginates. SOD activity in elevated IOP eyes declined to half of that in the fellow eye. CAT activity declined to approximately 75% of control at three weeks post IOP elevation. A compensatory 25% increase in GPx activity was observed in experimental eyes as compared to controls. In

spite of the presumed oxidative burden caused by IOP elevation, GSH levels were only moderately reduced in the experimental samples. TBARS levels, a measure of lipid peroxidation, increased to approximately 160% of controls. Melatonin is believed to increase the activity of GPx, raise SOD mRNA levels, and function as a direct antioxidant (Reiter et al., 1997; Barlow-Walden et al., 1995; Antolin et al., 1996). Its levels progressively declined to a minimum of approximately 25% of control. The most startling result of this study was the absent decline of GSH levels with adequate IOP elevation. The authors suggest that this result may represent an adaptation to redox imbalance with compensatory activation of GSH synthesis pathways. Although the mechanisms involved in the results of this study are still poorly understood, they are a large step forward in our understanding of the causal role that IOP elevation plays in glaucomatous vision loss. That IOP has a measurable effect on the redox status of the retina, and its responses to such challenges, may provide another avenue for the application of therapeutic tools such as antioxidants for the prevention of glaucomatous cell death (Moreno et al., 2004).

Proteomic analysis is another method of examining the potential of oxidative stress in glaucomatous neurodegeneration. With the Morrison model of IOP elevation in rats Tezel et al. (2005) studied the differential oxidation of retinal proteins. Retinal lysates were separated using the technique of 2D-oxyblots for the determination of oxidized proteins (containing carbonyl groups) as compared to fellow eyes. Mass

spectroscopic analysis, through peptide fingerprinting, identified three carbonylated proteins. The first of these was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a glycolitic enzyme, significant because oxidative modification of the active site enhances the binding of GAPDH to nucleic acids (Arutyunova et al., 2003). Subsequent nuclear accumulation of GAPDH bound DNA has been associated with proapoptotic signaling in neurons (Tatton et al., 2000) and neurodegenerative disorders including Alzheimer's, Huntington's, and Parkinson's disease (Chuang et al., 1996; Mazzola et al., 2001; Tatton et al., 2003). The second protein identified was HSP72, a stress response protein involved in cell survival; overexpression of heat shock proteins (HSP) has been noted in glaucomatous human eyes and *in vitro* (Tezel et al., 2000; Caprioli et al., 1996). Furthermore, stresses that induce endogenous HSP expression in experimental rat models of glaucoma confer neuroprotection (Ishii et al., 2003). Oxidative modification of HSPs may then attenuate the ability of eyes' endogenous neuroprotective pathways to respond to ocular hypertension. The final protein identified was glutamine synthetase located primarily in the Muller cell layer within the retina and is responsible for the conversion of extracellular glutamate into glutamine (Newman et al., 1996). Glutamine synthetase can be rapidly inactivated by oxidative modification, significantly reducing its ability to maintain the glutamate-glutamine cycle, and leading to the extracellular accumulation of neurotoxic glutamate (Levine et al., 1983). Inactivation may in turn enhance the hypothesized glutamate-induced excitotoxicity in glaucomatous eyes.

Immunohistochemical analysis identified these proteins to be of the highest density within the inner neural layer of the retina, suggesting that this layer is either more exposed or more susceptible to oxidative modification in ocular hypertension (Tezel et al., 2005). Similarly, Cummings et al. (2005) utilized mass spectroscopy to examine the differentially expressed proteins in the normal and pressure induced glaucomatous rat optic nerve heads. The proteins identified were transferrin, glutamate dehydrogenase-mitochondrial precursor, α-enolase, carbonic anhydrase, and β-crystallin-B chain (Cummings et al., 2005). The proteins transferrin and a-crystallin have been previously implicated oxidative damage in glaucoma (Farkas et al., 2004). The proteomics approach to glaucoma allows the identification of differential targets and may allow for insult directed therapies to be developed.

The enzyme responsible for the synthesis of nitric oxide (NO) has been shown to undergo overexpression in glaucomatous optic nerve heads (the confluence of the RGC layer within the retina) (Neufeld et al., 1999; Liu et al., 2000); excess NO produced then interacts with superoxides to produce peroxynitrites that yield nitrotyrosines (NT) on proteins (Huie et al., 1993; Ischiropoulos et al., 1992). The detection of NT in human glaucomatous optic nerve heads and blood vessels implicates oxidative stress in optic nerve head damage (Neufeld et al., 1999). Recent evidence suggests that neuronal death in glaucoma extends beyond the RGC layer and includes the lateral geniculate nucleus (LGN) (Weber et al., 2000; Yucel 2003). Utilizing NT as a marker for oxidative stress

Luthra et al., demonstrated positive immunohistochemical staining of blood vessels throughout the LGN thickness. with the affirmative nuclear and cytoplasmic staining of LGN neurons of the experimental glaucomatous primate eye. A significant increase in NT was also observed in the LGN layers connected to the fellow non-glaucomatous eye with respect to control. This observation is believed to be independent of RGC loss or IOP in fellow control eyes (Luthra et al., 2005).

As the role of oxidative stress in ocular hypertension and glaucomatous loss of RGCs becomes clearer, the precise etiologic mechanisms continue to elude investigators. A controversial yet pervasive theory is one of elevated intravitreal glutamate that is believed to produce excitotoxic oxidative stress. Based on the initial studies of Dreyer et al. (1996) elevated intravitreal glutamate concentrations were implicated in the excitotoxic loss of RGCs in glaucoma. They reported that vitreal glutamate concentrations were increased to twice that of control eyes in humans and nearly eight times control in monkeys with experimental glaucoma (Dreyer et al., 1996). Glutamate normally functions as the major excitatory amino acid neurotransmitter in the retina, but at high concentrations it becomes neurotoxic. When glutamate is injected into the vitreous in *in vivo* studies or when cultured retinal ganglion cells are treated with glutamate *in vitro*, retinal ganglion cells have been shown to undergo apoptosis (Lam et al., 1999; Lucas et al., 1957; Otori et al., 1998; Aoun et al., 2003; Pang et al., 1999). The apoptosis observed in such models has been attributed to the influx of calcium into cells

and the generation of oxidative free radicals. Pathologic quantities of nitric oxides (NO), produced due to elevated extracellular glutamate concentration, facilitate the production of superoxide free radical anions by the mitochondria, triggering cell death (Haefliger et al., 1999; Bonfoco et al., 1995). Additionally, oxidative stress can be imposed through the inhibition of glutamate/cystine antiporters, which decreases the availability of intracellular cysteine, a necessary precursor in glutathione synthesis (Murphy et al., 1989; Schubert et al., 2001; Atlante et al., 2001; Bridges et al., 2001). In the absence of adequate intracellular cysteine, cells cannot produce adequate glutathione to cope with the resultant overwhelming oxidative stress that glutamate challenge creates.

These findings spawned a flurry of *in vitro* and *in vivo* studies based on the excitotoxicity and oxidative burden produced by elevated intravitreal glutamate levels. Because of the tremendous importance of these findings Carter-Dawson et al. (2002) attempted to corroborate the primate data with a larger study of vitreal glutamate levels in experimental elevation of IOP in monkeys. The results of this study were a stark contrast to the findings of Dreyer et al. (1996). No difference in vitreal glutamate level was found in the glaucomatous eyes of monkeys, with respect to fellow controls eyes. As explained by the authors, it is important to note that this finding does not exclude glutamate-induced excitotoxicity from playing a role in glaucoma. Carter-Dawson's unpublished data demonstrate that glutamine (a metabolite of glutamate) and glutathione (the glutamate containing tri-aminoacid antioxidant) levels are significantly elevated in the

Muller cells in the eyes of monkeys' with experimental glaucoma (Carter-Dawson et al, 2002). Although Carter-Dawson's findings are contrary to those reported by Dreyer's, the possibility of glutamate-induced oxidative excitotoxic damage of RGCs in glaucoma cannot be dismissed.

The subjects of extracellular and retinal glutamate levels in glaucoma remain controversial, but recent data suggest that there is reason to believe that perturbations of glutamate levels may in fact be involved in the development of glaucoma. Low et al. (2006) compared the RGC layer of retinas of DBA/2J mice with glaucoma with control mice by immunostaining for glutamate to determine alterations in its concentrations. In the RGC layer of control mice, few damaged cells were observed and large amounts of glutamate were detected in the cells. In DBA/2J mice numerous damaged RGCs were observed and the level of glutamate immunoreactivity was high in only a fraction of cells within the damaged region of the retina. Moreover, many of the neurons with reduced amounts of intracellular glutamate in damaged regions did not appear damaged histologically. The loss of RGC glutamate in damaged retinal regions suggests that glutamate contributed to early retinal damage prior to changes in IOP (Low et al., 2006). Using an elevated IOP-induced ischemia model for retinal neurodegeneration in rats Nucci et al. (2005) demonstrated that systemic treatment with MK801 (NMDA receptor antagonist), GYK152466 (non-NMDA receptor antagonsist), or I-NAME (nitric oxide synthase inhibitor) prevent RGC loss observed 24 hours after IOP elevation. This

strongly suggests an excitotoxic, glutamate-mediated, mechanism of RGC death. This deduction is strengthened by the detection of glutamate levels increased by 90% over baseline by microdialysis probe placed directly into the retinas of rats bearing IOP elevation. These data suggest that acute elevation of IOP increases intraretinal levels of glutamate with subsequent activation of NMDA and non-NMDA glutamate receptors (Nucci et al., 2005). In a primate study conducted by Carter-Dawson et al. (2004), glutathione content in the Muller cells of monkeys with experimental glaucoma was investigated using immunocytochemistry and image analysis. As a constituent in GSH synthesis, alteration of intracellular concentrations can inhibit the rate of GSH synthesis. In this study Muller cells in glaucomatous retinas showed significantly greater immunoreactivity for glutathione than those of the fellow control retinas, increasing with the duration of elevated IOP. The presence of greater quantities of glutathione in the Muller cells of glaucomatous retinas was interpreted as being consistent with an increase in extracellular glutamate and an increase in transport and metabolism of glutamate (Carter-Dawson et al., 2004).

The studies presented in this section clearly indicate that elevated intravitreal glutamate concentrations and oxidative stress are involved in animal models of elevated IOP glaucoma. When comparing animal and human studies it becomes even clearer that there is an associate between elevated IOP and exaggerated oxidative stress. Animal studies support and corroborate the observations of increased lipid peroxidation,

decreased CAT activity, and compensatory increase of GPx activity made in human studies. Furthermore, proteomic analysis has identified proteins that are differentially translated and oxidized in animal models of glaucoma.

In vitro Studies

The mounting evidence from human and animal studies, demonstrating that oxidative stress plays a significant role in glaucomatous RGC death, lead to a rapid proliferation of *in vitro* models designed based on what is presumed to be occurring within the diseased eye. These models take a variety of approaches to promote oxidative stress and study the response of RGCs, many of them utilize anti-oxidants and neuroprotective drugs. Although these models are based on the best available data they are fallible and offer disparate evidence of glaucoma's pathophysiology.

Seminal in the *in vitro* study of retinal ganglion cell biology and glaucoma is the establishment of a clonal rat retinal ganglion cell line (RGC-5) by Krishnamoorthy et al. (2001). The RGC-5 cell line was identified positive for Thy-1, Brn-3C, Neuritin, NMDA receptor, GABA-B receptor, and synaptophysin expression; negative for GFAP, HPC-1, and 8A1. These results suggested the isolation of putative RGC clone. Serum deprivation of RGC-5 cells resulted in apoptosis; supplementation with BDNF and NT-4 in the growth media protected the RGC-5 cells from undergoing apoptosis. Differentiation of RGC-5 cells with succinyl concanavalin A (sConA) improved sensitivity to glutamate

toxicity, which could be reversed by inclusion of the glutamate receptor antagonist MK801 (Krishnamoorthy et al., 2001). The virtue of this cell line has been expanded by recent differentiation with the nonspecific protein kinase inhibitor staurosporine. This treatment arrested proliferation without inducing apoptosis, induced a neuron-like morphology, enhanced neuronal markers, and established outward rectifying channels (Frassetto et al., 2006). The establishment of this cell line, along with its differentiation and isolated primary RGCs, allows for the convenient study of various aspects of oxidative stress and retinal ganglion cell death.

The advent of the RGC-5 cell line spurred a flurry of investigations. One of the earliest was a model of elevated IOP-induced retrograde neurotrophin transport blockade via serum deprivation of RGC-5 cells. This insult perturbed the oxidative state of the cells as suggested by an increase in malonyldialdehyde (MDA, a lipid peroxidation product) and a decrease in reduced glutathione (GSH) levels in cell lysates. Serum deprivation was also associated with a loss of mitochondrial membrane potential, revealed by cytosolic release of cytochrome c and prolific JC-1 staining of mitochondria of dying RGC-5 cells. Furthermore, serum deprivation precipitated apoptosis of the cells and resulted in the activation of caspases-3, -8, and -9, and with increased levels of Bax with corresponding decreases in Bcl-2 levels and NF-kappaB binding activity. These findings illustrate that retrograde neurotrophin deprivation of retinal ganglion cell death can be modeled *in vitro*, and that oxidative stress is an integral part of the apoptotic

cascade (Charles et al., 2005).

Since the earliest studies of glaucoma pathobiology, elevated extracellular glutamate levels have been implicated in the formation of exaggerated oxidative stress and retinal ganglion cell loss. Utilizing the RGC-5 cell line Aoun et al. (2003) demonstrated that glutamate challenge of these cells resulted in more than 50% cell death that could not be prevented with the inclusion of glutamate receptor antagonist MK801 or glutamate transporter antagonist DL-TBOA. However, pretreatment with the anti-oxidants NAC and thiourea significantly attenuated RGC-5 cell death. It was further shown that inhibition of glutathione synthesis with BSO could also enhance the oxidative burden enough to promote cell death (Aoun et al., 2003). Further investigation into the effects of glutamate-induced oxidative stress by Kumar et al. (2005) revealed that glutamate challenge of RGC-5 cells promotes the formation of TBARS and induces cell death. These deleterious responses were prevented with the pretreatment of estrogens and non-feminizing estrogen analogues, drugs known to be neuroprotective anti-oxidants (Kumar et al., 2005). The response of RGC-5 cells to oxidative stress induced by three additional treatments – glutathione depletion, tert-butyl peroxide addition, and hydrogen peroxide – were examined and compared by Maher et al. (2005). These studies determined that all three inducers of oxidative stress share several features: perturbation of calcium homeostasis, pro-apoptotic caspase activation, depletion of glutathione levels, and increased DNA fragmentation, suggesting a final common pathway of oxidative

stress-induced cell death (Maher et al., 2005). The initial RGC-5 study by Maher was followed by an attempt to prevent these pro-apoptotic responses with flavanoids. This effort showed that various flavonoids could be used to protect retinal ganglion cells from each of the three inducers of oxidative stress. Furthermore, the flavonoids also induced the synthesis of proteins whose genes contain antioxidant response elements (Maher et al., 2005). A significant discrepancy in this study is the use of very high doses of glutamate as compared with the Aoun et al. (2003); Kumar et al. (2005) who used 5 mM glutamate as an LD_{50}. Even at that high concentration, Maher et al. (2005) observed neuroprotection by flavonoids.

The effects of exaggerated hydrostatic pressure on cultured RGC-5 cells have been investigated to elucidate the isolated effects of IOP elevation on retinal ganglion cells. The application of 100 mm Hg hydrostatic pressure induced TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) positive apoptosis. Proteomic analysis of hyper-pressure challenged RGC-5 cells determined 37 differentially expressed proteins under these culture conditions. GAPDH was expressed 10 times more in pressure challenged cells than in control cells; nuclear translocation was also enhanced. As described previously by Tezel et al (2005), increased expression and translocation of GAPDH to the nucleus is a hallmark of IOP elevation in rats. The correlation between *in vitro* and *in vivo* studies is significant, as additional studies may lead to the delineation of the pressure induced apoptotic cascades that have the potential to yield apoptosis directed therapies (Kim et al., 2006).

Primary cultures of RGCs are also used for *in vitro* experimentation. Insults that exaggerate oxidative burdens are commonly used to investigate the responses and mechanism of the responses of RGCs. In 2000, Kortuem et al. examined the differential susceptibility of retinal ganglion cells to ROS. Retrogradely labeled RGCs were dissociated and cultured with various ROS-generating systems. When compared with other retinal cells, RGCs were resistant to cell death induced by superoxide anion, hydrogen peroxide, or hydroxyl radical. The drugs aminotriaozle, l-buthionine sulfoximine, and sodium azide partially abrogated the RGC resistance to oxidative stress, suggesting that the observed resistance may be secondary to the protection conferred by anti-oxidant enzymes. These results hint that endogenous anti-oxidant defense systems may be exploited to protect the retina from oxidative stress in diseases in which perturbation of redox status has been implicated (Kortuem et al., 2000). Although RGCs may have the capability to defend themselves against some oxidative burdens, oxidative stress can still overwhelm their defense mechanisms. Lieven et al. (2003) examined the effects of oxidative stress on the mitochondrial membrane potential in RGCs. Treatment of retinal ganglion cells with H_2O_2 caused mitochondrial depolarization that was significantly reduced by bongkrekic acid. This suggested that H_2O_2-induced mitochondrial membrane depolarization may act through a permeability transition pore opening independent of thiol oxidation, and that it may be prevented with thiol reducing agents (Lieven et al., 2003). To elucidate the role that reactive oxygen species may have in signaling RGC apoptosis after axonal injury, a ubiquitous component of optic neuropathies, Nguyen et al. (2003) investigated the sequence of intracellular events

proximal to caspase activation. RGCs exposed to oxidative stress exhibited a secondary superoxide burst, which was not blocked by a broad-spectrum caspase inhibitor. This suggested that the secondary oxidative burst is proximal to caspase activation, and was inhibited by the protein synthesis inhibitor cyclohexamide. This result was consistent with RGC axonal injury inducing synthesis of proteins that mediate the amplification of oxidative stresses (Nguyen et al. 2003). A novel study by Lucius et al. (1996) examined the neuroprotection of primary RGCs against ROS-induced axonal degeneration by cocultured astrocytes. In this study, treatment of postnatal primary RGCs, with sydnonimine and iron salts, to catalyze the generation of oxidative radicals resulted in RGC axonal degeneration. Treatment of challenged cells with vitamin E and vitamin C attenuated axonal loss as did astrocyte coculture. The combination of astrocyte coculture with vitamins further improved the number and length of neuritis growing from the explanted retina. The results of this study indicate that astrocytes can protect retinal ganglion cells against ROS-induced oxidative stress, astrocytes secrete neurotrophic factors supporting RGC axonal regeneration, and free radical production after tissue injury may contribute to the failure of axonal regeneration in the mammalian nervous system (Lucius et al., 1996).

The *in vitro* studies reviewed here illustrate the diversity of approaches to elucidating the mechanisms of oxidative stress-induced ganglion cell death, but there are principles in common with all approaches. The first is that trophic support, via retrograde transport of neurotrophins or paracrine support from astrocytes, may be critical to the survival of RGCs. Secondly, elevated extracellular glutamate concentrations can induce

excitotoxic cell death via receptor-mediated and receptor-independent mechanisms. Lastly, flavanoids, estrogens, and various antioxidants can protect RGCs from various oxidative insults.

Conclusions

The studies presented, plus a host of others, provide the reader with a synopsis of the current evidence for the role of oxidative stress in glaucoma (Table 1). Although these studies are compelling, the cause of RGC death remains elusive. The fundamental questions remain unanswered. Is there an elevation of intravitreal and/or retinal glutamate levels in glaucoma patients? Is retrograde neurotrophin deprivation a cause or effect of RGC death? Are there progenitor cells that could be activated and differentiated to repopulate lost RGCs? Is oxidative stress the primary insult inducing RGC death? Proposed pathophysiologic pathways pertaining to RGC death in glaucoma are depicted in Figure 1. In spite of the ever-growing body of data, basic science has still to offer a mechanistic solution to the neurodegenerative loss of vision in glaucoma. A proposed solution is neuroprotection of retinal ganglion cells to prevent vision loss or attenuate visual field deterioration in affected patients. However, the basic therapeutic interventions available to clinicians have not changed in nearly fifty years. If the measure of progress is innovation, we have yet to move forward.

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Figure Legends

Table-1: Current evidence implicating oxidative stress in the pathophysiology of glaucoma. These studies represent investigations from human, animal, and *in vitro* based efforts.

Figure 1: Possible inducers and consequences of oxidative stress. Oxidative stress results from a number of insults that combine to induce cell death.

Figures

Tissue	Oxidative End Point	References
Aqueous Humor	Anti-oxidant Status/Enzyme activity Lipid Peroxidation	Ferreira et al., 2004 Bunin et al., 1992 Kurysheva et al., 1996
Trabecular Meshwork	DNA damage Aqueous Humor Viscosity Lipid Peroxidation/Aging Cell Adhesion TIGR	Izzoti et al., 2003 Sacca et al., 2005 Liu et al., 1984 Wang et al., 2001 Yan et al., 1991 Bunin et al., 1985 Babizhayev & Bunin, 1989 Russel et al., 1989 Zhou et al., 1999 Polansky et al., 1997 Lutjen-Dercoll et al., 1999
Serum	Glutathione Anti-oxidant Status/Enzyme activity	Gherghel et al., 2005 Yildrim et al., 2005
Retina	Anti-oxidant Status/Enzyme activity Lipid Peroxidation/Neurodegeneration Proteomic Analysis Apoptosis/Glutamate	Moreno et al., 2004; Sandback et al., 2001; Castagne et al., 2000; Castagne et al., 1999 Anderson et al., 1994 Lam et al., 1990; Reme et al., 1991; De LePex & Anderson, 1992; Organisciak &Winkler, 1994; Mainster et al., 1987 Tezel et al., 2005; Cummins et al., 2005; Farkas et al., 2004; Luthra et al., 2005 Levin et al., 1997 Dreyer et al., 1996; Carter-Dawson et al., 2002 & 2004; Low et a., 2006; Nucci et al.,

		2005; Guo et al., 2006; Hartwick et al., 2005; Madl et al., 2005; Wamsley et al., 2005; Levkovitch-Verbin et al., 2002;
RGC-5	Cell line Apoptosis/Neurotrophin Deprivation Glutamate	Krishnamoorthy et al., 2001; Frassetto et al., 2006 Charles et al., 2005; Kim et al., 2006 Aoun et al., 2003; Kumar et al., 2005; Maher et al., 2005; 2005; Agarwal et al., 2002; Dun et al., 2006
Primary RGCs	Apoptosis	Levin et al., 1999; Korteum et al., 2000; 2002; Geiger et al., 2002; Leiven et al., 2003; Nguyen et al., 2003; Lucius et al., 1998
Optic Nerve Crush	Free Radical Induced Cell Death	Lucius et al., 1998; Levkovitch-Verbib et al., 2000; Swanson et al., 2005

Table 1

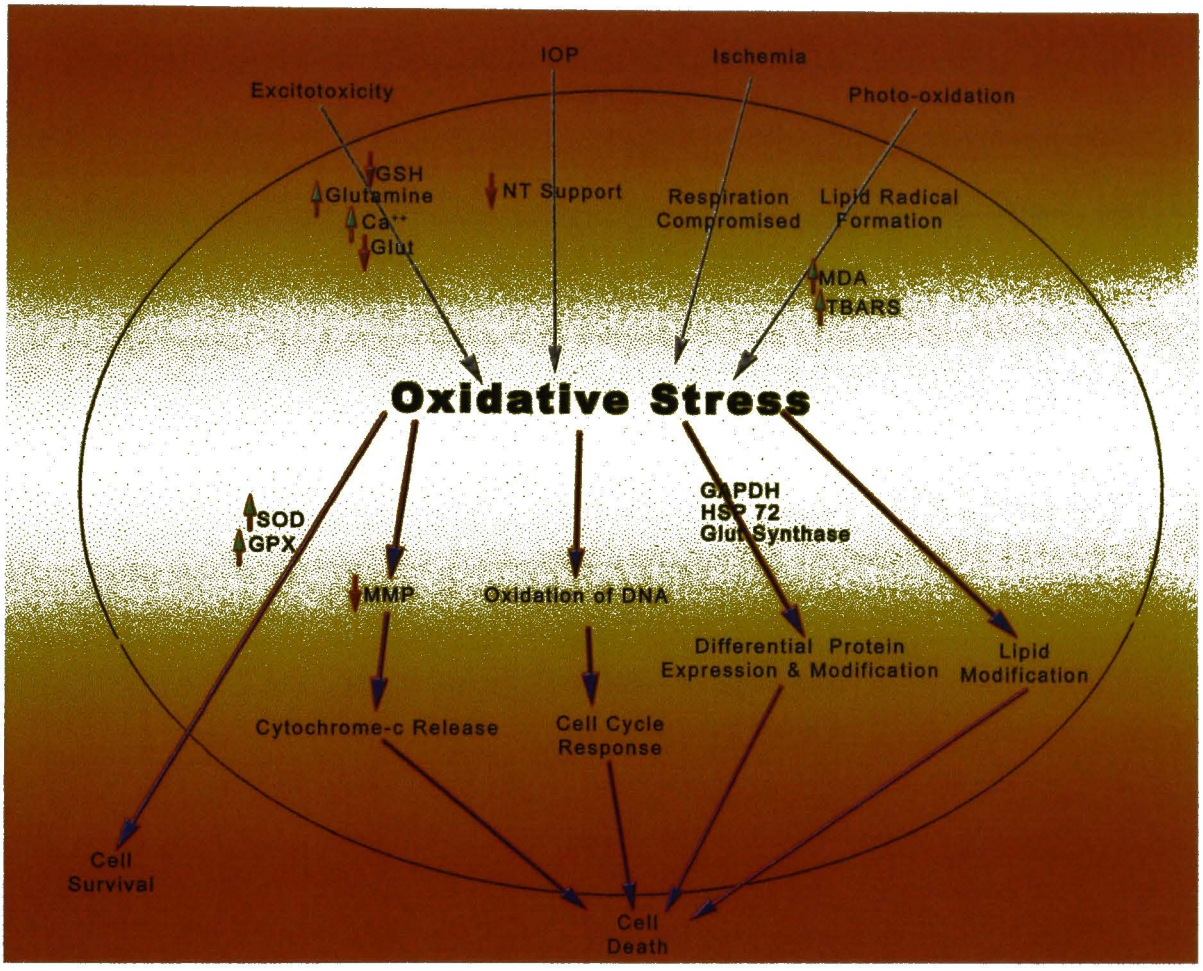
IOP
Ischemia
Excitotoxicity
Photo-oxidation
GSH
Glutamine
Ca++
Glut
NT Support
Respiration Compromised
Lipid Radical Formation
MDA
TBARS
Oxidative Stress
SOD
GPX
GAPDH
HSP 72
Glut Synthase
MMP
Oxidation of DNA
Differential Protein Expression & Modification
Lipid Modification
Cytochrome-c Release
Cell Cycle Response
Cell Survival
Cell Death

Figure 1

PREFACE TO APPENDIX B

The previous chapter delineated evidence of supraphysiologic accumulation of oxidative stress in glaucoma. In the following chapter we show that glutamate can be used to induce a lethal oxidative burden in RGC-5 rat retinal ganglion cells. This model has stirred controversy because of suggestions that glutamate levels may not be elevated in the glaucomatous retinas. Nonetheless, it serves as a reproducible method of inducing oxidative stress in RGC-5 cells.

APPENDIX B

Glutamate Induced Cytotoxicity of Transformed Rat Retinal Ganglion Cells As An Acute In vitro Model of Glaucoma

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While several etiologies of glaucoma exist, it is thought that the ultimate cause of vision loss is apoptosis of retinal ganglion cells (Quigley et al., 1995). Apoptosis of RGCs was first shown by axotomizing the optic nerve in the rat (Berkalaar et al., 1994; Garcia-Valenzuela et al., 1994; Rabacchi et al., 1994 a, b). Other investigators found apoptosis of RGCs in the monkey model of experimental glaucoma (Quigley et al., 1995; Garcia-Valenzuela et al., 1995). Studies in humans have shown that more than 50% of the patients with POAG shown terminal d-UTP nick end labeling (TUNEL) positive labeling in the retinal ganglion cell layer (Kerrigan et al., 1998).

Several stimuli may account for the induction of apoptosis of retinal ganglion cells in glaucoma (Nickells, 1996). These include either neurotrophin deprivation caused by blockade of retrograde axonal transport or glutamate toxicity caused by ischemia, suggesting that reduced blood flow to the tissue surrounding the optic disc may result in the generation of reactive oxygen intermediates causing oxidative damage, leading to apoptosis of RGCs (Nickells, 1996).

The pathologic changes observed in glaucomatous retinas are attributable to apoptosis of retinal ganglion cells within the optic nerve head, resulting in characteristic cupping of the optic disk (Wygnanski et al., 1995; Neufeld et al., 2003; Danias et al., 2003; Quigley et al., 1995). Experimentally a variety of pathologic situations may be modeled to study the loss of retinal ganglion cells; one such model is a glutamate cytotoxicity induced apoptosis of these cells (Goldblum et al., 2002).

Glutamate acts as a normal neurotransmitter in the retina, but its high levels may be neurotoxic both *in vivo* when injected in vitreous or in *in vitro* treatment of cultured

retinal neuronal cells and results in apoptosis of retinal ganglion cells (Lam et al., 1999; Lucas and Newhouse, 1957; Otori et al., 1998; Vorwerk et al., 1996). Although controversial, elevated glutamate levels may exist in the vitreous humor of patients with glaucoma, and the major causes of cell death from glutamate are the influx of calcium into cells and the generation of free radicals (Brooks et al., 1997; Carter-Dawson et al., 2002; Dkhissi et al., 1999; Dreyer et al., 1996; Levkovitch-Verbin et al., 2001; Osborne et al., 1999). Pathologic quantities of nitric oxides (NO) are produced which facilitates the production of free radical superoxide anions by the mitochondria, ultimately triggering cell death (Haefliger et al., 1999; Bonfoco et al., 1995). Additional oxidative stress is imposed through the inhibition of glutamate/cystine antiporters, which decreases the availability of intracellular cysteine, a necessary precursor in glutathione synthesis (Murphy et al., 1989; Schubert et al., 2001; Atlante et al., 2001; Bridges et al., 2001). Taken together, excessive glutamate concentrations pose an overwhelming oxidative stress on retinal ganglion cells that leads to their death.

We have shown that the cytotoxic effects of glutamate on RGC-5 cells cannot be reversed by pretreatment with MK801 or DL-TBOA, suggesting that glutamate toxicity is not mediated through a NMDA receptor and/or glutamate transporter. Furthermore, pretreatment with the antioxidants NAC and thiourea reversed the glutamate cyotoxicity observed in RGC-5 cells (Aoun et al., 2003). In the present study transformed rat retinal cells, RGC-5s, were used in a glutamate induced cell death model of glaucoma. Here we show that RGC-5 cells are susceptible to glutamate cytotoxicity in a dose dependent fashion through mechanisms that perturb glutathione production. The data represented in

Figure 1 demonstrates that RGC-5 cells are susceptible to glutamate cytotoxicity with an LD_{50} of 2mM glutamate.

As extracellular levels of glutamate rise, the function of glutamate/cystine antiporters on the cell surface is inhibited and may be reversed. This results in reduced cystine uptake, and in extreme cases, a net efflux of cystine from cells. Glutamate challenged cells cannot then continue to produce glutathione in cystine depleted states, the concentrations of which are dependent upon intracellular cysteine levels (Murphy et al., 1989; Schubert et al., 2001; Atlante et al., 2001; Bridges et al., 2001). Consequently, cells exposed to elevated extracellular glutamate concentrations not only produce an immense superoxide burden, and the available amount of the cells' ultimate reducing agent is also significantly depleted. Figure 2 depicts the effects of elevated extracellular glutamate concentrations on the function of glutamate/cystine antiporters and how a paucity of available cystine leads to reduced glutathione production.

The ability of RGC-5 cells to take up cystine and synthesize glutathione was examined in our acute *in vitro* model of glaucoma. 24 hours following 5mM glutamate treatment the ability of RGC-5 cells to take up ^{35}S-cystine was examined. An approximate three-fold reduction in the ability of RGC-5 cells to take up ^{35}S-cystine was observed. To correlate the ^{35}S-cystine uptake findings to glutathione production, the intracellular concentration of reduced glutathione (GSH) was measured following a 24-hour 5mM glutamate challenge. As compared to control RGC-5 cells, GSH was found to be three times less abundant in cells treated with glutamate. These finding support the concept that elevated extracellular concentrations of glutamate may inhibit the function

of glutamate/cystine antiporters and reduce the ability of RGC-5 cells to produce glutathione when cystine uptake is inhibited by glutamate treatment.

Furthermore, the levels of the regulatory subunit of GCS were increased by 5mM glutamate treatment, but the levels of the catalytic subunit were not changed following 24-hour glutamate challenge (Kumar et al., unpublished data). The use of glutamate as a cytotoxin is supported by the work of groups such as Carter-Dawson's, who have observed increases in glutamine, glutathione, and glutamate/aspartate transporter levels in experimental glaucomatous monkey retinas, indicating an elevation in extracellular glutamate and enhanced glutamate transport and metabolism (Carter-Dawson et al., 2002). Taken together these studies suggest that glutamate may act through an oxidative pathway to influence the survival of retinal ganglion cells in glaucoma.

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Figure Legends

Figure 1: Dose dependent reduction of RGC-5 cell viability following 24 hour glutamate treatement. An LD_{50} of 2mM gltuamate was observed in this study. 5mM glutamate treatment resulted in an approximate 75% reduction in cell viability as compared to control conditions. Data are the mean +- SEM; n = 3. *P<0.05 versus control.

Figure 2: A model of elevated extracellular glutamate levels on glutamate/cystine antiporter function and glutathione production. High levels of extracellular glutamate block glutamate/cystine antiporters, thereby inhibiting cystine uptake by the cells.

Figures

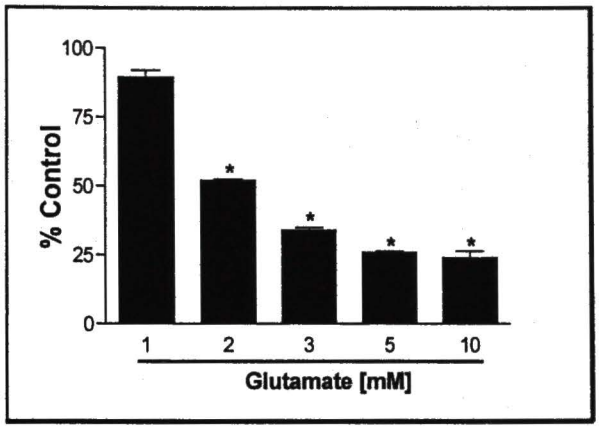

Figure 1

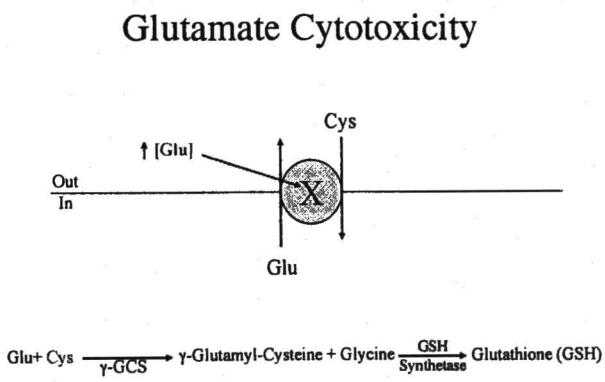

Figure 2

PREFACE TO APPENDIX C

The previous chapters demonstrated that oxidative stress, regardless of etiology, create a lethal burden for RGCs. Here we examine the role of estrogens in the retina with a focus on their neuroprotective effects in glaucoma. Current studies and our own data are reviewed to provide evidence supporting their use in studies of neuroprotection.

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Please Note:

Appendix C has been retracted from this dissertation.

Appendix C

Estrogens as Retinal Neuroprotectants

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Abstract

Estrogens play a critical role in the normal growth and development of humans and in recent years our understanding of their effects in the central nervous system (CNS) have been advancing rapidly. It is now known that estrogens influence synaptic plasticity, brain development, memory, and have been shown to be neuroprotective in degenerative disorders. The understanding of the influences of estrogens in the retina, as a component of the CNS, has not kept pace with the advances in understanding of the brain. The studies that have addressed the effects of estrogens on the retina, specifically focusing on glaucoma, are examined here.

Introduction

Glaucomatous visual field deterioration is a neurodegenerative process commonly associated with an insidious increase in intraocular pressure (IOP), with characteristic optic disc and retinal ganglion cell nerve fiber layer damage (Wygnanski et al., 1995; Van Buskirk et al., 1992). World Health Organization statistics indicate that glaucoma accounts for blindness in at least 5 million people, causing 13.5% of worldwide blindness (Thylefors et al., 1995). In the United States, glaucoma is the second leading cause of blindness and the second most frequent reason for ambulatory visits to physicians by Medicare beneficiaries (Javitt, 1994). Extrapolating to the entire Medicare population, blindness and vision loss are associated with greater than $2 billion in non-ophthalmologic related costs. The staggering healthcare and economic burdens make vision loss a medical imperative (Javitt et al., 2007).

In recent years the evidence supporting the use of estrogens as neuroprotectants has become compelling (reviewed by Hoffman et al., 2006; reviewed by Simpkins et al., 2005; Simpkins et al., 2004; Rau et al., 2003; reviewed by Merchanthaler et al., 2003; reviewed by Behl et al., 2002; reviewed by Amantea et al., 2005; reviewed by Marin et al., 2005). However, conclusions from the Women's Health Initiative Study indicate that conjugated equine estrogens and estrogen plus medroxyprogesterone acetate cause excess increases in the risk of ischemic stroke in generally healthy postmenopausal women (Hendrix et al., 2006; Wassertheil-Smoller S, et al., 2003). Additional findings indicate that low doses of 17β-estradiol may in fact protect against ischemia and reperfusion injury (Macrea et al., 2006; reviewed by Wise et al., 2005). A correlation between

glaucoma and ophthalmic artery blood flow changes with hormone therapy (HT), female reproductive factors, as well as HT and IOP clearly illustrate that estrogens influence the health of the retina (Hulsman et al., 2004; Battaglia et al., 2004; Sator et al., 1997; Nirmalan et al., 2004; Lee et al., 2003). Nonetheless, the use of estrogens as neuroprotectants is complicated by the natural feminizing sequelae that would be undesired in male patients, but this opens the door for investigation of synthetic non-feminizing estrogen analogues. Here we examine if estrogens can be effective neuroprotectants in the retina, with an emphasis on glaucoma as the primary retinal neurodegenerative disease.

Estrogen Receptor Expression in the Retina

Estrogen receptors α and β (ERα and ERβ) are involved in classical receptor mediated genomic pathways as well as alternative mechanisms of orchestrating neuroprotection (Hall et al., 2001; Marin et al., 2005; Amantea et al., 2005). Bovine and rat retinas have been shown to express both receptor isoforms (ERα predominates in rat) throughout the retinal thickness, seen in greatest density within the ganglion cell layer, the inner nuclear layer, and outer portion of the outer nuclear layer (Kobayashi et al., 1998; Kumar et al., 2005). Human donor eyes from premenopausal women express ERα in the neurosensory retina and retinal pigment epithelium, but receptors could not be detected in tissues dissected from men and postmenopausal women (Ouegta et al., 1999). Another study of ER expression in humans showed that ERβ protein was localized to the ganglion cell layer and choroid. However, at the transcriptional level both ERα and ERβ were observed with local differences in expression, suggesting variability in the ratio of ERα:ERβ (Munaut et al., 2001). ERs have also been detected in the neuroendocrine secretory and metabolic ciliary epithelium. 17β-hydroxysteroid dehydrogenases (17HSDs), involved in the biosynthesis and inactivation for sex steroids, were shown to be under direct paracrine influence of 17β-estradiol, evidence of estrogen modulating its own fait within the eye (Coca-Prados et al, 2003). The presence of estrogen receptors and metabolic machinery within the eye suggest that estrogens play more than a passive role in the retina. Although estrogen receptors in humans were only detected in tissue taken from fertile female donors, below we will also examine the receptor independent effects that estrogens can produce.

Retinal Estrogenic Effects

Given the intimate association between estrogens, their receptors, and their metabolism in the eye, is it possible that estrogens could have deleterious effects similar to those seen with HT in the WHI? Rather than producing hypercoagulability, it appears that HT in older women does not have a long-term vasodilatory effect on retinal arterioles. This was a theory taken from coronary vessel and incidence of myocardial infarction studies in fertile women, and does not support the hypothesis that exogenous estrogen exposure accounts for greater observed retinal arteriolar diameters in women (Leung et al., 2003). Curiously, blood viscosity is decreased and ocular vascularity is improved in women with glaucoma who receive HT (Battaglia et al., 2004). Neither of these studies clearly indicate what, if any role, HT plays in glaucoma. Is it possible then that gender differences or exposure to endogenous feminizing hormones could affect IOP and the development of glaucoma?

This question was the basis for investigations that examined the effect of HT on IOP in a single case study and three large international studies examining female reproductive factors as risks for the development of open angle glaucoma (OAG). An early case study examined IOP changes with initiation of HT through 12 weeks of treatment. In a single glaucoma patient treated for menopausal symptoms with HT for the first time, IOP was reduced by an average of 4.5mmHg at four weeks and by an average of 4mmHg at 12 weeks after initiating treatment (Sator et al., 1998). In a larger cross-sectional controlled study, 107 women receiving HT (with 107 controls) underwent IOP assessment, cup-to-disc ratio assessment, and completed comprehensive medical and

family history questionnaires. The two groups did not differ in mean IOP, cup-to-disc ratios, prevalence of elevated IOP, or prevalence of glaucoma. Only a personal history of ischemic heart disease was a clear risk factor for increased IOP (Abramov et al., 2005). The Rotterdam study published findings on the association between early menopause and the development of OAG. This population-based study concluded that early menopause (≤45 years of age), age-adjusted and controlled for HT use, was a significant risk factor for the development of OAG (Hulsman et al., 2001). The Blue Mountain Eye study examined the association between endogenous estrogen exposure and OAG determining that early menarche (age ≤ 12 years) and increasing parity significantly increased the risk for OAG. Additionally, an insignificant risk increase was noted for an early natural menopause (≤45 years of age), also observed in the Rotterdam study (Lee et al., 2003). A similar study conducted in rural southern India determined that there was no association between female reproductive factors and OAG (Nirmalan et al., 2005). This disparate finding may be explained by significantly greater phytoestrogen content in the predominantly vegetarian south Indian diet, possibly masking what may otherwise have been a remarkable association between female reproductive factors and OAG. This theory is supported by a study that examined retinal thickness in male and female rats whose diet was supplemented with soy phytoestrogens. Male rats that were fed a phytoestrogen fortified diet and female rats that were fed a standard diet showed greater retinal thickness than their respective controls. Although this data suggests that phytoestrogens influence rat retinal characteristics in a sexually dimorphic manner, an influence is clearly demonstrated (Lund et al., 2003). These studies equivocally suggest

that HT and length of endogenous estrogen exposure may influence the development of OAG.

These studies suggest that estrogens serve roles within the retina and may be factors in development, prevention, and treatment of glaucoma. With an understanding of the presence of estrogen receptors in the retina, their influence on blood viscosity and ocular vascularity, the impact of HT on IOP, and the influence of female reproductive factors on OAG, the neuroprotective effects of estrogens will now be discussed.

Estrogens as Neuroprotectants

Estrogen receptor independent and dependent neuroprotective mechanisms have been well established in the brain (Marin et al., 2005; Amantea et al., 2005; Simpkins et al., 2005; Brann et al., 2007; Singh et al., 2006), but similar studies in the retina have not been conducted or are very limited. Those studies that demonstrate neuroprotective efficacy in models of retinal disease are examined here.

One of the pioneering studies in estrogen-mediated retinal neuroprotection examined the efficacy of a synthetic estrogen analogue in an *in vivo* model of retinitis pigmentosa (RP) and in an *in vitro* model of NMDA-induced excitotoxic glaucomatous retinal ganglion cell death. In the RP model, treatment with the an estrogen analogue at post natal day 9 yielded an outer nuclear layer, containing the photoreceptors cells lost in RP, that was nearly twice the thickness in untreated controls. In the glaucoma model, primary RGCs treated with the same estrogen analogue were protected from NMDA-induced excitotoxic death. Furthermore, the *in vitro* study demonstrated maintenance of mitochondrial stability and inhibition of lipid peroxidation. These findings were in accord with previous reports from brain parenchymal studies, offering a novel approach to neuroprotection in degenerative diseases of the retina (Dykens et al., 2004).

Another study examined protection of cultured retinal pigment epithelial cells (RPE), the pathologic target in age-related macular degenerative vision loss, by 17β-estradiol against hydrogen peroxide-induced cell death. This study not only demonstrated significant protection of RPE cells, but also showed that 17β-estradiol quenched hydrogen peroxide-induced up-regulation of apoptosis-related proteins (Yu et al., 2005).

The large cross-sectional Salisbury Eye Evaluation Project supported these *in vitro* findings. This study evaluated the effects of HT and female reproductive factors on age related macular degeneration (AMD), showing that current HT was associated with lower odds of large drusen predictive of advanced AMD (Freeman et al., 2005). Together, these studies suggest that estrogen treatment may be effective in AMD, in addition to RP and glaucoma as described above.

Oxidative stress, induced by ischemia-reperfusion or other perturbations, is a ubiquitous pathway of degenerative vision loss. The effects of 17β-estradiol on leukocyte accumulation have been evaluated during ischemia–reperfusion injury and retinal damage after transient retinal ischemia. Treatment with 17β-estradiol significantly inhibited leukocyte accumulation and subsequently improved retinal function as assessed by electroretinogram (Nonaka et al., 2000). Another ischemic retinal study showed that 17β-estradiol protected RGCs from early changes in synaptic connections that are associated with ischemia preceding apoptosis and ischemia-induced global apoptosis (Kaja et al., 2003).

These studies created the foundation for the field and led to the examination of estrogen-mediated neuroprotective mechanisms. Estrogens are classically known to effect transcriptional modulation via receptor mediated genomic pathways, but they may also alter intracellular signaling cascades (reviewed by Singh et al., 2006). One study approached this subject by examining estrogen-mediated retinal neuroprotection in an *in vitro* hydrogen peroxide-induced model of retinal neurodegeneration along with an *in vivo* model of light-induced photoreceptor degeneration. Both 17β-estradiol and 17α-

estradiol protected retinal neurons *in vitro*, with 17β-estradiol activating the phosphoinositide 3-kinakse (PI3K) pathway, transiently increasing phospho-Akt levels. The estrogen receptor antagonist tamoxifen did not reverse the protective effect of 17β-estradiol, but inhibition of the insulin receptor beta blocked the PI3K mediated protective effects. This finding suggested that neuroprotection with 17β-estradiol may be independent of its receptors, but dependent on the PI3K signaling pathway that is known to promote neuronal survival. Systemic administration of 17β-estradiol, in the *in vivo* arm of the study, demonstrated activation of insulin receptor beta as well, with a transient increase in PI3K activity and phosphorylation of Akt, protecting rat photoreceptor cells (Yu et al., 2004).

Similarly, two recent studies addressed the neuroprotective effect of 17β-estradiol via extracellular signal-regulated kinase (ERK) pathway induction. In the first, retinas were harvested from female rats, with and without ovariectomy, to determine the effect of optic nerve transection on RGC survival. RGC survival was significantly decreased in ovariectomized rats, but prevented with intravitreal 17β-estradiol injection. Protection was mediated via the ERK signal transduction pathway. Interestingly, ERK inhibitor U0126 inhibited the neuroprotective effect observed (Nakazawa et al., 2006). In the second study, the neuroprotective effect of 17β-estradiol against N-methyl-D-aspartate (NMDA)-induced retinal neurotoxicity was examined. Here, retinal pretreatment with 17β-estradiol silastic implants attenuated RGC death due to intravitreal injection of NMDA. However, coadministration of U0126 or estrogen receptor antagonist ICI 182,780 with NMDA completely abolished the protective effect of 17β-estradiol.

Moreover, NMDA treatment alone significantly increased the levels of phosphorylated ERK (p-ERK) that increased further with 17β-estradiol pretreatment. 17β-estradiol induced increases in p-ERK levels were attenuated with administration of U0126 and ICI 182,780 (Hayashi et al., 2007).

Our own studies showed that 17β-estradiol and the synthetic estrone-derived non-feminizing estrogen analogue ZYC-3 are effective neuroprotectants against glutamate-induced cytotoxicity of RGC-5 rat retinal ganglion cells. 17β-estradiol and ZYC-3 afforded complete protection against glutamate-induced RGC-5 cytotoxicity. As opposed to 17β-estradiol, ZYC-3 had no appreciable affinity for either estrogen receptor isoform, but was nearly 20-fold more effective at inhibiting lipid peroxidation (Kumar et al., 2005). We also determined that ZYC-3 pretreatment of glutamate insulted RGC-5 cells elicits activation of a host of anti-apoptotic signal transduction pathways.

Signal transduction pathways that are known to affect the survival of stressed neurons were examined in RGC-5 cells treated with glutamate. Phosphorylated-Akt (P-Akt), also known as PKB or Rac, plays a critical role in the balance between survival and apoptosis, and is activated by a host of survival factors (Franke et al., 1995&1997; Burgering et al., 1997). The p42 (Erk1) and p44 (Erk2) MAP kinase proteins (activated by phosphorylation) also play critical roles in growth and differentiation regulated by a host of factors including neurotransmitters and neurotrophins (Marhsall et al., 1995; Hunter et al., 1995; Hill et al., 1995; Cowley et al., 1994). At 24 hours post glutamate challenge in phosphorylated Akt (PKB) and Erk1/2 (p44/42) levels declined sharply; this decline was not observed with ZYC-3 pretreatment (Figure 1). Activation of

phosphorylation cascades in these pathways has been shown to promote survival of retinal ganglion cells in a variety of models of RGC injury and protection (Nakazawa et al., 2005; Nakanishi et al., 2006; Kilic et al., 2006; Koriyama et al., 2006). The p90 S6 ribosomal kinsase family of proteins responds to numerous growth factors and is activated by phosphorylation down stream of Erk1 and Erk2 (Lazar et al., 1999; Frodin et al., 1995). Phosphorylated activation of the p90 ribosomal protein indicating sustained activation of this survival promoting pathway in insulted cells pretreated with ZYC-3, not seen in glutamate challenged cells alone (Figure 1) (Nguyen et al., 2005). A similar pattern of phosporylation was seen for S6 ribisomal protein (P-S6) indicative of an increase in translation necessary for sustained cell growth and division (Figure 1) (Peterson et al., 1998). Additionally, phosphorylation of p53 was increased with ZYC-3 pretreatment (Figure 1). Phosphorylated activation of tumor suppressor protein p53 plays a major role in response to DNA damage by arresting the cell cycle and initiating DNA repair or apoptosis (Levine et al., 1997; Meek et al., 1994; Milczarek et al., 1997). This may indicate cell cycle arrest for DNA repair to promote RGC-5 cell survival. The pro-apoptotic p38 MAP kinase pathway serves as a gateway to cytokine and stress responses, with activation via phosphorylation leading to cell death (Han et al., 1994; Lee et al., 1994; Rouse et al., 1994; Fresheny et al., 1994). This pathway displayed a predictable pattern of activation with insult and neuroprotection. Basal levels of phosphorylated p38 in control samples were minimally detectable, but glutamate treatment resulted in a sharp increase in p38 phosphorylation that was inhibited with ZYC-3 pretreatment (Figure 1). In models of axotomy-induced apoptosis, phosphorylation of p38 peaked at one day post

axotomy, inhibition of p38 phosphorylation attenuated RGC loss, and selective inhibition of NMDA receptors showed dose dependent attenuation of p38 activation resulting in protection of RGCs (Kikuchi et al., 2000; Castagne et al., 1999).

These studies demonstrate that natural estrogens and synthetic analogues are effective neuroprotectants, an outcome classically attributed to receptor mediated gene expression, but that these effects may also be due to receptor independent activation of signal transduction pathways. Estrogens may be promising treatments for degenerative retinal disorders including AMD, RP, and glaucoma. However promising, most of these studies were conducted with natural estrogens and are subject to criticisms that have led to a fear of HT within the general population. An alternative approach would exploit the neuroprotective virtues of estrogens while attenuating their classic receptor mediated feminizing effects. One of the key receptor-independent mechanisms by which estrogens afford neuroprotection occurs through scavenging and resonance stabilization of free radicals. Below we present our findings based on the screening of such drugs in an *in vitro* study of glutamate-induced cytotoxicity of RGC-5 rat retinal ganglion cells as a model for glauocomatous RGC death.

The Future of Neuroprotection

Oxidative stress is recognized as a fundamental pathogenic mechanism of RGC death in glaucoma (Kumar et al., 2007; Sacca et al., 2007; Tezel et al., 2007). Conversely, estrogens have been identified as potent antioxidants (Prokai et al., 2003,2006,2007; Blair 2006; Vina et al, 2006; Singh et al., 2006). Simple principles of organic chemistry suggest that synthetic estrogens have a future as designer neuroprotective drugs. Key to their receptor-independent neuroprotective effects is their ability to function as direct antioxidants by quenching free radicals and terminating their propagation (Czlonkowska et al., 2003; Behl et al., 1998; Behl et al., 2002). This mechanism involves donation of a hydrogen atom from the phenolic group found on the A ring of natural estrogens (Figure 2B), to the free-radical (Figure 2C), resonance stabilizing the free radical and terminating chain reactions (Figure 2D). This process leaves behind a phenoxyl radical that can be regenerated *in vivo* (Prokai et al, 2006). Regeneration of phenolic antioxidants occurs via three known pathways utilizing ascorbic acid, glutathione-dependent free-radical reductase, and a recently discovered NADPH-mediated reductive aromatization (X in Figure 2) (Packer et al., 1979;McCay et al., 1989;Prokai et al., 2003). This mechanism is key to their function as direct antioxidants.

Based on these principles, the efficacy of synthetic estrogen derivatives designed to harness the antioxidant capabilities of estrogens without their receptor mediated feminizing effects will be the choice of future neuroprotective drugs in this class of compounds. Prodrug quinolic variants of some compounds have also been shown to attenuate estrogen receptor binding and is believed to enhance the ability of the

compound to traverse the lipid bilayer (Figure 2A) (Prokai et al., 2003). The studies were conducted based on previously established protocols for treatment of RGC-5 with glutamate to induce an increased oxidative burden to examine the neuroprotective efficacy of novel compounds (Kumar et al., 2005). The drug ZYC-3 was identified as the most effective neuroprotectants and its mechanisms of action are the subject of a portion of our work in this field.

Conclusions

Estrogens clearly impact the health of the retina. Current evidence indicates that estrogen receptors are found throughout the retinal thickness, concentrated prominently in the ganglion cell and nerve fiber layers. At least in part, receptors mediate the influence of estrogens on blood viscosity and ocular vascularity. Estrogens and female reproductive factors may also impact IOP and the development of OAG. The observation that estrogens may protect against degenerative vision loss in RP, AMD, and glaucoma is suggestive of their retinal neuroprotective capabilities, but also paves the way for the development of non-feminizing estrogen-like drugs that can be used in patients regardless of sex or health predispositions. Although not all novel drugs examined produced complete protection, even partial protection shows that outcome directed drug design can be an effective method of developing neuroprotecitve drugs. These results may then be used to refine the design of compounds to improve their efficacy. Utilizing the capabilities of medicinal, quantitative, and computational chemistry to model free radical scavenging and resonance stabilization will the refine the design of synthetic estrogens. Reexamination of such compounds in selective high throughput *in vitro* experiments could rapidly produce a host of efficacious drugs.

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Figure Legends

Table 1. Examination of the neuroprotective efficacy of estrogens and analogues. Maximal efficacy, with dose of drug, was examined in a glutamate-induced cytotoxicity model of *in vitro* retinal ganglion cell death utilizing the RGC-5 cell line. This screening process was used to guide drug selection and design, and to identify highly efficacious compounds for detailed studies of mechanism of action.

Figure 1. Activation and inhibition of signal transduction cascades that promote cell survival.

The modulation of key signaling cascades was examined at 24 hours post glutamate treatment as a parallel assessment of cell death versus survival along with prior data by Western blot analyses using phospho-specific antibodies against signaling proteins. The standard treatment of cells used in all prior experiments, with corresponding controls, were applied here. Changes in phosphorylation status of these signal transduction intermediates was interpreted as a modulatory effect.

Figure 2. Termination, Stabilization, and Recycling of free-radical by phenolic estrogen derived drugs. (adapted from Prokai et al., 2007)

Structure A represents the quinolic ring that can spontaneously convert to phenolic ring found in the structure of estrogens. The phenol (B) can then scavenge free-radicals (C) and resonance-stabilize them (C-D) until reduced (X). Reduction of phenoxy radicals (C) is an enzyme-mediated process utilizing ascorbic acid, glutathione-dependent free radical

reductase, and a newly discovered NADPH-mediated reductive aromatization.

Figures

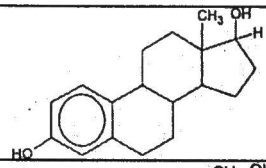
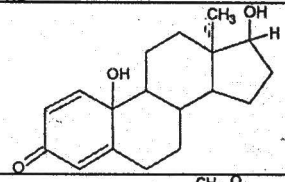
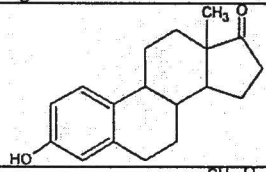
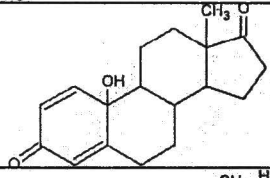
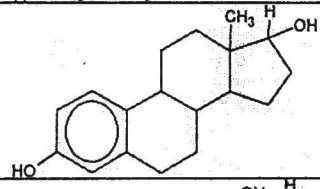
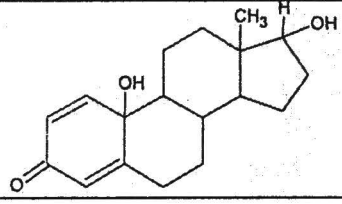
Compound	Structure	Maximum efficacy (% Control Cell viability)	Dose producing Maximal efficacy
17β-estradiol (E2)		100%	1μM
E2Q		75%	2μM
Estrone (E1)		100%	1μM
E1Q		75%	2μM
17α-estradiol		25%	2μM
17α-estradiol-quinol		60%	2μM

Table 1

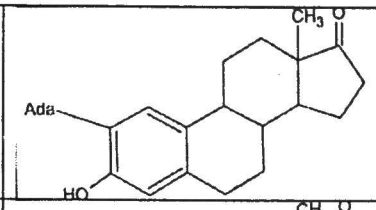
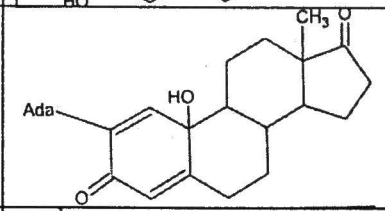
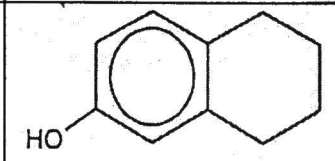
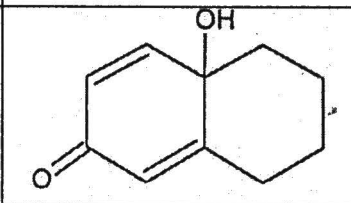
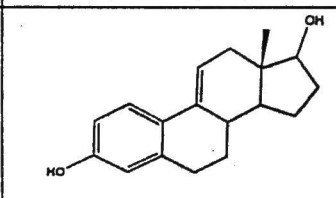
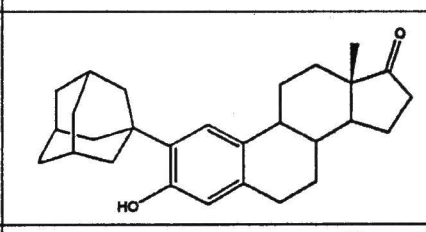
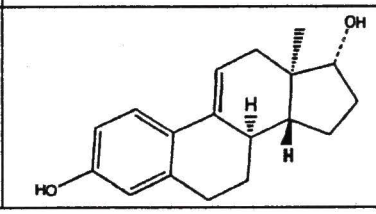
ADAE1		100%	1μM
ADAE1-Q		25%	1μM
THN		10%	1μM
THNQ		40%	1μM
ZYC-1		75%	0.5μM
ZYC-3		100%	1μM
ZYC-10		45%	1μM

Table 1

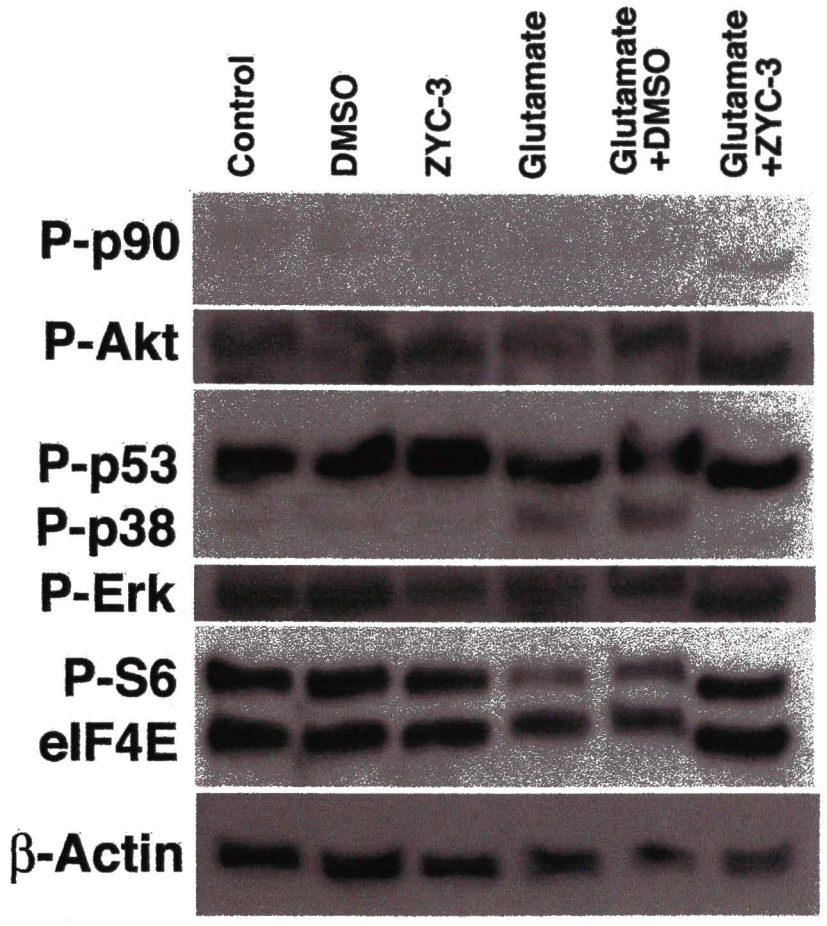

Control
DMSO
ZYC-3
Glutamate
Glutamate +DMSO
Glutamate +ZYC-3
P-p90
P-Akt
P-p53
P-p38
P-Erk
P-S6
eIF4E
β-Actin

Figure 1

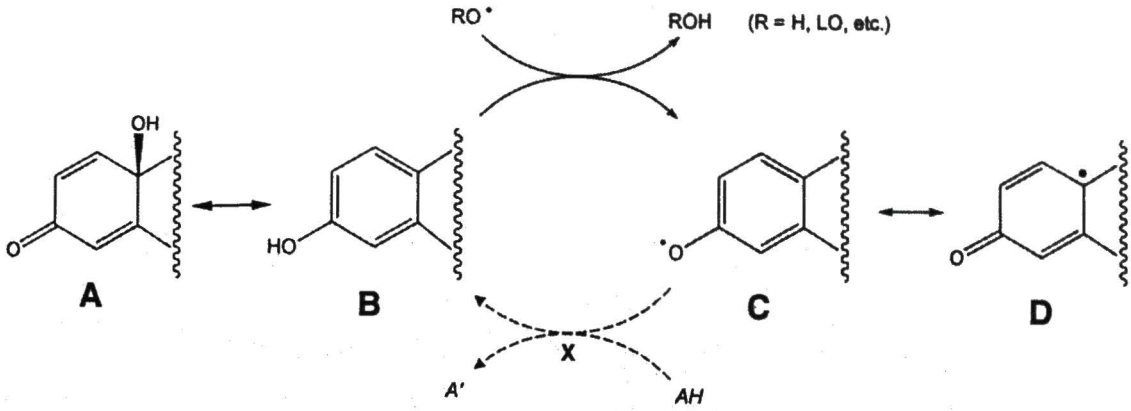


Figure 2

PREFACE TO APPENDIX D

The previous chapter examined the role of estrogens in retinal health from the perspective of glaucomatous neuroprotection. Non-feminizing estrogen analogues were proposed as neuroprotectants. Here we more closely examine the potential role of synthetic estrogens in optic neuropathies.

APPENDIX D

Non-Feminizing Estrogen Analogues as Neuroprotectants in Optic Neuropathies

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Retinal ganglion cell death has been determined to be the final common pathway in glaucoma. The continuous loss of retinal ganglion cells results in irreversible progressive visual field deterioration that culminates in blindness, yet no effective therapy is currently available to reverse retinal ganglion cell loss. Therefore, preventing retinal ganglion cell death is a logical approach to maintaining vision in effected individuals. Neuroprotection has been investigated as a therapeutic option for a host of neurodegenerative diseases including ocular disorders such as glaucoma (Kuehn et al., 2005). One potential medical intervention that provides a great deal of promise is neuroprotection of retinal ganglion cells with estrogens and synthetic estrogen analogues. Although the larger body of data demonstrates that such an approach could have a tremendous impact on how glaucoma and ocular diseases are treated, basic science and clinical ophthalmologist have been delayed in their investigation of this treatment paradigm.

The neuroprotective efficacy of estrogens was first demonstrated more than a decade ago and has since spurred a flurry of research into steroid hormones and neuroprotection. It is believed that the intrinsic neuroprotective capacity of estrogens is within the phenolic A ring, a structural component of estrogens. As proposed by Green et al. (2000) the phenolic A ring hypothesis for the neuroprotective effects of estrogens are based upon the following observations. First, the structure-activity relationship shows that a phenolic A ring and at least two additional rings are required for neuroprotection, but the structural requirements for feminizing sex steroid activity are more stringent. Second, neuroprotection with phenolic A ring compounds may occur in cells that lack

estrogen receptors and are not attenuated by estrogen receptor antagonists. Third, phenolic A ring containing estrogen compounds may rapidly activate signal transduction pathways that are known to be involved in cellular homeostasis. Finally, *in vivo* treatment with estrogens results in a neuronal type-independent neuroprotection from ischemic insults. The potential mechanisms of action that may be involved in the neuroprotective effects of phenolic A ring containing estrogen compounds include, but are not limited to: 1) estrogen-mediated free radical termination and cycling that potently inhibits oxidative stress, 2) interactions with estrogen receptor mediated and estrogen receptor independent anti-apoptotic signal transduction pathways, 3) induction of anti-apoptotic proteins, 4) regulation of intracellular calcium concentrations (Green et al., 2000). These neuroprotective mechanisms become even more diverse when examining receptor independent estrogens, the non-feminzing estrogen analogues.

In light of the growing body of data investigating the effects of conjugated equine estrogens on women's health (Women's Health Initiative Study, WHI), the role for non-feminizing, estrogen receptor independent neuroprotectants becomes more intriguing. However, natural estrogens still form the basis from which such investigations have been initiated and the results of these studies are compelling.

Green et al. (2001) demonstrated that a synthetic enantiomer of 17β-estradiol (Ent-E2) can be at least as effective and potentially more potent than natural 17β-estradiol (E2) at completely attenuating SK-N-SH neuroblastoma cell loss when exposed to H_2O_2. Ent-E2 was also effective at reducing rodent middle cerebral artery occlusion-induced lesion size by 60%. Furthermore, Ent-E2 did not stimulate uterine growth,

indicating that the *in vitro* and *in vivo* neuroprotective effects can be disassociated from the estrogenic effects of E2 (Green et al., 2001).

These findings were corroborated and expanded by Simpkins et al in 2004. By introducing chiral modifications or by adding bulky groups on the A-ring of the estrogen backbone, the estrogenicity of the novel compounds was reduced or eliminated (Figure 1). These synthetic estrogens were then screened in an *in vitro* model to identify the neuroprotective analogues. Those estrogen analogues identified with neuroprotective capacity were then utilized in a rodent model of stroke induced via transient middle cerebral artery occlusion (MCAO). One such compound reduced the ischemic volume by more than 50% and was even more effective than the naturally occurring prototype E2. In this study, modification eliminated estrogenicity of the parent steroid while enhancing its neuroprotection. This allows for neuron protection without the potential associated side effects of chronic hormone use. Additionally, such drugs would allow both men and women to be treated without the fear of the feminizing effects of naturally occurring estrogens (Simpkins et al., 2004). In yet another neuronal model, Cordey et al demonstrated that removing two of the usual three double bonds in the A-ring of the estrogen backbone significantly reduced the new estren compounds' affinity for estrogen receptors α and β, and did not exert proliferative effects on reproductive tissues. These compounds were then demonstrated to be effective in protecting primary cultures of rat cerebrocortical neurons against β-amyloid toxicity, an Alzheimer's disease related insult. Curiously the effect of these non-receptor binding estrens was dependent upon the rapid and transient activation of the protein kinase C signaling pathways (Cordey et al, 2005).

As polycyclic phenols, the synthetic non-receptor binding estrogen analogues do not interact with estrogen receptors α and β making their likely mechanism of action non-genomic. Dykens et al. (2003) demonstrated that such compounds work in part by attenuating ATP losses and allowing the maintenance of mitochondrial membrane potential in neuronal cell lines against *in vitro* insults. These results emphasize that synthetic estrogen analogues, without affinity for estrogen receptors, may still be very effective at protecting neuronal metabolic pathways through a mitochondrial mechanism (Dykens et al., 2003).

Although the compounds discussed thus far have all been phenols, the quinolic form of estrogens may also confer neuroprotection with reduced estrogenicity. Prokai et al demonstrated the quinol of estrone (E1-Q) inhibits the formation of H_2O_2 in rat brain homogenates and reduces the ischemic lesion volume in transient rodent MCAO. It is believed that the quinols of estrogens are part of a unique NADPH requiring redox cycle that allows estrogens to access the large reducing potential of cells and apply it to the savaging of membrane lipid radicals (Prokai et al., 2003).

Two studies directly address the use of synthetic estrogens in neuroprotection of the retina. The first of these utilizes the transgenic rat model of human rhodopsin S334ter mutant that induces rapid photoreceptor degeneration. In this study Dykens et al., demonstrated that the proprietary compound MITO-4565 reduced gross lipid peroxidation approximately three-fold, which is further reduced with the inclusion of exogenous glutathione. Furthermore, photoreceptors cells in the S334ter mutant were protected and the outer nuclear layer thickness of the retina was maintained *in vivo* by

MITO-4565. Treatment of RGC primary cultures with MITO-4565 protected the cells from NMDA mediated excitotoxic cell death (Dykens et al., 2004). The final study addressed the efficacy of synthetic non-receptor binding estrogen analogues in an *in vitro* model of glutamate-induced RGC death. In this study we demonstrated that three synthetic non-receptor binding estrogen analogues effectively inhibited lipid peroxidation, had no proliferative effect, conferred neuroprotection of RGCs inspite of estrogen receptor antagonism, and had little appreciable affinity for estrogen receptors α and β. Importantly, the surviving RGCs maintained the morphology and cellular density of healthy, uninsulted cells (Kumar et al., 2005).

In conclusion, these studies provide the basis for the investigation of estrogens and synthetic estrogen analogues in the neuroprotection of retinal neurons. Furthermore, the *in vivo* studies provide compelling data for the pursuit of their efficacy in primates and eventually clinical use in humans. Understanding how these drugs regulate cellular destructive and protective mechanisms could lead to further innovations in drug design and in methods to prevent retinal ganglion cell degeneration. *In vivo* studies of these drugs may then form the bridge to a better clinical approach to managing ocular disorders in which retinal neurons are lost.

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Figure Legends

Figure 1: **(A)** 17β-Estradiol is the natural prototypical estrogen used in neuroprotective studies. By convention the rings are lettered. The 'A' and 'B' rings are believed to be the estrogen receptor binding structures. These rings are the targets for synthetic modification to alter estrogen receptor affinity and enhance antioxidant capacity. **(B)** ZYC-3 is a synthetic variant of estrone. The addition of a bulky electron donating adamantly group in the C2 position, ortho to the hydroxyl moiety, on the 'A' ring of estrone serves as an example of structural modification to attenuate affinity for estrogen receptors as well as enhance the ability of the compound to scavenge free radicals.

Figure 2: Conversion of the quinol to phenol. NADPH serves as a critical cofactor in the intracellular conversion of quinolic estrogens to phenolic estrogens. These phenolic estrogens may then scavenge free radicals and can be recycled by the cells antioxidant defense mechanisms. Adapted from Prokai et al., 2003.

Figures

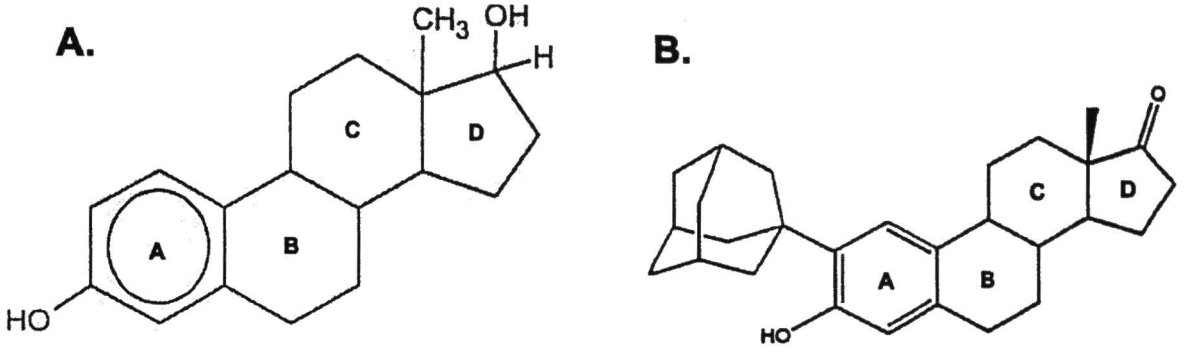

Figure 1

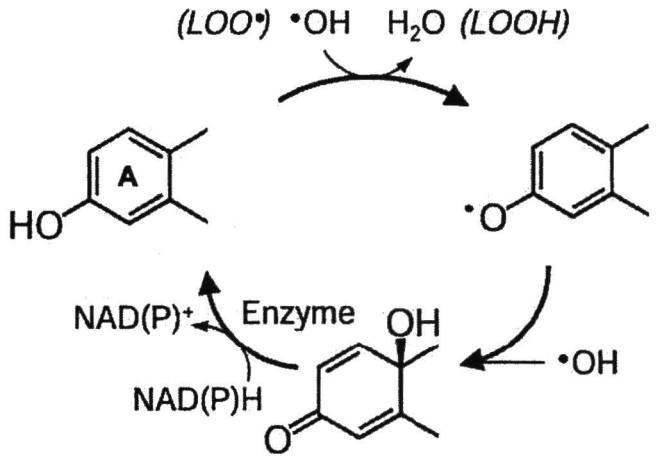

Figure 2