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Purpose: Glutamate has been implicated in many pathologies affecting the Central Nervous System including those in the retina, but the exact nature of the role of glutamate in neuronal degeneration remains unclear. In the retina, Müller cells are resistant to glutamate insults that are normally toxic to other cells of the retina, however the molecular and biochemical mechanisms that control their death or survival are not well understood. We used a series of pharmacological inhibitors and molecular biology agents on cultured Müller cells to dissect two key signaling pathways normally involved in cell survival, the Mitogen Activated Protein Kinase - Extracellularly Regulated Kinase (MAPK(ERK) pathway and the Phosphatidylinositide 3 Kinase (PI3K) pathway. Since preliminary data in our laboratory showed that Müller cells upregulate their secretion of neurotrophins including Brain Derived Growth Factor (BDNF) in response to glutamate treatment, we also examined the effect of BDNF on the activation of these two signaling pathways. **Methods:** Early passaged Müller cells were treated with various concentrations (5 nM – 50 μM) of inhibitors of the MAPK(ERK) pathway (GW5074, U0126, and PD98059) or with various concentrations (1 – 50 μM) of inhibitors of the PI3K pathway (LY294002 or Akt inhibitor) in the presence and absence of 50 ng/ml of BDNF for 24 hours. These experiments were repeated in Müller cells transfected with either NFκB or Bcl2 DNA. Cell cultures were then analyzed for surviving cells with an

MTS/PMS assay, a colorametric method for determining the number of viable cells in a proliferation assay. **Results:** The MAPK(ERK) inhibitors PD98059 and GW5074 both resulted in decreases in Müller cell survival. PD98059 did not decrease Müller cell survival until concentrations were high enough to suppress ERK2 phosphorylation. Müller cells transfected with NFκB or Bcl2 DNA were able to resist treatment with concentrations of PD98059 that reduced cell number in untransfected cells. The PI3K inhibitor LY294002 also resulted in significant decreases in Müller cell survival in both untransfected cells and cells transfected with NFκB or Bcl2 DNA. Treatment with an inhibitor farther down in the PI3K pathway, Akt inhibitor, did not significantly decrease Müller cell survival. Finally, BDNF was not able to increase cell survival in Müller cells treated with PD98059 or U0126, although it did increase the survival of cells treated with GW5074. BDNF was also able to reverse the decrease in cell survival caused by LY294002 in both untransfected Müller cells or Müller cells transfected with NFκB or Bcl2 DNA. **Conclusions:** Our data shows that Mitogen Activated Protein Kinase - Extracellularly Regulated Kinase (MAPK(ERK) and Phosphatidylinositide 3 Kinase (PI3K) are both essential for Müller cell survival. There is modulation between the pathways and they may be interconnected far upstream at a protein previously associated with only the MAPK(ERK) pathway. These results are consistent with a role for both pathways in Müller cell survival.

BRAIN DERIVED NEUROTROPHIC FACTOR REGULATES MÜLLER CELL SURVIVAL VIA MAPK AND PI3K PATHWAYS

Sara A. Taylor, B.S.

APPROVED:

Major Professor

Committee Member

Committee Member

Committee Member

University Member

Chair Department of Cell Biology and Genetics

Dean, Graduate School of Biomedical Sciences

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THESIS

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Sara A. Taylor, B.S.

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CHAPTER I

INTRODUCTION AND BACKGROUND

Introduction

Numerous studies implicate glutamate, the principal neurotransmitter in the brain and spinal cord, in diverse pathologies affecting the Central Nervous System (CNS), including those in the retina (55, 45). However, in spite of ample evidence supporting a role for glutamate toxicity in many of these diseases, the exact nature of the role of glutamate in the degenerative changes to CNS neurons remains to be elucidated. Similarly, in the retina, glutamate appears to be most lethal to retinal ganglion cells (RGCs) as compared to other retinal cell types (55, 66), and yet its role in the pathophysiology of glaucoma, a retinal disease primarily affecting RGCs, remains in question. This is attributed mainly to findings that while glutamate exhibits direct toxicity to RGCs in vitro and in vivo, glutamate levels in glaucomatous retinas remain below toxic levels (6). To begin to resolve these conflicting reports with regards to the role of glutamate in the development of glaucoma, we focused our investigations on the retinal Müller cells, the retinal cell type involved in the processing of extracellular glutamate in the retina. We hypothesized that Müller cells, which are responsible for uptake and detoxification of toxic levels of extracelllular glutamate might mediate the

toxic effects of glutamate on RGCs. Understanding the effects of glutamate on retinal Müller cells could shed light on this seeming paradox.

Müller cells, the predominant glial cell type in the retina, span the entire thickness of the mammalian retina with their processes interdigitating with retinal neurons including RGCs (39). Such intimate contact between Müller cells and retinal neurons suggests a protective function for Müller cells in the retina. Indeed it has been well-documented that glutamate/aspartate transporters (GLAST) in Müller cells are responsible for removal of glutamate from the synaptic space, and thus contribute towards keeping extracellular glutamate concentrations low (30). In the retina, glutamine synthetase, an enzyme involved in transforming glutamate to glutamine, is primarily expressed in Müller cells (21). Studies have also shown that glutamate toxicity to RGC can be modulated by the presence of Müller cells in co-culture (21, 30, 45) such that the presence of Müller cells results in increased numbers of surviving RGCs during glutamate treatment.

The Mammalian Retina

The eyeball is a spherical structure that is divided into three tunics or layers: the outermost fibrous tunic (the cornea and the sclera); the middle vascular tunic (the choroid, ciliary body and iris), and the innermost nervous tunic (the retina) (Fig. 1a). The sclera, the superficial coat of the posterior part of the eyeball, is a tough, avascular layer consisting of collagen fibers and fibroblasts that gives the eyeball its shape. The anterior portion of the sclera becomes the transparent cornea to allow light into the eyeball. The middle layer of the eyeball actually consists of three parts: the choroid, the ciliary body,

and the iris. The choroid is the posterior portion of the vascular tunic and underlies most of the sclera. It is highly vascularized and provides nutrients to the posterior surface of the retina. Anteriorly, the choroid becomes the ciliary body, which consists of ciliary processes and ciliary muscle. The iris consists of circular and radial smooth muscle fibers enclosing a hole in the center known as the pupil. The function of the iris is to control the amount of light entering the eyeball by constricting and dilating the pupil. The retina or the innermost layer of the eyeball lines the posterior portion of the choroid. The retina consists of ten layers, from outermost to innermost they are: the retinal pigment epithelium (RPE), the inner and outer photoreceptor segment layer, the outer limiting membrane (OLM), the outer nuclear layer (ONL), the outer plexiform layer (OPL), the inner nuclear layer (INL), the inner plexiform layer (IPL), the ganglion cell layer (GCL), the nerve fiber layer (NFL) and the inner limiting membrane (ILM) (Fig 1b). There are six types of cells located in the ten layers of the retina, excluding the neuroglia. The RPE layer is made up of pigmented epithelial cells that are in close contact with the rods and cones of the photoreceptor cells. The soma of the photoreceptor cells are located in the ONL and they synapse with processes of the horizontal and bipolar cells in the OPL. The soma of the horizontal, bipolar, amacrine cells are located in the INL. These cells in turn synapse with the processes of the RGC in the IPL, the soma of the RGCs being in the GCL.

Retinal Müller Cells

Neurons have traditionally been viewed as the active cells of the nervous system, responsible for conducting nervous impulses that form networks to process sensory information and initiate motor action. Another group of cells that form a supportive matrix to the neurons have been recognized and termed neuroglia. In 1851, Heinrech Müller described a radiating cell in the vertebrate retina that is now known as the Müller cell. Heinrech Müller recognized that these cells were probably not neurons, but he was making his observations several years prior to the concept of a neuroglial component in nervous tissue (49). Today, the neuroglia are a recognized cellular component of the nervous system that actually far outnumber the neurons. The neuroglia were long believed to be a mostly passive collection of accessory cells that provided physical and perhaps some metabolic support to neurons, but their role in the nervous system is probably much more extensive

Most vertebrate retina have three types of glia - Müller cells, astrocytes, and microglia (3). A fourth glial cell type - oligodendrocytes are found in rabbit retinas, a species with myelinated ganglia in the retina (13). Müller cells are the predominant glial cells in the retina, comprising about 90% of the total retinal glia. They are oriented radially, with their soma in the INL and their processes extending in both directions to span the entire thickness of the retina and interdigitating and forming extensive contacts with soma and processes of retinal neurons especially with photoreceptor cells and RGCs (39, 59). Such extensive contacts between the Müller cells and retinal neurons suggest a supportive, or even protective, function for Müller cells. Indeed, Müller cells are

purported to be protective to retinal neurons, although this has not been fully elucidated. A major function of Müller cells is to remove extracellular glutamate via Excitatory Amino Acid Transporter 1, also known as EAAT1 or glutamate/aspartate transporters also known as GLAST (12, 38, 66). Müller cells are believed to act as a cellular sponge, soaking up glutamate from the synaptic space. Once glutamate has been taken up into the Müller cell, glutamine synthetase converts glutamate to a relatively non-toxic amino acid, glutamine. Since excessive glutamate is believed to be excitotoxic, this Müller cell function serves to protect various neurons in the retina.

Müller cells have been shown to display neuroprotective functions exhibited by astrocytes and microglia in other parts of the CNS. Neuronal cultures (unrelated to Müller cells) that were incubated with microglial conditioned medium and then exposed to glutamate showed an increase in survival over neurons exposed to glutamate alone (68). RGCs co-cultured with Müller cells have also been shown to be more resistant to glutamate insult than neurons cultured by themselves alone (30). This protective function may be due to the Müller cell's ability to remove glutamate from the synaptic space, but might also be due to paracrine effects of growth factors, such as neurotrophins, secreted by Müller cells. Astrocytes and microglia have previously been shown to increase their production of neurotrophins after ischemic insult (54) or in response to glutamate or ischemic stress (44, 52), respectively.

Glutamate

Glutamate is an excitatory amino acid neurotransmitter in the central nervous system (CNS); it is the principal neurotransmitter for fast excitatory signaling (42). Glutamate has been implicated in neuronal death found in several pathologies of the CNS including Amyotrophic Lateral Sclerosis (ALS), hypoxic-ischemic brain injury (7), epilepsy (62), Huntington's disease (57), Parkinson's disease (33) and Human Immunodeficiency Virus (HIV) related dementia (14). Diseases of the retina in which glutamate toxicity appears to play a role include glaucoma, trauma to the optic nerve and diabetic retinopathy (55, 45). Although glutamate has been indirectly implicated in many CNS pathologies, its detrimental role remains to be more precisely elucidated.

In neurons, glutamate toxicity is mediated by Ca^{++} (55, 66). Normally, the intracellular Ca^{++} is maintained at very low levels (~ 0.1 μM) and deviation from physiologic levels can affect many different targets. Persistent glutamate activation of N-methyl-D-aspartic acid (NMDA) receptors with simultaneous membrane depolarization (a result of glutamate's role as a ligand for α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors with their associated Na^{+} channels) leads to a prolonged opening of N-methyl-D-aspartic acid receptor (NMDAR) channels and permits a massive Ca^{++} influx (19). The depolarization that results from Na+ influx through AMPA receptors is also believed to allow additional Ca++ entry into cell through voltage dependent Ca^{++} channels (VDCCs) (5). NMDA receptor activation and concurrent VDCC permeability trigger Ca^{++} dependent responses in cell which either stimulate protective mechanisms to the cell (2^{nd} messenger signaling) or promote injury and cell death.

Second messenger signaling that enhances cell survival includes the activation of Ca^{++}/Calmodulin protein kinases resulting in the phosphorylation/activation of cAMP response element binding protein (CREB), a transcription factor that upregulates survival proteins (56). Brain-derived neurotrophic factor (BDNF) DNA binding to Camkinase IV-phosphorylated CREB promotes increased expression of BDNF mRNA (27). Ca^{++} dependent apoptotic responses include mitochondrial damage and activation of proteases, endonucleases and Nitric Oxide Synthase (NOS) (29).

Neurotrophins

Neurotrophins consist of a family of growth factors that are similar in molecular structure and elicit similar responses in target cells. These include BDNF, nerve growth factor (NGF), neurotrophin-3 (NT3), and neurotrophin-4 (NT4). While their target cells may differ, neurotrophins can be responsible for differentiation, outgrowth, synaptic modulation, gene regulation, and survival in neurons when they bind to their high affinity receptors (17). The high affinity receptors for BDNF, NGF, NT3 and NT4 are tyrosine kinase receptors called TrkB, TrkA, TrkC and TrkB, respectively. The low affinity neurotrophin receptor is also a death receptor called p75NTR that lacks the kinase domain of the Trk receptors, having a death domain instead (23). In the absence of their high affinity receptor, neurotrophins can bind to p75NTR, causing the activation of cell death machinery (23), but p75NTR may have a more dynamic role in cell death mediated by early precursor forms of neurotrophins.

Recently various cells of the CNS such as cortical neurons and the RPE have been shown to produce an early precursor form of neurotrophins called proneurotrophins. These proforms can be processed (cleaved) extracellularly by proteases such as plasmin and matrix metalloproteases to produce the smaller mature neurotrophin forms (35). Lee et al. (2002) showed that proforms of NGF and BDNF bind p75NTR with greater affinity than do the mature forms and resulted in apoptosis in sympathetic neurons. As part of our data we will show that under certain conditions Müller cells increase their expression of high molecular mass neurotrophin proteins that very likely are proneurotrophins.

Survival Pathways: MAPK and PI3K

Phosphatidylinositide 3 Kinase (PI3K) and Mitogen Activated Protein Kinase (MAPK) have both been implicated in neuronal survival. Both pathways mediate a number of anti-apoptotic responses in the cell (Fig 1c). pAkt in the PI3K pathway and pERK in the MAPK(ERK) pathway can both phosphorylate Bad (26) as well as activate nuclear factor kappa B (NFκB) and CREB (2). Activated CREB promotes the transcription of survival factors such as Bcl-2 (16) and NFκB has been shown to suppress the signals for cell death (41). There is considerable crosstalk between these two pathways. PI3K appears to be able to modulate the MAPK(ERK) pathway with possible substrates including Raf and MEK (32, 61). Conversely, Akt has been shown to have an inhibitory effect on Raf (32). Proteins in the MAPK pathway can have regulatory effects on the PI3K pathway as well. For instance, Ras has been shown to activate PI3K (16, 32).

MAPK(ERK) signaling pathway

MAPK cascades are well conserved and have a key role in the regulation of gene expression. MAPK cascades are organized in a three kinase hierarchy consisting of a MAPKKK (a MEK activator, Raf in the MAPK(ERK) pathway), a MAPKK (a MAPK activator, MEK in the MAPK(ERK) pathway), and a MAPK (ERK in the MAPK(ERK) pathway)(32). In mammals, there are 5 MAPKs - ERK1/2, JNK MAPK, P38 MAPK, ERK 3 and ERK 5 (10). ERK 1/2 regulates cell differentiation, proliferation and survival, JNK MAPK and p38 MAPK regulate the stress response, inflammation and apoptosis, ERK 5 is believed to regulate proliferation and the function of ERK 3 remains unclear (50). However in different cell types or under the influence of different physiological conditions MAPK is capable of inducing different cell reactions. For example, ERK generally controls proliferation while JNK usually mediates the inflammatory response to stress. However, in some cells, JNK has been shown to induce proliferation (50). Temporal limitations may also control the activity of MAPKs. In PC12 cells, transient activation of ERK results in proliferation, while persistent ERK activation results in cell differentiation (50). Thus it can be seen that the regulatory mechanisms of MAPK signaling as well as its interactions with other cell signaling pathways are complex and varied with a great deal of overlap.

PI3K(Akt) signaling pathway

The Phosphatidylinositide 3 Kinase (PI3K) pathway is a complex signaling pathway that is crucial to anti-apoptotic function in the cell (Fig 1c). PI3K functions as both a

lipid and a protein kinase (61). As a lipid kinase, PI3K phosphorylates the 3'-OH of the inostitol ring of an inositol phospholipid (PIP, PIP2, or PIP3) which in turn modifies/activates protein kinase-B (PKB) also known as Akt (61, 27). Phosphorylated/activated Akt has been shown to act on many substrates to inhibit apoptosis. In many cases, Akt phosphorylation inhibits proteins that are proapoptotic in their unphosphorylated state (such as Bad) while in some cases, phosphorylation by Akt activates proteins that are anti-apoptotic. Examples of this latter function include the NFκB and CREB transcription factors (63). The phosphorylation and activity of NFκB can lead to increased expression of antiapoptotic proteins and the phosphoyrlation of CREB can lead to upregulation in Bcl2 protein which has antiapoptotic function.

In addition to being a lipid kinase, PI3K possesses protein kinase activity (8). Putative subtrates include Raf and MEK (61, 32) signaling molecules in the MAPK(ERK) pathway.

Purpose of the Study and Hypothesis

Glutamate has been implicated in neuronal death in several pathologies of the CNS including ALS, Alzheimer's, hypoxic-ischemic brain injury, epilepsy, Huntington's, Parkinson's and HIV related dementia. Diseases of the retina in which glutamate toxicity appears to play a role include glaucoma, trauma to the optic nerve, and diabetic retinopathy (55, 45, 1).

Neuronal death associated with increased glutamate has been hypothesized to be due to the excitotoxic nature of glutamate (15), however Müller cells have long been known

to remove excess glutamate from the synaptic space, thereby lowering the extracellular levels of glutamate in the retina. Glutamate has been shown to be toxic to both neurons and glia in the CNS (45), but the effects of glutamate on Müller cells have not been fully investigated. This study was initiated to determine the effects of glutamate treatment on Müller cell survival. When we began the project, we assumed that glutamate would be lethal to Müller cells, however, Müller cells exhibited resistance to glutamate toxicity and glutamate-treated Müller cells increased their secretion of neurotrophins including BDNF. We, therefore, investigated the intrinsic survival mechanisms in Müller cells and the effect of BDNF on these survival pathways. The ***hypothesis*** was that BDNF activation of PI3K and MAPK pathways regulates Müller cell survival during stress to the cell. We investigated the following **specific aims** to answer the hypothesis:

1. To determine the role of the MAPK(ERK) signaling pathway in the survival of cultured Müller cells.
2. To determine the role of the PI3K signaling pathway in the survival of cultured Müller cells.
3. To determine the effect of BDNF on the activation of MAPK and PI3K signaling pathways in cultured Müller cells.

CHAPTER I FIGURES

Figure 1a. *Cross section of the human eye showing the anatomy of the eyeball.*

Figure 1b. *Cells and layers of the retina. The retina is arranged in ten layers consisting of six cell types – photoreceptors, horiazontal, amacrine, bipolar, retinal ganglion cells and Müller cells.*

Figure 1c. *Diagram of the MAPK(ERK) and PI3K pathways demonstrating mechanisms of survival and crosstalk between the pathways.*

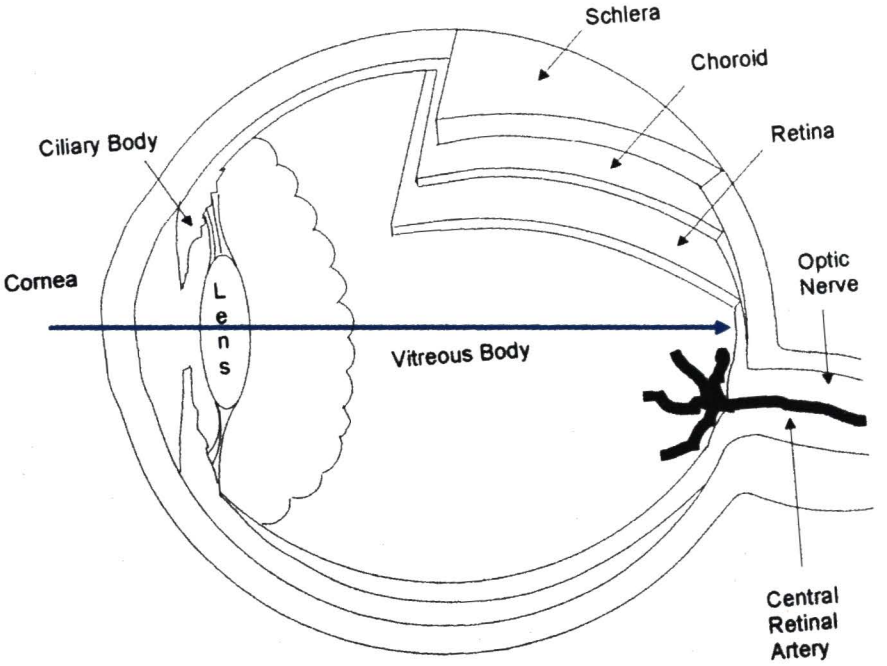


Figure 1a. Cross section of the human eye
(Modified from Fundamental Neuroscience, Churchill Livingstone. Inc, Pub. 1997)

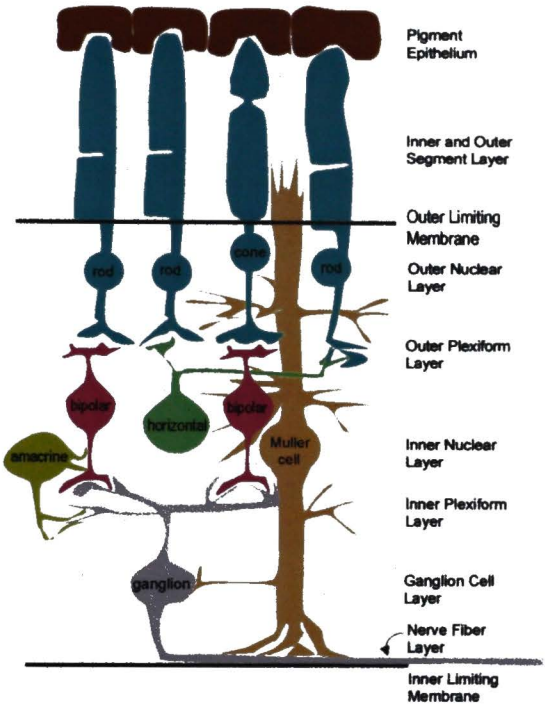


Figure 1b. Cells and layers of the retina

(Modified from Fundamental Neuroscience, Churchill Livingstone. Inc, Pub. 1997)

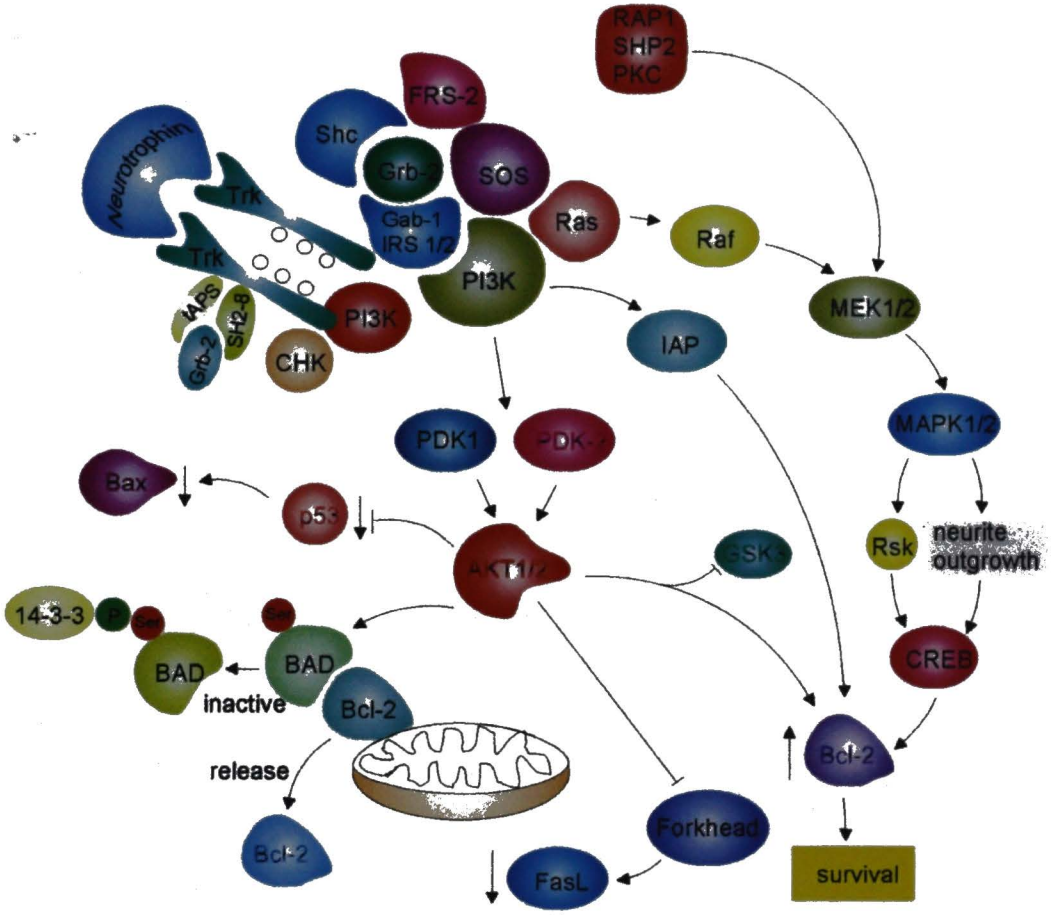


Figure 1c. MAPK(ERK) and PI3K signaling pathways

(Modified from Cell Signaling Technology, Inc. Copyright 2000).

CHAPTER II

MATERIALS AND METHODS

Müller Cell Cultures

The Müller cells used for this project were from an established cell line. Sprague-Dawley rats, 8-10 days old, were sacrificed in accordance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 80-23). Eyeballs were removed and Müller cells isolated as described previously (47). Briefly, eyeballs were soaked in serum-free DMEM (Delbeccos' Modified Eagles Medium with 4 mM L-glutamine, 1 mg/ml glucose, 100 units/ml of penicilllin, 100 μg/ml streptomycin and 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH 7.6 (HEPES) buffer for 12-16 hours followed by incubation in DMEM containing 1 mg/ml trypsin, 0.4 mg/ml ethylenediaminetetraacetic acid (EDTA) and 70 units/ml collagenase at 37^0 C for 1 hour. Retinas were isolated, separated manually by repeated pipetting and cultured in DMEM supplemented with 10% FBS (Fetal Bovine Serum). Cells were grown into confluence and passaged every 7 days. Aliquots from this cell line were frozen in 10%dimethylsulfoxide(DMSO)/70%DMEM/20%FBS and maintained in liquid nitrogen. Upon use the aliquots were thawed rapidly in a 37^0C water bath and plated on T25 flasks (Costar, Cambridge MA) in 5 ml DMEM supplemented with 10% FBS. Cultures were

grown in a humidified atmosphere of 95% air, 5% CO_2 at 37^0 C. Culture media and supplements were obtained from Sigma (St. Louis, MO).

Immunocytochemistry

To determine if the Müller cells used in this project continue to express Müller cell markers or produce endogenous neurotrophin proteins, immunocytochemistry was done. Müller cells were plated on 8 well Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL) at a plating density of 15,000/well in basal media (DMEM with 100 units/ml penicillin, 100 μg/ml streptomycin and 15 mM HEPES) supplemented with 10% FBS. Cells were allowed to attach overnight at 37^0 C in 5% CO_2 and then media supplemented with 10% FBS was removed and replaced with basal media. The next day, cells were washed with 0.01 M phosphate buffered saline (PBS) for 5 min and then fixed with 2.0% paraformaldehyde (Fischer Scientific, Pittsburgh, PA) in PBS for 20 min. Slides were washed with 0.01M PBS 3 times, and incubated in a blocking solution containing 5% normal goat serum (Pierce, Rockford, Ill.) for 1 hour. The goat serum was suspended in a 0.01 M PBS buffer containing 0.5% bovine serum albumin (Fisher Scientific) and .05% Triton-X 100 (Bio-Rad, CA). Blocking solution was removed and primary antibodies diluted in the blocking solution were used to determine the expression of cellular retinaldehyde binding protein (CRALBP) (Jack Saari, PhD), glial fibrillary acidic protein (GFAP) (Zymed, San Francisco CA), S-100 (Sigma Immunochemical, St Louis, MO), carbonic anhydrase C (CA) (Chemicon, Temecula CA), BDNF, NGF, NT3, and NT4 (Santa Cruz Biotechnology). Primary antibodies were diluted (1:200) in

0.01MPBS/0.5%BSA/0.5%TritonX. Primary antibodies were applied overnight at 4^0C, then washed 5 times with 0.01MPBS/0.5%BSA. Anti-rabbit or anti-mouse Alexa-fluor 488 labeled secondary antibodies (Sigma Immunochemicals, St Louis, MO) were diluted 1:200 in 0.01MPBS/0.5%BSA/0.5%TritonX and applied for 1 hour, then washed 5 times with 0.01MPBS/0.5%BSA and once with 0.01M PBS. The control was immunocytochemistry done without the primary antibody. Coverslips were applied to samples and viewed under a microscope with an epifluorescence attachment. Immunucytochemistry experiments were done at least 3 times to confirm results.

Cell counts (growth curve)

Müller cells were treated with glutamate (1mM) for an extended period of time, and then cell counts were done to determine the ability of Müller cells to survive chronic glutamate treatment. Müller cells were plated at a density of 20,000 cells/well on 12 well (surface area 3.8 cm^2) tissue culture plates (Costar, Cambridge, MA) in 1.0 ml basal media (DMEM with 100 units/ml penicillin, 100 μg/ml streptomycin and 15 mM HEPES)(Hy-Clone, UT) supplemented with 10% FBS (Hy-Clone). Cells were allowed to attach overnight at 37^0C in 5% CO_2. After being washed 2 times with basal media, cells were incubated in 1.0 ml fresh basal media in the presence or absence of 1.0 mM L-glutamate (Sigma Chemical, St. Louis MO) for the duration of the experiment. To compensate for glutamate uptake in the cultures, L-glutamate (1.0 mM) was added every 24 hours to glutamate-treated cultures, while the media was changed every 3 days. The numbers of surviving cells from triplicate wells were counted daily. Cells from triplicate

wells were incubated in 500 µl 0.02% EDTA (Sigma) for 5 min. at 37^0C. EDTA was removed and 200 µl PET (Panceatin, EDTA, Trypsin) was used to detach the cells from the wells. Within 5 min of addition of PET, 50 µl of FBS was added to stop dissociation. 250 µl of Trypan Blue (Sigma) was added to the trypsinized cells. An aliquot of this suspension was deposited on a hemocytometer underneath a coverslip, filling the counting grids. The number of cells were counted in each grid and the average number was multiplied by the dilution factor (2) and by 10^4 to obtain the number of cells/ml. To obtain a total cell count, the number of cells/ml was multiplied by the original volume of fluid of the cell sample (.25 ml). Cell count assays were done 4 times in triplicate to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

MTS/PMS assay

MTS assays were performed on Müller cells treated with various concentrations of glutamate (0.1 mM to 20.0 mM) to determine the effect of glutamate on Müller cell survival. This is based on a reaction between MTS and mitochondrial dehydrogenase produced by living cells.

$$\text{MTS} + \text{NADPH} \xrightarrow{\textit{Mitochondrial dehydrogenase}} \text{Formazan} + \text{NADP}$$

After the MTS reagent has been applied to the cells, the absorbance of formazan is measured on a spectrophotometer. PMS is an electron coupling reagent, it facilitates the transfer of electrons (H^+) from NADPH to MTS to yield formazan & NADP.

Separate proliferation assays were done on Müller cells or Müller cells transfected with Bcl2 or NFκB gene treated with inhibitors of cell signaling to determine the role of the components of key signaling pathways in Müller cells. Müller cells were plated on 96 well plates (Costar, Cambridge, MA) at a density of 10,000 cells/well in 100 µl basal media (DMEM with 100 units/ml penicillin, 100 µg/ml streptomycin and 15 mM HEPES) supplemented with 10% FBS. Cells were allowed to attach overnight at 37^0C in 5% CO_2. The next day, after being washed 2 times with basal media, cells were incubated in 100 µl basal media overnight (at least 17 hours). The next day cells were treated with glutamate (0.0 - 20.0 mM in 100 µl basal media) or LY294002 (10, 20, 50 µM), PD98059 (10, 20, 50 µM), U0126 (50, 100, 500 nM), Akt inhibitor (1, 5, 10 µM) or GW5074 (5, 10, 20 nM) in 100 µl basal media with each experimental condition being performed in triplicates. Glutamate concentrations were chosen to determine the effect of high concentrations of glutamate on Müller cell survival. Inhibitor concentrations were chosen to include treatments below and above the IC_{50} for each inhibitor. Since DMSO was the diluent used in the inhibitor reagents, DMSO controls at the most concentrated levels of DMSO used (1:1000) were run to show that DMSO did not have significant effect on Müller cell survival. K252a controls were also included in the assays to show that K252a at concentrations used in the experiment (100 nM) had no significant effect on Müller cell survival. After 48 hours of treatment cell survival was determined using a

non-radioactive cell proliferation (MTS) assay (Cell titer 96; Promega Corp., Madison, WI). Briefly, the basal media was removed from the cells and each well received 100 µl DMEM without phenol red (Hy-Clone), 20 µl (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS)(Promega Corporation, Madison WI) and 1 µl phenazine methosulfate (PMS)(Promega). The cells were allowed to incubate in the MTS/PMS for 1 hour at 36^0C. Absorbances were then read on a Vmax/kinetic microplate reader (Molecular Devices) at 490 nm at 15 min intervals for 1 hr. Background absorbance readings were used to correct test absorbance readings by preparing a triplicate set of control wells without cells containing the same volumes of culture medium and combined MTS/PMS solution as in the experimental wells. The average 490 nm absorbance from the control wells lacking cells was subtracted from all other absorbance values to yield corrected values. Absorbance readings were converted to cell counts based on standard curves. Standard curves were generated by plating cells in 96 well plates at a density of 1,000 to 40,000 cells/well in 100 µl basal media, allowing them to attach for 2 hours and then incubating them in MTS/PMS substrate solution for 60 min., with absorbance readings at 490 nm taken every 15 min. After it was determined which of the pathways were crucial for Müller cell survival, proliferation assays were repeated with the effective inhibitor in the presence of BDNF (50 ng/ml) or in the presence of BDNF and K252a (50 ng/ml and 100 nM respectively) as part of the experimental conditions. MTS assays were done at least 3 times in triplicate. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

Sample collection

Samples of conditioned media or cell lysate of Müller cells treated with various concentrations of glutamate (0.0, 0.1 and 1.0 mM) were collected for ELISA and Western blot analysis of proteins that we hypothesized might change expression due to glutamate treatment (neurotrophins, TrkB and NMDA receptors, glutamate transporters, pERK and Bcl2). Müller cells were plated at equal densities on 6 well plates (3.5 cm diameter, Corning Glass Works, Corning NY), T25 or T75 flasks (Costar, Cambridge, MA), in 1 – 15 ml basal media (DMEM with 100 units/ml penicillin, 100 μg/ml streptomycin and 15 mM HEPES) supplemented with 10% FBS. Cells were allowed to attach overnight at 370C in 5% CO_2 and grown to confluence. Cells were then placed in basal media overnight (at least 17 hours) and then the next day, were incubated in basal medium supplemented with various treatments (see specific experiments to follow) for 2 - 48h depending on the experiment. Conditioned media was collected for ELISA and Western blot. Conditioned media was concentrated (60X in Centricon centrifugal filter devices fitted with a YM3 filter (Millapore)) and protein concentrations measured with spectrophotometry using the Bio-RAD DC Protein Assay kit (Bio-RAD Laboratories, Hercules, CA) (see procedure to follow). Conditioned media had protease inhibitors added (1mM phenylmethanesulfonyl fluoride (PMSF), 1mM aprotinin, 1mM pepstatin A, and 1mM leupeptin) and was stored at -80^0 C until use. Cell lysate was collected for RT-PCR, immunoprecipitation, and Western blot. Cell lysate was collected by washing cells once with cold 1X PBS for 5 minutes. PBS was removed and replaced with 150 – 300 μl ice-cold protein lysis buffer. Cells were detached from flasks with cell scrapers (VWR

Scientific Products, NJ) and the cell lysate was placed in 1.7 ml microcentrifuge tubes (VWR Scientific Products, NJ). Cell lysate was sonicated in a 50 sonic dismembrator (Fisher Scientific, Pittsburgh, PA) for 10 seconds on ice. Before being used in RT-PCR, immunoprecipitation, and Western blot, the cell lysate had protein concentrations measured with spectrophotometry using the Bio-RAD DC Protein Assay kit (Bio-RAD Laboratories, Hercules, CA). Cell lysate was stored at -80^0 C until use. Protein lysis buffer: 50 mM Tris-HCl (pH 7.4), NP-40 1%, Na-deoxycholate 0.25%, NaCl 10 mM, EDTA 1 mM, PMSF 1mM, Aprotinin 1 μg/ml, Na3VO4 1 mM, NaF 1 mM.

Bradford (Bio-Rad) protein quantitation

Protein concentrations of conditioned media and cell lysate were measured to ensure equal loading of proteins during testing. Protein concentrations were measured with spectrophotometry using the Bio-RAD DC Protein Assay kit. (Bio-RAD Laboratories, Hercules, CA). 100 μl protein standard bovine serum albumin (BSA) or sample was pipetted into clean, dry test tubes. 500 μl of BioRad working reagent A (contains copper tartrate) was added and the mixture vortexed briefly, about 3 seconds. Next, 4.0 mL of BioRad working reagent B (contains Folin) was added and the mixture vortexed briefly, about 3 seconds. A blank was generated by adding only reagent A and reagent B, omitting the addition of protein. After a 15 minute room temperature incubation, the OD was measured on a Beckman Du64 spectophotometer at 750 nm, using the blank sample to calibrate the instrument to 0.000. To prepare a standard curve for the assay procedure, three dilutions of protein were made containing 5, 10, and 20 μg of BSA standard/ml.

Using Microsoft Excel, a standard curve was prepared by plotting the blank corrected absorbance readings from the standard samples on the y-axis versus the known protein concentration (5, 10, and 20 μg/ml) on the x-axis. Protein concentrations of the unknowns were determined by plotting the absorbance readings of the unknowns on the standard curve.

Reverse Transcritpion-Polymerase Chain Reaction

RT-PCR was done on RNA collected from Müller cells treated with various concentrations of glutamate (0.0, 0.1, and 1.0 mM) to determine the effect of glutamate treatment on endogenous neurotrophin message in Müller cells.

Total RNA extraction. Müller cells were plated on 6 well plates, 3.5 cm diameter (Corning Glass Works) at a plating density of 200,000 cells/well in 3 ml of basal media supplemented with 10% FBS. The cells were allowed to attach overnight at 37^0 C in 5% CO_2. The next day, the media was removed and replaced with 3 ml basal media supplemented with various concentrations of glutamate (0.0, 0.1, and 1.0 mM) and again incubated overnight. The following day total RNA from Müller cells was extracted using Ultraspec RNA isolation system (Biotecx Laboratories, Houston TX). The cells were lysed directly in the culture dish by first removing basal media and then adding 1 ml Utraspec RNA solution and passing the cell lysate through a pipette several times. The cell lysate was transferred immediately into 1.7 ml microcentrifuge tubes. The homogenate was then incubated for 5 minutes at 4^0 C. Next, 0.2 ml of chloroform/ml homogenate was added and the microcentrifuge tubes were shaken vigorously for 15

seconds before incubating on ice for 5 minutes. The homogenate was centrifuged at 12,000xg at 4^0 C for 5 minutes. After centrifugation, the homogenate formed two phases; a lower organic phase and the upper aqueous phase separated by an interphase. DNA and protein were in the organic phase and the interphase respectively, and the RNA was in the aqueous phase. The aqueous phase was transferred to a new microcentrifuge tube and an equal volume of isopropyl alcohol was added, taking care not to shake. The samples were incubated for at least 10 minutes at -20^0 C, and then centrifuged for 10 minutes at 12,000xg at 4^0 C. The RNA precipitate formed a white pellet at the bottom of the microcentrifuge tube. The supernatant was removed and the pellet was washed 2 times by adding 1 ml of 75% ethanol, vortexing briefly and centrifuging at 7,500xg for 5 minutes at 4^0 C. After the second wash, the pellet was dried under a vacuum for 5-10 minutes, not allowing the RNA pellet to dry completely. The RNA pellet was dissolved in 50 ml diethylpyrocarbonate (DEPC) treated water or distilled water that had been autoclaved for 60 minutes.

First Strand complementary DNA Synthesis. cDNA was synthesized from the RNA by random priming. 10 µg of total RNA for a 100 µg volume reaction was incubated with 1.5 µg random primers (Promega) at 85^0 C for 3 min. 2.0 µl Rnasin (Promega), 10 µl 5X reverse transcription buffer (Promega), 20 µl dNTPs and 4.0 µl reverse transcriptase (RT) was added to the reaction mixture. DEPC treated water was added to bring the final volume to 100 µl. Reaction mixture was incubated at 42^0C for 30 min, then 94^0C for 2 min. cDNA sample was stored at – 80^0 C until use.

Polymerase Chain Reaction. PCR analysis of Müller cell expression of neurotrophin message was performed using the Taq Start Antibody Hot Start Method (Clontech laboratories Inc, Palo Alto, CA). Primers were designed using the computer program Oligo 4.0 (National Biosciences, Plymouth MN). Upper primer for NT3 was 5'GATCCAGGCGGATATCTTGA 3', lower primer was 5' AATCATCGGCTGGAATTCTG 3'. Upper primer for NT4 was 5'TCCTGAGT GGGACCTCTTG 3', lower primer was 5' CACTCACTGCATCGCACAC 3'. Upper primer for NGF was 5' CAGGCAGAACCGTACACAGA 3', lower primer was 5' GTCTGAAGAGGTGGGTGGAG 3'. Upper primer for BDNF was 5' ATGACCATCCTTTTCCTTACTATGGT 3', lower primer was 5' CTTCCCCTTTTAATGGTCAATGTAC 3'. Cycle parameters were 94^0C for 5 min., 94^0C for 20sec., 60^0C for 1 min., 72^0C for 30sec. and a final extension at 72^0C for 5 min. Control reactions were done for each primer pair without mRNA. PCR for 18S was used as an internal control. PCR amplified products were separated by agarose gel electrophoresis on a 2% agarose gel containing 5 μg/ml ethidium bromide stain to facilitate visualization of bands under UV illumination. PCR experiments were done at least 3 times to confirm results.

Enzyme Linked Immunosorbent Assay

To determine the total amounts of neurotrophins secreted from glutamate treated Müller cells, ELISA using the antibodies against BDNF, NGF, NT3 and NT4 was performed on conditioned media collected from Müller cells treated with various

concentrations of glutamate (0.0, 0.1, and 1.0 mM). ELISA kits were purchased from Promega (Madison, WI) and the immunoassay was performed according to manufacturer's instructions. Briefly, the assay was done as follows. A 96 well plate was prepared by coating each well of the plate with 100 µl of Anti-neurotrophin/Carbonate buffer (10 µl of the Anti-neurotrophin mAb to 10 ml of carbonate coating buffer (0.025M sodium bicarbonate and 0.025M sodium carbonate)). After an overnight incubation at 4^0C all wells were washed once with 1X TBST wash buffer (20 mM Tris (pH 7.6), 150 mM NaCl, .05% Tween-20) and blocked with the 1X Blocking & Sample buffer solution supplied with the kit for 1hour at room temperature without shaking. Following plate blocking, the plate was washed once with 1X TBST wash buffer. A standard curve ranging from 0-500 pg/ml was prepared using the neurotrophin standard provided with the kit and was designated to two columns of wells in the plate. Three serial dilutions of control or treated Müller cell lysate was made with 1X Blocking & Sample buffer and 50 µl of diluted samples were applied to test wells. Plates were sealed and incubated for two hours at room temperature with shaking (400 rpm). After incubation the plate was washed five times with 1X TBST wash buffer. A 1:500 dilution of Anti-human (recognizes rat neurotrophins) neurotrophin pAb was prepared with 1X Blocking & Sample buffer and 100 µL was added to all wells. The plates were incubated at room temperature for 2 hours with shaking (400 rpm). After washing five times with 1X TBST, 100 µl of a 1:200 dilution of Anti-IgY HRP conjugate was added to all wells and incubated for 1 hour at room temperature with shaking (400 rpm). After incubation, the plate was washed five times with 1X TBST wash buffer. 100 µl room temperature TMB

One Solution (supplied with the kit) for color development was applied to all wells. Incubation was at room temperature with shaking (400 rpm) for 10 min. The reaction was stopped with 100 µl of 1N HCl added to all wells. The plate was read on a microplate reader (Spectramax 340PC, Molecular Devises) within 30 minutes of stopping the reaction at a 450 nm wavelength. ELISA experiments were done 3 times in triplicate to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Western blotting was done on conditioned media, cell lysate, and TrkB immunoprecipitate from glutamate treated (0.0, 0.1 and 1.0 mM) Müller cells with antibodies to neurotrophins, TrkB and NMDA receptors, glutamate transporter, NFkB and Bcl2 proteins to determine the effect of glutamate treatment on these proteins. Conditioned media, cell lysate, or immunoprecipitate samples were prepared (see sample collection, above and immunoprecipitation to follow). For BDNF secretion, conditioned media was collected after cells were treated with glutamate (0.0, 0.1, 1.0 mM) for 48 hours. For TrkB expression, cell lysate was collected after cells were treated with glutamate (0.0, 0.1, 1.0 mM) for 48 hours. For TrkB binding to BDNF and TrkB activation, immunoprecipitate (see immunoprecipitation to follow) was collected after cells were treated with glutamate (0.0, 0.1, 1.0 mM) for 48 hours. For pERK and Bcl2 expression after BDNF treatment cell lysate was collected after BDNF only treatment (50 ng/ml) or BDNF in the presence of K252a (50 ng/ml and 100 nM respectively) for 2

hours (pERK) and 48 hours (Bcl2). The samples were separated by SDS-PAGE using a stacking polyacrylamide gel of 4% and a separating gel of 10 - 12%, depending on the protein of interest to be separated. Lower percent acrylamide gels were used for higher molecular weight proteins such as the receptor proteins and higher percent acrylamide gels were used for proteins of lower molecular weight such as the neurotrophins to allow for distinct separation. Protein samples were loaded onto the stacking gel after protein estimation was done to ensure equal loading of proteins. Separation was done in Mini-Protean II Electrophoresis Cell (Bio-RAD) according to manufacturer's directions in 1X Tris/Glycine/SDS buffer with a voltage setting of 100 volts.

Western Blot

After separation, the proteins were electrophoretically transferred onto Immobilon-P membranes (Millapore) using the Minitrans-Blot Electrophoretic Transfer Cell (Bio-RAD) according to manufacturer's directions. Following electrophoresis, the gels were equilibrated in transfer buffer containing 25 mM Tris, 192 mM glycine and 20% v/v methanol, pH 8.3 for 20 minutes. The Immobilon-P membranes were cut to the dimensions of the gel and activated by submerging in 100% methanol for 20 seconds. Following methanol activation, the membranes were submerged in dH2O for 2 minutes. Next the membrane was soaked in transfer buffer for 15 – 30 minutes to eliminate trapped air bubbles in the matrix. Filter paper cut to exceed the dimensions of the membrane and gel and the fiber pads were also soaked in the transfer buffer. A sandwich was made with the gel and the membrane at the center encased in two filter papers which

in turn were encased between two fiber pads. The fiber pads, filter paper, gel and membrane were placed in a gel holder cassette so that the gel was placed on the cathode (-) side, and the membrane was placed on the anode (+) side. The cassette was placed in an electrode module so that the black panel of the cassette faced the black cathode electrode panel of the electrode module. The electrode module was placed in a buffer tank filled with transfer buffer to just above the level of the top row of circles on the gel holder cassette. An ice pack was placed alongside the cassette, and a stir bar was placed in the bottom of the buffer tank. The buffer tank was placed on a magnetic stirrer and the buffer tank was covered with the assembly lid so that the electrode wires on the lid were attached to the appropriate pins of the electrode module (black wire to cathode and red wire to anode). The magnetic stirrer was turned on and the power supply was turned on to supply 100 volts for 1 hour. After the transfer was completed, membranes of Western blots of BDNF secretion, TrkB expression and TrkB binding to BDNF were blocked with 5% skim milk at room temperature for 1 hour, and then were incubated in 1:200 – 1:1000 dilutions of BDNF or TrkB primary antibodies overnight at 4^0 C while rotating (antibody concentration is 200 μg/ml)(Santa Cruz Biotechnology). For Western blots of pERK1/2, Bcl2, and NFκB expression, the membranes were blocked with 5% skim milk at room temperature for 1 hour and then were incubated in 1:500 (pERK1/2) or 1:200 (Bcl2 and NFκB) dilutions of primary antibodies overnight at 4^0 C while rotating (antibody concentration is 200 μg/ml)(Santa Cruz Biotechnology). For Western blots of NMDAR and GLAST, blocked membranes were incubated in 0.5 mg/ml (NMDAR) or 1:1000 (GLAST) dilutions of primary antibodies overnight at 4^0 C while rotating. The next day,

membranes were washed in 1X PBST 3 times for 5 minutes. Membranes were then be incubated in 1:1000-1:3000 dilutions of horseradish peroxidase conjugated secondary antibody. Antibody was either against mouse or rabbit depending on the primary antibody (antibody concentration is 200 μg.ml) (Santa Cruz Biotechnology). Secondary antibodies were incubated with rotation at room temperature for 1 hour. Membranes were washed in 1X PBST 2 times for 5 minutes and then washed in 1X PBS 2 times for 10 minutes. The proteins were visualized by chemilluminescent detection. Equal volumes of ECL detection reagent A and ECL reagent B (Amersham Pharmacia Biotech) were mixed together and used to cover the membrane. After a 1 minute incubation at room temperature while rotating, the membranes were exposed to X-ray film for varying exposure times to obtain images of the protein bands. X-ray film was developed in a film processor (Konica). To standardize loading, samples had protein estimation done prior to loading, but protein loading was confirmed by reprobing the blots with antibody to β-tubulin (1:500, antibody concentration = 200μg/μl)(Santa Cruz Biotechnology). Immunoblots for each protein were done at least 3 times to confirm results. Immunoblot bands were analyzed by NIH Scion Image to determine fold difference from control.

Immunoprecipitation

Immunoprecipitation of glutamate-treated (0.0, 0.1, and 1.0 mM) Müller cell lysate with TrkB antibody followed by immunoblot with an antibody for BDNF was done to determine the effect of glutamate treatment on BDNF signaling in Müller cells. Müller cells for immunoprecipitation were plated on a 35 mm culture plate at a plating density of

200,000/plate in 3 ml basal media supplemented with 10% FBS. Cells were grown to confluence at 37^0 C in 5% CO_2. Serum supplemented media was removed and replaced with 3 mls basal media overnight (for at least 17 hours). The next day, cells were treated for 48 hours with 3 ml of basal media supplemented with glutamate (0.0, 0.1 and 1.0 mM). After treatment, culture media was removed and the cells were washed once with 1 ml of ice cold 1X PBSfollowed by the addition of 200 µl of ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), NP-40 1%, Na-deoxycholate 0.25%, NaCl 10 mM, EDTA 1 mM, PMSF 1mM, Aprotinin 1 µg/ml, Na_3VO_4 1 mM, NaF 1 mM) was added to the culture plate, and shaken for 10 minutes. Lysate was then transferred to a 1.7 ml microcentifuge tube and sonicated for 2 seconds. Lysate was centrifuged at 10,000xg for 10 minutes at 4^0 C. For every 100 µl of Müller cell lysate, 10 µl Protein G agarose conjugate (Santa Cruz Biotechnology) was added. The mixture was incubated for 1 hour at 4^0 C while being shaken, followed by centrifugation at 10,000xg for 5 minutes at 4^0 C. Supernatant was removed, and 1 µl anti-TrkB IgG (antibody concentration = 200 µg/µl) (Santa Cruz Biotechnology) added. After overnight incubation at 4^0 C with shaking, 10 µl Protein G agarose conjugate was added (for every 100 µl of Müller cell lysate) followed by incubation for 1 hour at 4^0 C while shaking. Immunoprecipitate mixture was centrifuged at 10,000xg at 4^0 C for 10 minutes and then washed three times with 1X PBS. After each wash, the immunoprecipitate was centriguged at 10,000 x g for 10 minutes at 4^0 C. The supernatant was decanted and the pellet was collected after the last wash and resuspended in 40 µl Laemmli's sample buffer with 5% β-mercaptoethanol. Immunoprecipitate sample was boiled for 5 minutes before use and stored at -20^0 C until use.

Transfection

Müller cells were transfected with Bcl2 and NFκB plasmid so that downstream components of the MAPK(ERK) and PI3K pathways could be examined by proliferation assays performed with cell signaling inhibitors. On the day before transfection, Müller cells were plated in 24 well plates so that they were 90 -95% confluent on the day of transfection, 25,000 cells/well. Cells were grown in basal media supplemented with 10% FBS but were placed in 500 µl basal media before the transfection procedure. For each well to be transfected, 2 µl of LF2000 reagent (Invitrogen Life Technologies) was added to 50 µl of basal media and incubated for 5 min. at room temperature. Diluted LF2000 reagent (above) was added to 1.0 µg DNA plasmid containing Bcl2 or NFκB (N. Agarwal, Ph.D. UNTHSC, Fort Worth, TX) in 50 µl of basal media. After being combined, the diluted LF2000 reagent and the DNA plasmid were incubated at room temperature for 20 minutes to allow DNA-LF2000 reagent complexes to form. 100 µl of the DNA-LF2000 reagent complexes were added directly to each well containing Müller cells to be transfected. Negative control consisted of omitting the addition of DNA plasmid. Cells were incubated at 37^0 C in 5% CO_2 for 24-48 hours. The cells were then passaged in fresh media supplemented with 10% FBS. Plasmids contained a resistance gene for geneticine, successfully transfected Müller cells were selected for with daily treatment of geneticine (800 µg/ml)(Gibco, Grand Island NY). Cells were incubated at 37^0 C in 5% CO_2 until confluent at which time cell lysate was collected and run on a Western blot with antibody to Bcl2 or NFκB to check for successful transfection.

CHAPTER 2 TABLES AND FIGURES

Antibody	*Concentration*	*Source*	*Application*
BDNF	1:200 1:500 1:200 – 1:1000	Santa Cruz Biotechnology Promega Santa Cruz Biotechnology	Immunocytochemistry ELISA Western Blot
NGF	1:200 1:500 1:200 – 1:1000	Santa Cruz Biotechnology Promega Santa Cruz Biotechnology	Immunocytochemistry ELISA Western Blot
NT3	1:200 1:500 1:200 – 1:1000	Santa Cruz Biotechnology Promega Santa Cruz Biotechnology	Immunocytochemistry ELISA Western Blot
NT4	1:200 1:500 1:200 – 1:1000	Santa Cruz Biotechnology Promega Santa Cruz Biotechnology	Immunocytochemistry ELISA Western Blot
CRALBP	1:200	Jack Saari, Ph.D.	Immunocytochemistry
GFAP	1:200	Zymed	Immunocytochemistry
S-100	1:200	Sigma Immunochemical	Immunocytochemistry
Carbonic Anhydrase	1:200	Chemicon	Immunocytochemistry
TrkB	1:200-1:1000 1:100	Santa Cruz Biotechnology Santa Cruz Biotechnology	Western Blot Immunoprecipitation
NMDAR	0.5 μg/ml	Chemicon	Western Blot
GLAST	1:1000	Susan Amara, Ph.D.	Western Blot
NFκB	1:200	Santa Cruz Biotechnology	Western Blot
Bcl2	1:200	Santa Cruz Biotechnology	Western Blot
pERK	1:500	Santa Cruz Biotechnology	Western Blot

Table 1. Table summarizing the antibodies used in the study.

Neurotrophin	*Primer type*	*Primer Sequence*
BDNF	upper lower	5'ATGACCATCCTTTTCCTTACTATGGT3' 5'TCTTCCCCTTTTAATGGTCAATGTAC3'
NGF	upper lower	5'CAGGCAGAACCGTACACAGA3' 5'GTCTGAAGAGGTGGGTGGAG3'
NT3	upper lower	5'GATCCAGGCGGATATCTTGA3' 5'AATCATCGGCTGGAATTCTG3'
NT4	upper lower	5'TCCTGAGTGGGACCTCTTG3' 5'CACTCACTGCATCGCACAC3'

Table 2. Table summarizing the PCR primers used in the study.

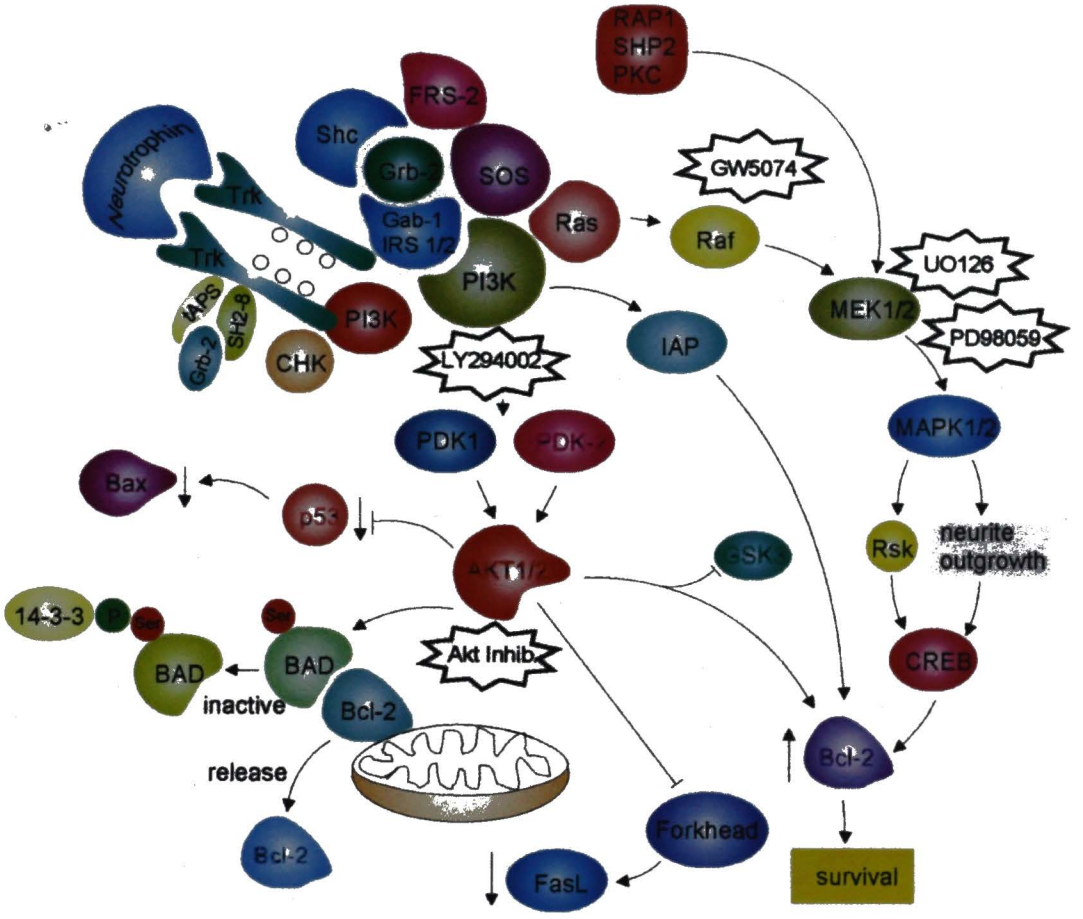


Figure 2a. Diagram of site specific action of MAPK(ERK) and PI3K inhibitors.

CHAPTER III

PRELIMINARY DATA:

Effects of Glutamate on Cultured Müller Cells

Because glutamate has been implicated in many diseases of the CNS including diseases of the retina and because the effect of glutamate has not been well studied in Müller cells, we decided to begin this project by investigating the effect of glutamate treatment on cultured Müller cells. After establishing that our cell line consisted of normal Müller cells, we examined the effect of glutamate on Müller cell expression of neurotrophin message RNA, secretion of neurotropins, intrinsic BDNF signaling, expression of glutamate receptor and glutamate transporter proteins, and on Müller cell survival.

Retinal cell line expresses Müller cell markers

Before investigation of glutamate treatment on Müller cells could begin, the cell line that was used in this projected was characterized to show that it consisted of normal Müller cells. The cell line that was used for this project was developed as described previously in the Methods section of this thesis (47). One ml aliquots of low passage numbers of the Müller cell line were frozen in DMEM containing 10%dimethylsulfoxide(DMSO) and 20%FBS and stored under liquid nitrogen. Aliquots

that were thawed and used for this project were characterized to ensure that that they were normal Müller cells by immunocytochemistry with antibodies to known Müller and glial cell markers. Antibodies to Cellular Retinaldehyde Binding Protein (CRALBP), Glial Fibrillary Acidic Protein (GFAP), S-100 and Carbonic Anhydrase C (CA), were used to characterize the cell line as Müller cells (Fig 3a). CRALBP is the specific marker for Müller cells (Roque et al. 1992). GFAP is an astrocyte marker and Müller cells should be negative however GFAP is present in glial cells undergoing gliosis and since these cells are in culture, they are likely to express GFAP (47). Labeling by the markers was primarily cytoplasmic although nuclear staining can be seen as well. GFAP showed typical staining of the filamentous structures in the cytoplasm. Cells were negative for known markers of neurons (neurofilament 200 kDa), endothelial cells (Factor VIII related antigen) and RPE cells (cytokeratin)(data not shown). In addition to expressing known glial cell markers as well as specific markers of Müller cells, the cells showed the normal spindle shaped morphology characteristic of Müller cells.

Cultured Müller cells express neurotrophin message RNA and protein

To determine the presence of endogenous neurotrophin protein in Cultured Müller cells grown in basal media without glutamate supplement, immunocytochemistry was done with antibodies to BDNF, NGF, NT3 and NT4. The results showed that cultured Müller cells express endogenous protein for all four neurotrophins. Labeling was primarily cytoplasmic (Fig 3b).

To examine the effect of glutamate treatment on neurotrophin message in Müller cells, mRNA was harvested from cultured Müller cells after treatment with glutamate (0.0, 0.1 and 1.0 mM) for 48 hours. cDNA obtained from mRNA was amplified by PCR using primers to BDNF, NGF, NT3 and NT4. Although the PCR is not quantitative, visualization of the PCR product on an agarose gel showed correctly sized bands for all four neurotrophins. Thus endogenous neurotrophin message RNA is present in Müller cells and is not ablated by glutamate treatment (Fig. 3c).

Glutamate increases neurotrophin expression in cultured Müller cells

Having first established that these cultured Müller cells express endogenous neurotrophin message and protein, we found that in response to glutamate treatment (0.0, 0.1, and 1.0 mM), cultured Müller cells increased their expression and secretion of BDNF, NGF, NT3 and NT4. ELISA performed on glutamate treated Müller cell conditioned media showed that Müller cells increased secretion of BDNF, NGF, NT3 and NT4 in response to glutamate treatment (Fig 3d). NT3 and NT4 control levels were 1.4 and 3.5 pg/ml respectively while NGF control levels were ~ 18.0 pg/ml and BDNF in the control samples was the highest of the neurotrophins at 70 pg/ml, almost 20–50 fold higher than NT3 and NT4. There was a dose dependent increase in all of the neurotrophins tested. NT3 increased ~ 34 fold to 48 .0 pg/ml with glutamate treatment (1.0 mM), NT3 increased ~ 10 fold to 35 pg/ml, NGF increased ~ 3 fold to 59 pg/ml and BDNF secretion by Müller cells treated with 1.0 mM glutamate increased ~ 4 fold to 286 pg/ml. Although NT3 showed the greatest increase foldwise during glutamate treatment,

BDNF remained the major neurotrophin secreted by Müller cells. With levels of 285 pg/ml secreted by glutamate treated Müller cells, BDNF was ~ 4 – 6 times higher than any of the other neurotrophins. ELISA does not distinguish between small and large forms of BDNF so the ELISA graph represents an increase in total BDNF secreted.

The ELISA results were substantiated with immunoblots done on Müller cell conditioned media after glutamate treatment. Glutamate treatment (0.0, 0.1 and 1.0 mM) for 48 hours followed by immunoblotting of Müller cell conditioned media with antibodies to BDNF, NGF, NT3 and NT4 indicate that in response to glutamate treatment Müller cells increased their secretion of NGF, NT3 and NT4 as indicated by the presence of appropriately sized bands of ~ 13 – 14 kDa (Fig 3e). All of the neurotrophins showed a dose dependent increase in secretion with glutamate treatment (0.1 – 1.0 mM) ranging from 3 – 8 fold increases over control treatment (no glutamate). Interestingly two bands were visible when probed with an antibody to BDNF. The smaller of the two bands was ~ 14 kDa, the expected size of this neurotrophin. The larger of the two bands was a slower migrating band of ~ 36 kDa.

BDNF was able to bind TrkB receptor throughout glutamate treatment

Because Müller cells secreted large amounts of BDNF during glutamate treatment, and because Müller cells are known to express the high affinity BDNF receptor, TrkB (40, 46, 64), we felt that BDNF signaling must be important to to Müller cells. Accordingly we next tested the effects of glutamate on the expression and activity of TrkB receptor. Cell lysate collected from glutamate treated (0.0, 0.1 and 1.0 mM) Müller

cell cultures followed by immunoblot with an antibody to TrkB showed correctly sized bands for the TrkB receptor, ~ 120 kDa. During glutamate treatment, TrkB expression remained at ~ 90% of the expression in the control (Fig 3f), indicating that in response to glutamate, Müller cell expression of TrkB receptor shows no apparent change. Immunopreciptation of the cell lysate with an antibody to TrkB followed by immunoblot with an antibody to BDNF showed that BDNF binding to TrkB was not compromised by glutamate treatment, the amount of BDNF bound to TrkB receptor remained constant throughout glutamate treatment, only increasing during glutamate treatment by about 10% (Fig 3g). Moreover, the BDNF reactive band was observed at the size expected for a BDNF dimer, rather than at the size expected if the bound protein was proBDNF. The persistent expression of TrkB and BDNF binding to TrkB suggest that BDNF has an important function Müller cells, possibly maintaining intrinsic signaling that is critical for Müller cell survival.

Glutamate treatment modulates EAAT1 and NMDAR expression in Müller cells.

Glutamate receptors (NMDAR) play a crucial role in mediating gluamate excitotoxicity in neurons (55, 65), and glutamate transporters (GLAST) are active in removing toxic glutamate from the synaptic space (12, 38, 66). Therefore, the next step in our investigation of the effects of glutamate on Müller cells was to examine the effects of glutamate on the expression of these two proteins. Cell lysate was collected from Müller cells treated for 48 hours with varying concentrations (0.1 and 1.0 mM) of glutamate. The cell lysate was separated by SDS-PAGE and transferred to a

nitrocellulose membrane for immunoblotting with antibodies to EAAT1 and NMDAR. In response to glutamate treatment, Müller cells produced appropriately sized bands for NMDAR at ~ 120 kDa and GLAST at ~ 65 kDa. The glutamate transporter expression increased by up to 25 fold over the control (Fig 3h). In contrast, the NMDAR receptor expression in glutamate treated cells decreased by up to 6 fold from control levels (Fig. 3h).

Glutamate treatment is not toxic to cultured rat Müller cells

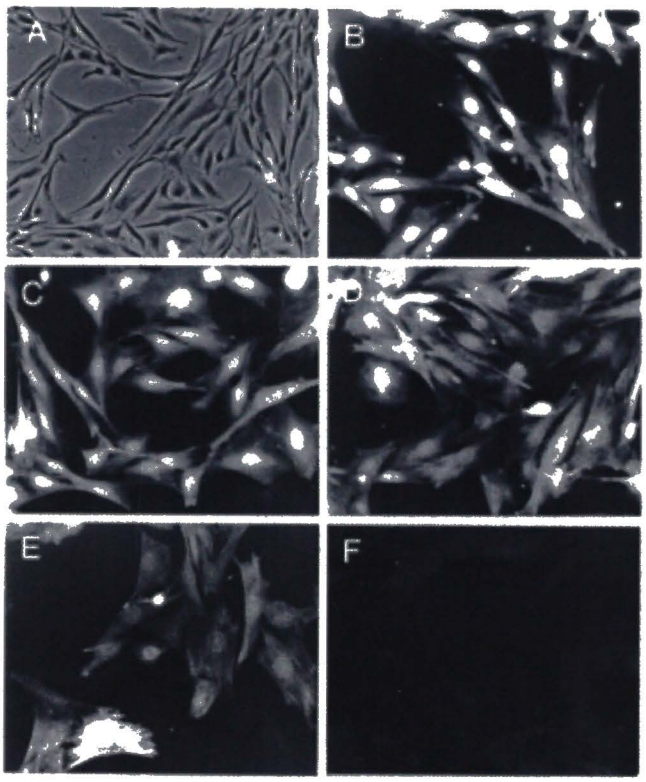
Glutamate has been implicated in many pathologies of the CNS, but its neurotoxic effect remains to be elucidated. Müller cells appear to be immune to death in retinal pathologies (4) , so we wanted to examine the effect of glutamate treatment on Müller cell survival. First we examined the effects of prolonged glutamate treatment on Müller cells. Cultured Müller cells were grown in basal medium in the presence or absence of glutamate (1.0 mM) for 10 days. To compensate for glutamate taken up by Müller cell glutamate transporters, exogenous glutamate (1.0 mM) was added every 24 hours; and the medium was changed every 3 days. Cell numbers were analyzed with daily cell counts using trypan blue exclusion and a hemocytometer. There was no significant difference ($p < 0.05$) in cell counts between the cell cultures that received glutamate supplementation as compared with cultures grown in basal medium only after 10 days of treatment (Fig. 3i). The lack of Müller cell susceptibility to glutamate toxicity was confirmed by the absence of significant ($p < 0.05$) cell death when cells were exposed to

varying concentrations of glutamate (0.1 - 20.0 mM) for 48 hours and then analyzed by a cell proliferation assay (Fig 3j).

Summary/Concluding Remarks

The Müller cells used in this project were determined to be a normal Müller cell line as they expressed known glial and specific Müller cell markers. The Müller cells exhibited characteristic Müller cell spindle shaped morphology, and they were excluded from being endothelial cells, RPE cells and neurons (RGCs) as they were negative for the characteristic markers for these types of cells. The Müller cells were shown to express endogenous neurotrophins (BDNF, NGF, NT3, and NT4) in untreated cells as well as neurotrophin message RNA for all four neurotrophins. The Müller cells were shown to increase their secretion of all four neurotrophin proteins in response to glutamate treatment. Müller cell expression of TrkB receptor and binding of BDNF to TrkB proved not to be compromised in glutamate treated Müller cells, suggesting that BDNF signaling remained active throughout treatment. Finally, cultured Müller cells were resistant to decreases in survival caused by either prolonged or acute (high concentrations) of glutamate treatment.

CHAPTER III FIGURES



(A) Phase Contrast (B) S-100 (C) Carbonic Anhydrase
(D) CRALBP (E) GFAP (F) Negative Control

Figure 3a. Cultured Müller cells express Müller cell markers.

Immunocytochemistry was done on Müller cells plated on chamber slides with primary antibodies to S-100 (1:200), Carbonic Anhydrase C (1:200), CRALBP (1:200) and GFAP (1:200). Phase contrast showing the typical spindle shaped morphology of Müller cells (A). S-100 (B), Carbonic Anhydrase C (C) and GFAP (E) are glial cell markers. CRALBP (D) is a specific cell marker for Müller cells. Negative control (F) was done without a primary antibody. Figures show representative immunocytochemistry, experiments were done 3 times to confirm results.

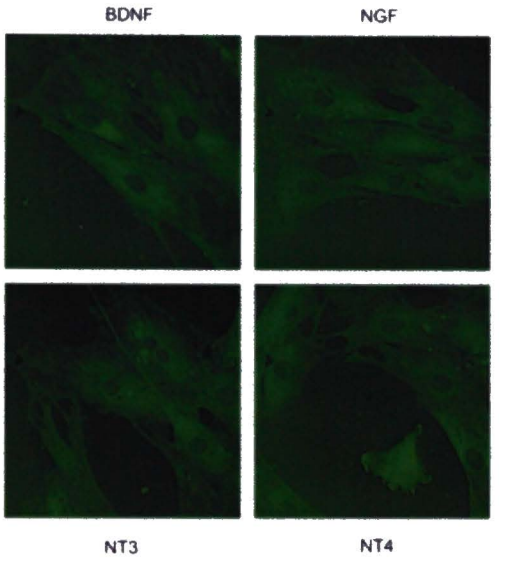


Figure 3b. Müller Cells express neurotrophin protein.

Immunocytochemistry was done on Müller cells (not treated with glutamate) plated on chamber slides with primary antibodies to BDNF, NGF, NT3 and NT4. Müller cells express endogenous protein for BDNF, NGF, NT3 and NT4 (staining is primarily cytoplasmic). Figures show representative immunocytochemistry, experiments were done 3 times to confirm results

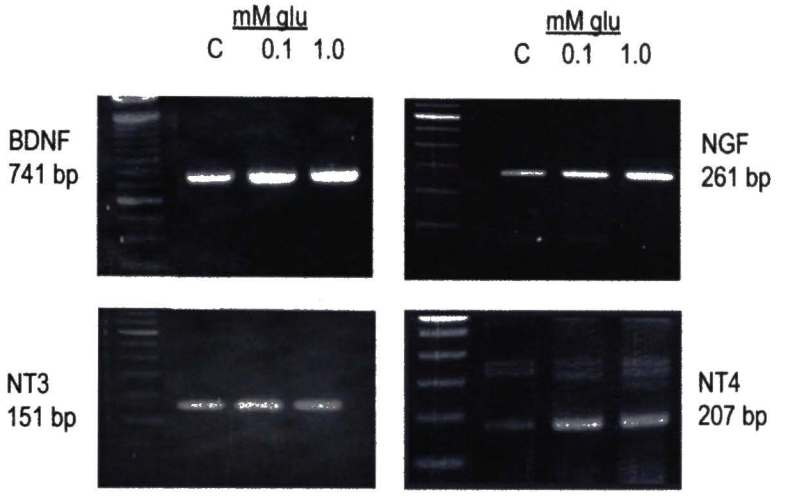


Figure 3c. Müller Cells express neurotrophin mRNA.

Semi-quantitative RT-PCR was done on cDNA made from RNA collected from Müller cells treated with various concentrations of glutamate (0.0, 0.1, and 1.0 mM) for 48 hours. Primers to BDNF, NGF, NT3 and NT4 were designed using the computer program Oligo 4.0 (National Biosciences, Plymouth MN) and were used to determine the presence of neurotrophin message RNA in glutamate treated Müller cells. Figures show representative PCR gels, experiments were done 3 times to confirm results.

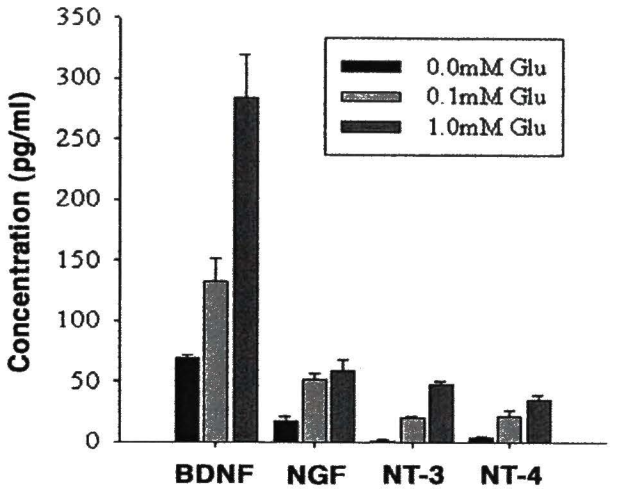


Figure 3d. Glutamate stimulates neurotrophin secretion in cultured Müller cells.

Conditioned media was collected from Müller cells treated with various concentrations of glutamate (0.0, 0.1, and 1.0 mM) for 48 hours and concentrated 10X prior to being analyzed by ELISA using antibodies to BDNF, NGF, NT3 and NT4. Significant dose-dependent increases in all the secreted neurotrophins were seen with glutamate treatment as compared to untreated cultures ($p < 0.05$) and BDNF was secreted from Müller cells in markedly greater amounts than the other neurotrophins. Bars represent mean $\pm$ standard devivation (SD). ELISA was done 3 times in triplicate to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

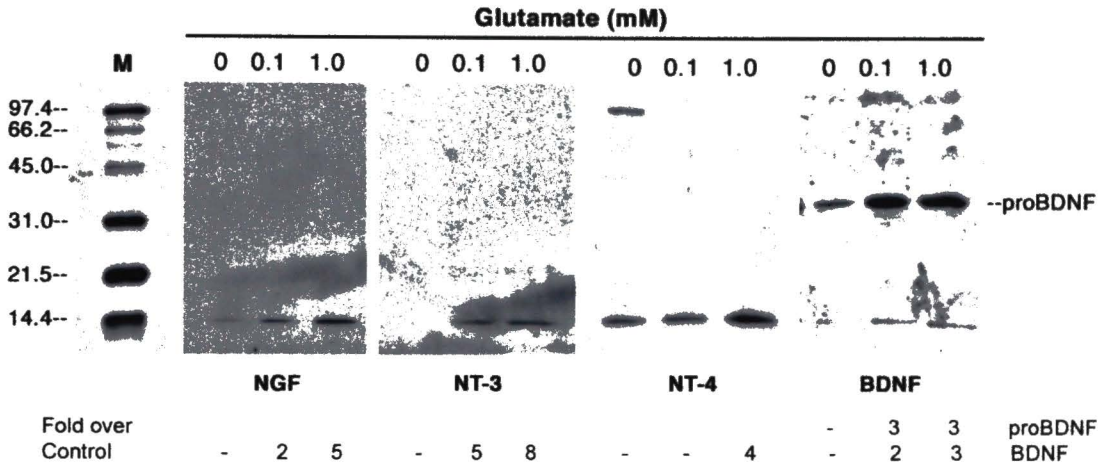


Figure 3e. Glutamate promotes neurotrophin secretion in cultured Müller cells.

Conditioned media was collected from Müller cells treated with varying concentrations of glutamate (0.0, 0.1, and 1.0 mM) for 48 hours and concentrated 60X. Conditioned media was separated by SDS-PAGE followed by immunoblot with antibodies to BDNF, NGF, NT3 and NT4 using chemiluminescence to detect bands. Increases in monomeric bands (~ 14 kDa) were observed for all neurotrophins as a result of glutamate treatment. BDNF immunostaining demonstrated a second, slower migrating band of ~ 36 kDa (possibly a pro-form of BDNF) which also increased as a result of glutamate treatment. Figure shows a representative blot, immunoblots were done 3 times to confirm results.

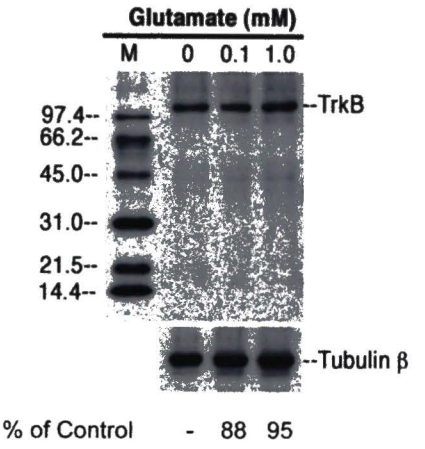


Figure 3f. TrkB expression in glutamate treated Müller cells.

Cell lysate was collected from Müller cells treated with varying concentrations of glutamate (0.0, 0.1, and 1.0 mM) for 48 hours prior to being separated by SDS-PAGE followed by immunoblot with an antibody to TrkB receptor. The immunoblot showed no apparent change in the expression of TrkB receptor in the presence or absence of glutamate treatment. β-Tubulin was used to normalize sample loading. Figure shows a representative blot, immunoblot was done 3 times to confirm results.

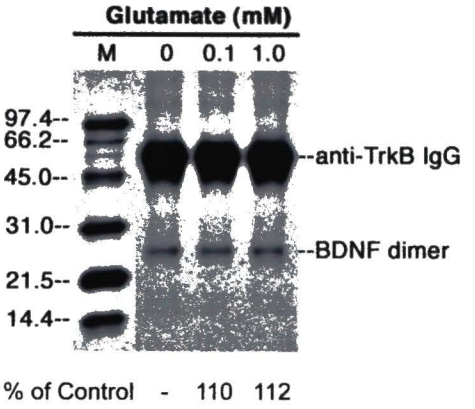


Figure 3g. BDNF–TrkB binding in glutamate treated Müller cells.

Cell lysate was collected from Müller cells treated with varying concentrations of glutamate (0.0, 0.1, and 1.0 mM) for 48 hours prior to immunoprecipitatation with TrkB antibody followed by immunoblot with antibody to BDNF. The immunoblot showed no apparent change in the expression of BDNF bands of ~ 26 kDa across all treatment conditions. No other bands such as ~36 kDa bands for proBDNF were observed except for very large bands at ~ 50 kDa which were consistent with the heavy chain of the ant-TrkB IgG used for immunoprecipitation. Figure shows a representative blot, immunoblot was done 3 times to confirm results.

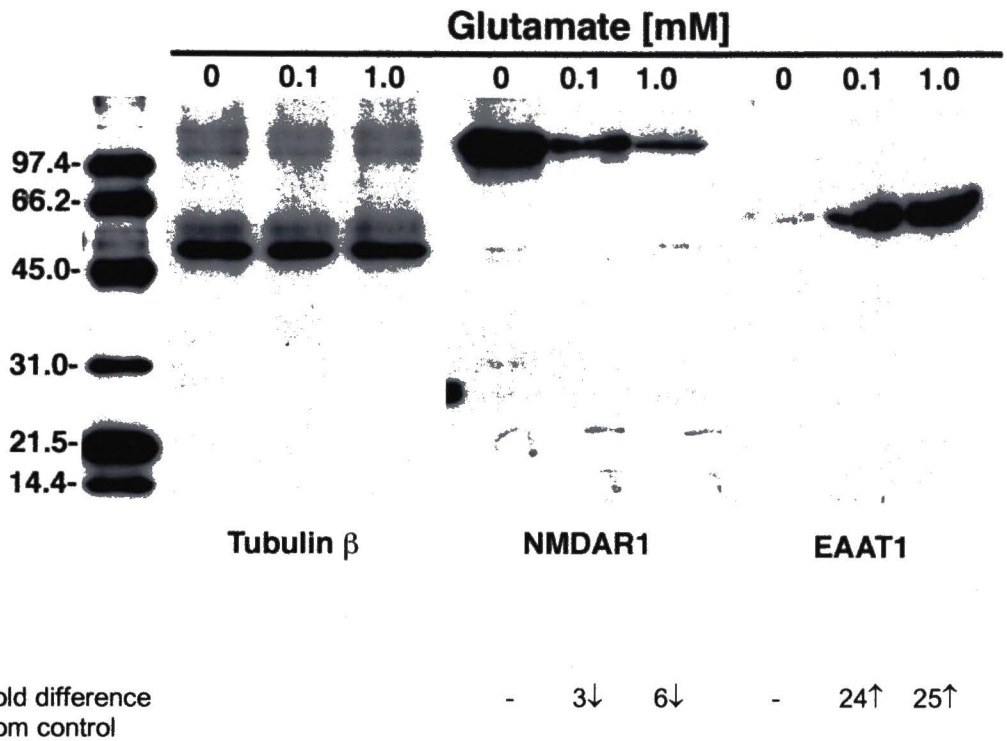


Figure 3h. Effect of glutamate on expression of NMDAR and EAAT1 in Müller cells.

Cell lysate collected from Müller cells treated with varying concentrations of glutamate (0.0, 0.1, and 1.0 mM) for 48 h prior to separation by SDS-PAGE and immunoblot with antibody to EAAT1, shows appropriately sized bands for NMDAR at ~ 120 kDa and GLAST at ~ 65 kDa Glutamate treatment of Müller cell cultures promoted decreased NMDAR and increased GLAST levels in immunoblots, as compared with untreated cutures. β-Tubulin was used to normalize sample loading. Figure shows a representative blot, immunoblot was done 3 times to confirm results.

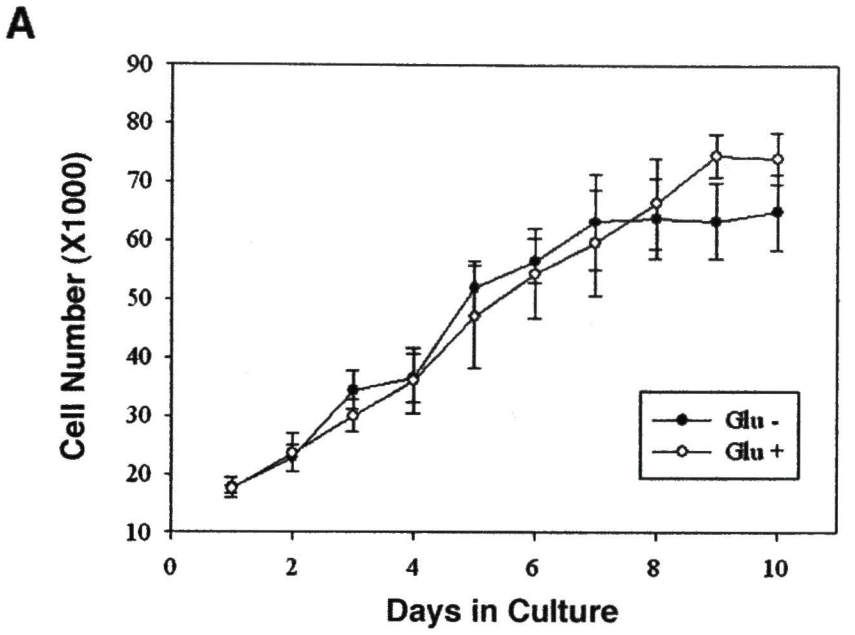


Figure 3i. Effect of prolonged glutamate treatment on Müller cell survival.

Müller cell cultures were treated with 1.0 mM glutamate or kept in basal medium for up to 10 days. 1.0 mM glutamate was added every 24 h to glutamate treated cultures to compensate for glutamate uptake and synthesis into glutamine. Medium in all cultures was changed every 3 days. The number of surviving cells were counted daily using trypan blue exclusion and a hemocytometer. No significant differences ($p < 0.05$) were observed between the cultures. Graph represents mean cell counts $\pm$ SD. Cell counts were done 4 times in triplicate. Statistical analysis was done by student's *t* test using Systat Version 7.0 statistical software package.

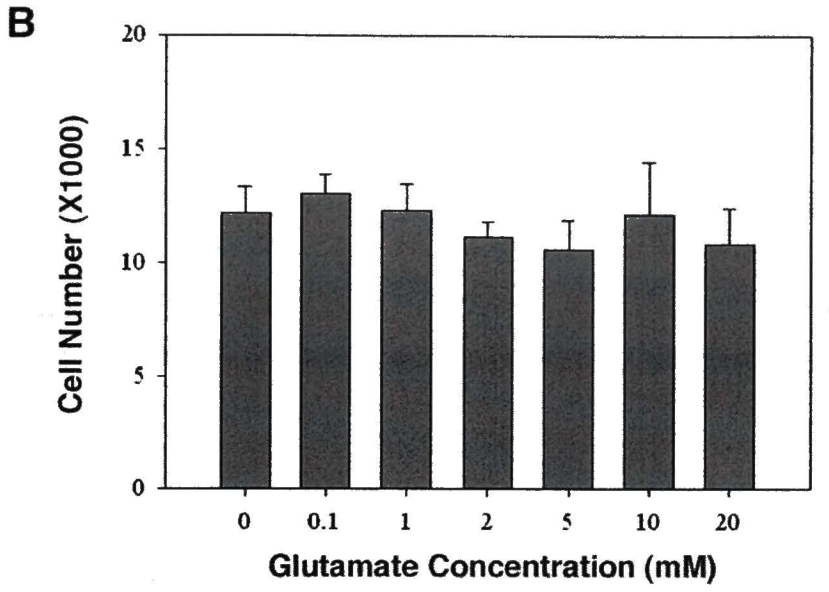


Figure 3j. Effect of varying concentrations of glutamate on Müller cell survival.

Müller cell cultures were treated with varying concentrations of glutamate (0.0 –20 mM) for 48 h prior to having MTS/PMS proliferation assays performed to determine Müller cell survival in the presence of glutamate. No significant differences ($p < 0.05$) were observed in the number of Müller cells incubated in various concentrations of glutamate as compared with untreated cells. Bars represent mean cell counts $\pm$ SD. Proliferation assays were done 4 times in triplicate to confirm results. Statistical analysis was done by student's *t* test using Systat Version 7.0 statistical software package.

CHAPTER IV

RESULTS-SPECIFIC AIM 1:

MAPK(ERK) Pathway in the Survival of Cultured Müller Cells

Taken all together, the results of our preliminary data suggest that there is constitutive BDNF signaling in Müller cells that could activate survival pathways in Müller cells. Because the MAPK(ERK) and the PI3K pathways are two key signaling pathways concerned with cell survival (16, 25, 31, 36, 37), we decided to study their intrinsic roles in Müller cell survival. Our examination of intrinsic cell signaling pathways began with an examination of the MAPK(ERK) pathway. Cultured Müller cells were treated with inhibitors of the Ras-Raf-MEK-ERK1/2-Bcl2 pathway to determine the role of this pathway to Müller cell survival. Table 3 (p.63) summarizes the experimental design of Specific Aim 1.

Effect of Raf inhibition on Müller cell survival

To examine the effects of blocking the MAPK(ERK) pathway upstream in the signaling pathway, cultured Müller cells were treated with with GW5074 (5 –20 nM), an inhibitor of the upstream signaling protein Raf. Treatment resulted in a significant ($p < 0.05$) dose-dependent decrease (up to 50%) in Müller cell survival (Fig 4a). In this

experiment, and proliferation assays to follow, since DMSO was the diluent used in the inhibitor reagent preparation, DMSO controls at the most concentrated levels of DMSO used in the experiment (1:1000) were run to show that DMSO did not have a significant ($p < 0.05$) effect on Müller cell survival. The concentrations of GW5074 used in this experiment were based on IC_{50} values for GW5074 of 9 nM (Product specification, product # 1381, Tocris Cookson, Ellisville, MO).

Effect of MEK inhibitors on Müller cell survival

Treatment of the Müller cells with an inhibitor farther down in the MAPK(ERK) signaling pathway – the MEK1/2 inhibitor U0126 (50 – 500 nM) for 24 h , resulted in a dose-dependent decrease (up to 20%) in Müller cell survival when assayed for cell survival with an MTS/PMS survival assay (Fig 4b). There was no significant ($p < 0.05$) decrease in cell survival at the lowest concentration of U0126 used (50 nM, but there was a significant ($p < 0.05$) decrease in cell survival at the higher contrations (100 and 500 nM) of U0126 used. The IC_{50} of U0126 is 90 nM (Product specification, product # 1144, Tocris Cookson, Ellisville, MO) so a significant loss of cell survival was seen near the IC_{50} of U0126.

Cell lysate was collected from Müller cells treated with varying concentrations of another MEK1/2 inhibitor PD98059 (10 and 50 μM) for 2 h and then immunoblotted with an antibody to pERK to show that the PD98059 inhibitor reagent was active. Since ERK is downstream of MEK in the MAPK(ERK) signaling pathway, and since PD98059 is a MEK1/2 inhibitor, PD98059 should be able to downregulate the expression of pERK.

Immunoblots of Müller cell lysate treated with PD98059 and then probed with pERK antibody resulted in two appropriated sized bands for pERK, one at ~ 44 kDa (pERK1) and one at ~ 42 kDa (pERK2) (Fig 4c). The immunoblot showed that a low concentration of PD98059 (10 μM) resulted in levels of pERK1/2 that remained at 50 and 80% of the control treatment respectively, but a higher concentration of PD98059 (50 μM), downregulated pERK1/2 expression by more than 75%.

Having established that the PD98059 inhibitor was active, Müller cells were treated with PD98059 at various concentrations (10 – 50 μM) for 24 hours and then assayed for cell survival with an MTS/PMS survival assay in order to investigate the effect of MEK inhibition on Müller cells. Treatment of the Müller cells with PD98059 at lower concentrations (10 and 20 μM) did not result in significant ($p < 0.05$) decreases in survival, however at a higher concentration of 50 μM PD98059, there was a significant ($p < 0.05$) decrease (30%) in survival (Fig 4d). The IC_{50} of MEK1 is 5–10 μM whereas the IC_{50} of MEK2 is 50 μM (Product specification, product # 1213, Tocris Cookson, Ellisville, MO) so significant decreases in cell survival were seen at concentrations of PD98059 that inhibit the activity of pERK2.

Effect of MEK inhibitors on Bcl2 and NFkB transfected Müller cell survival

To further elucidate the role of MAPK signaling in Müller cells we used Bcl2 and NFκB transfected cells in survival assays since these transcription factors are downstream proteins in the MAPK signaling pathway (15, 40, 43). Müller cells were transfected with Bcl2 or NFκB plasmid DNA and grown in culture. After selection with

geneticine, the success of the transfection was verified by probing cell lysate with antibodies to Bcl2 (1:200) and NFκB (1:200). The resulting immunoblots showed appropriately sized bands for Bcl2 of ~ 29 kDa and for NFkB of ~ 65 kDa. (Figs 4e,f). Cultures of successfully transfected cells were treated with various concentrations of PD98050 (10 – 50 μM). As opposed to nontransfected cells, Bcl2 transfected Müller cells were resistant to treatment with PD98059 even at a concentration of 50 μM (Fig 4g). Similarly, NFκB transfected cells treated with PD98059 showed nearly the same pattern of resistance to treatment with PD98059 as the Bcl2 transfected cells (Fig 4h).

Summary/Concluding Remarks

It is clear from the results of the experiments done for Specific Aim 1 that the MAP(ERK) signaling pathway is essential for Müller cell survival. Inhibition of a protein near the beginning of the MAP(ERK) pathway – Raf, resulted in decreased Müller cell survival. Inhibition farther down in the pathway of MEK enzymes also resulted in decreases in cell survival. In the case of PD98059, significant decreases in cell survival were not seen until the concentration of the MEK inhibitor was great enough to inhibit the phosphorylation and activity of pERK2. This doesn't preclude the necessity of pERK1 to Müller cell survival, but it does suggest that pERK2 is an essential component of MAPK(ERK) signaling that contributes to survival. Because Bcl2 and NFκB transfected cells were able to withstand treatment that resulted in decreases in cell survival in non-transfected cells, it seems likely that the MAPK(ERK) pathway mediates cell survival via these two proteins.

CHAPTER IV TABLES AND FIGURES

Inhibitor	Cell Type	Target	IC_{50}	Concentration used in study	Sig. decease in survival seen at:
GW5074	Müller	Raf	9 nM	5 – 20 nM	10 nM
U0126	Müller	MEK	90 nM	50 - 500 nM	100 nM
PD98059	Müller	MEK1 MEK2	5-10 μM 50 μM	10 – 50 μM	------- 50 μM
PD98059	Bcl2 X	MEK1/2	5-10 μM 50 μM	10 – 50 μM	------- -------
PD98059	NFκB X	MEK	5-10 μM 50 μM	10 – 50 μM	-------

Table 3. Table summarizing MAPK(ERK) inhibitor treatment and effect

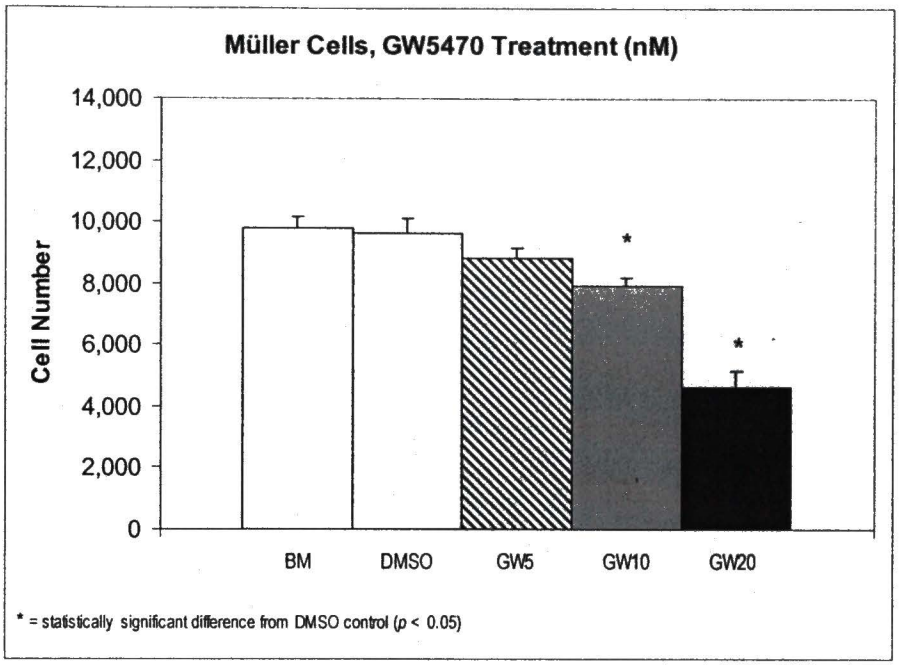


Figure 4a. Effect of GW5074 on Müller cell survival.

Müller cells were treated with 5, 10 or 20 nM GW5074 for 24 h and then assayed for survival with a MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. GW5074 caused a significant ($p < 0.05$) dose dependent decrease in Müller cell survival as compared to DMSO treated cells at 10 and 20 nM GW5074. Bars represent mean $\pm$ SD. * = statistically significant difference from DMSO control ($p < 0.05$). GW5, GW10, and GW20 = treatment with 5, 10, and 20 nM GW5074 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

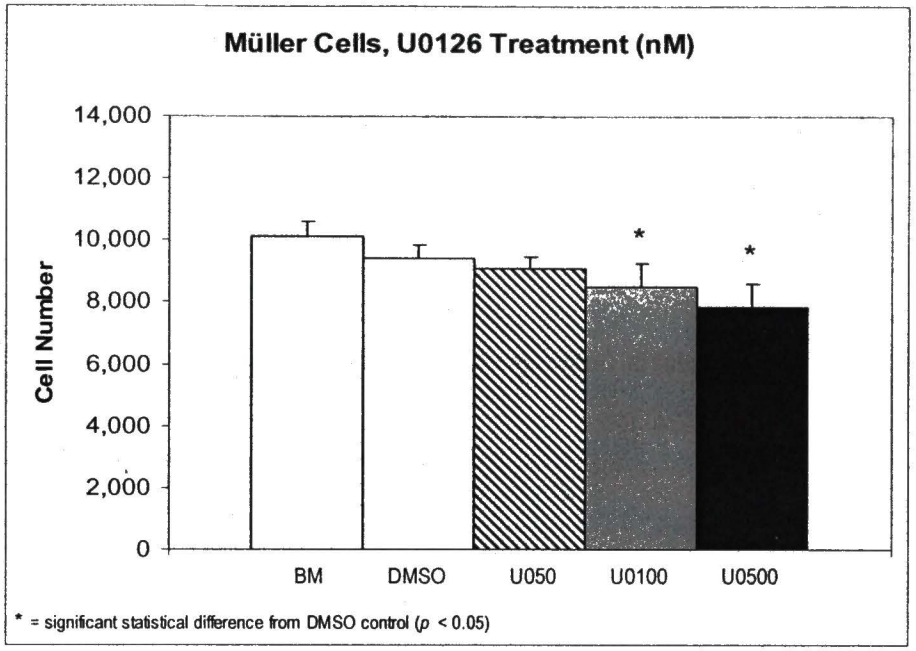


Figure 4b. Effect of U0126 on Müller cells survival.

Müller cells were treated with 50, 100 and 500 nM U0126 for 24 h and then assayed for survival with a non-radioactive MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. U0126 caused a significant ($p < 0.05$) decrease in Müller cell survival at 100 and 500 nM as compared to DMSO treated cells. Bars represent mean $\pm$ SD. * = statistically significant difference from DMSO control ($p < 0.05$). U050, U0100, and U0500 = treatment with 50, 100, and 500 nM U0126 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

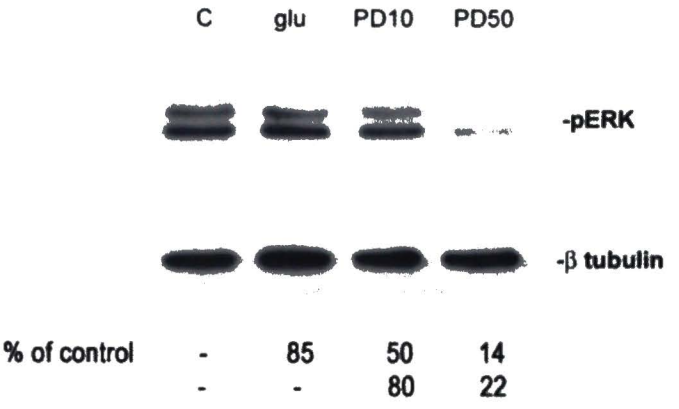


Figure 4c. Effect of PD98059 on pERK expression.

Cell lysate was collected from Müller cells treated with varying concentrations of PD98059 (10, 20 and 50 □M) for 2 h. Cell lysate was separated by SDS-PAGE followed by immunoblot with an antibody to pERK1/2 (1:500). Treatment with PD98059 resulted in a decrease in pERK1/2 expression by more than 75%. Figure shows a representative blot, immunoblot was done 3 times to confirm results.

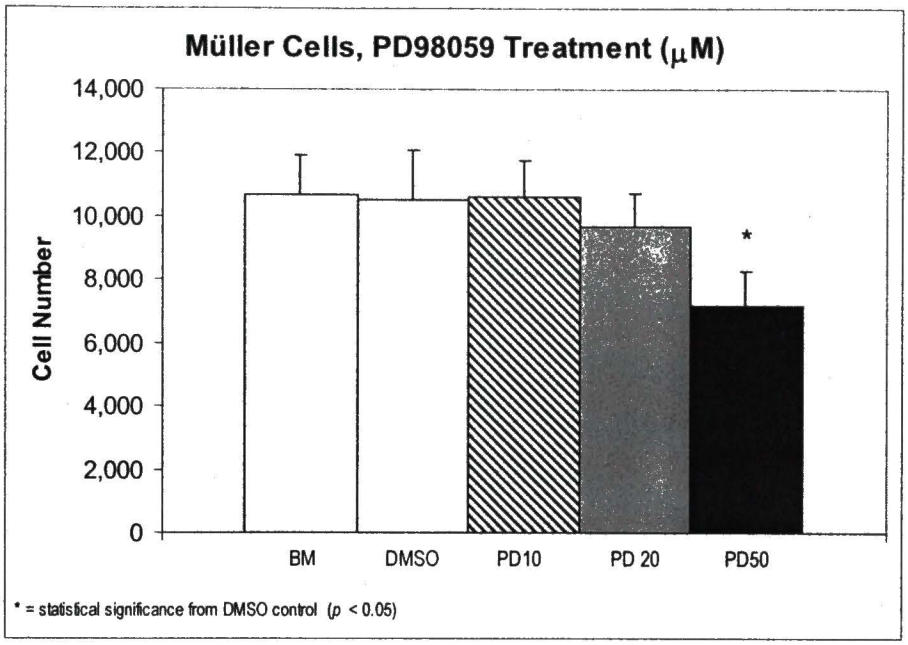


Figure 4d. Effect of PD98059 on Müller cell survival.

Müller cells were treated with 10, 20 or 50 □M PD98059 for 24 h and then assayed for survival with a non-radioactive MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. PD98059 did not decrease Müller cell survival significantly ($p < 0.05$) until a concentration of 50 □M PD98059 was used. Bars represent mean + SD. * = statistically significant difference from DMSO control ($p < 0.05$). PD10, PD20, and PD50 = treatment with 10 mM, 20 mM, and 50 mM PD98059 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

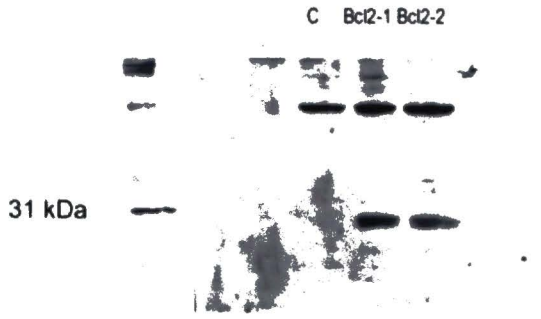


Figure 4e. Bcl2 Transfection.

Müller cells were transfected with Bcl2 plasmid DNA. Cell lysate was collected from transfected cells, separated by SDS-PAGE followed by immunoblot with an antibody to Bcl2 (1:200) to verify the success of the transfection.

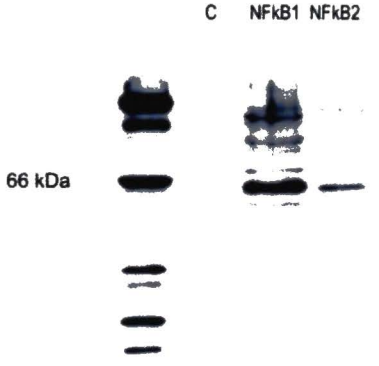


Figure 4f. NFkB Transfection.

Müller cells were transfected with NF□B plasmid DNA. Cell lysate was collected from transfected cells, separated by SDS-PAGE followed by immunoblot with an antibody to NFκB (1:200) to verify the success of the transfection.

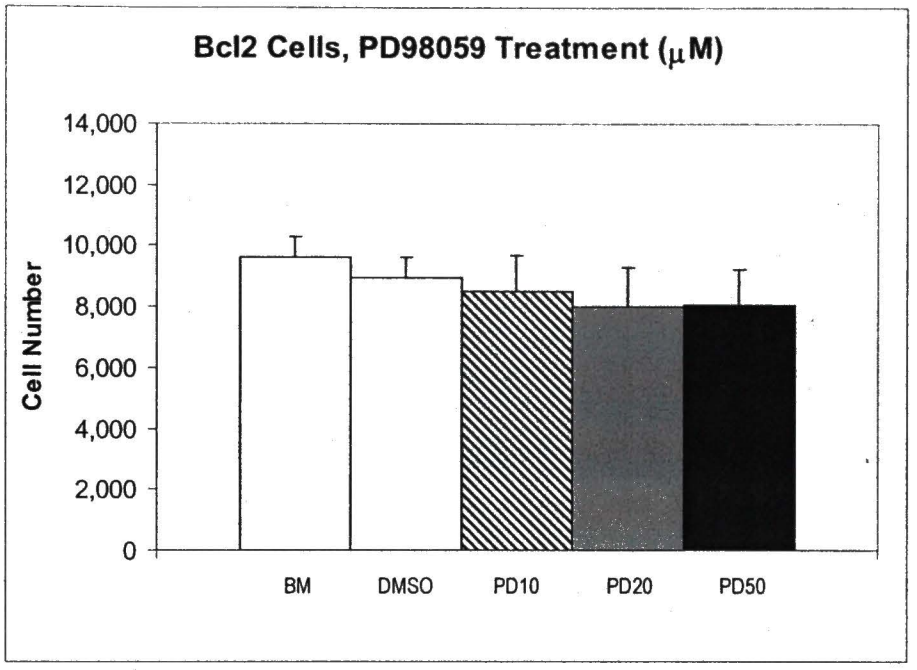


Figure 4g. Effect of PD98059 on Bcl2 transfected Müller cell survival.

Müller cells transfected with Bcl2 plasmid were treated with 10, 20 or 50 □M PD98059 for 24 h and then assayed for survival with MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine the number of surviving cells in treated Müller cell cultures. PD98059 did not decrease Müller cell survival significantly ($p < 0.05$) at any of the concentrations of PD98059 used in the experiment. Bars represent mean + SD. PD10, PD20, and PD50 = treatment with 10 mM, 20 mM, and 50 mM PD98059 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

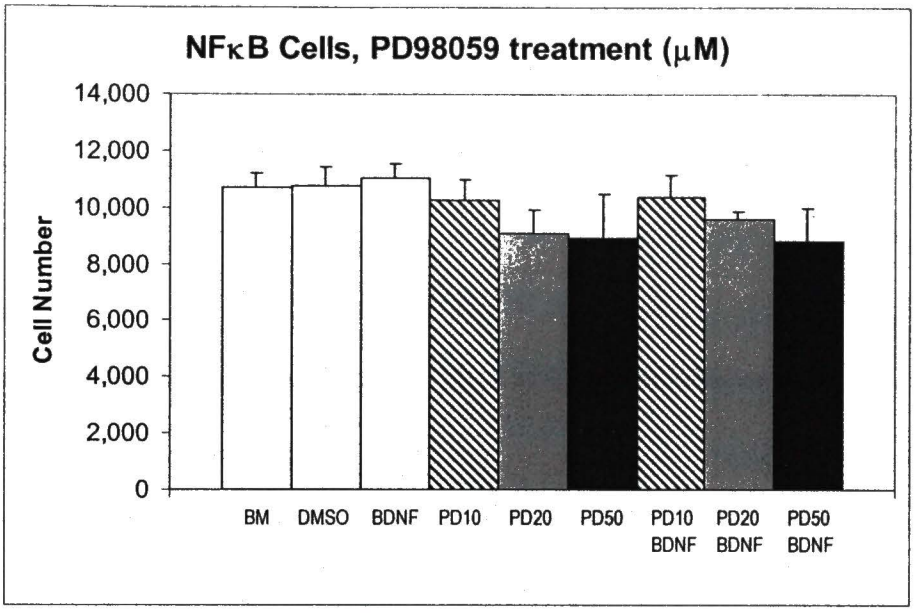


Figure 4h. Effect of PD98059 on NFkB transfected Müller cell survival.

Müller cells transfected with NFκB plasmid were treated with 10, 20 or 50 μM PD98059 for 24 h and then assayed for survival with MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. PD98059 did not decrease NFκB transfected Müller cell survival significantly ($p < 0.05$) at any of the concentrations used in the experiment. PD10, PD20, and PD50 = treatment with 10 mM, 20 mM, and 50 mM PD98059 respectively. Bars represent mean $\pm$ SD. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

CHAPTER V

RESULTS-SPECIFIC AIM 2:

PI3 (Akt) Pathway in the Survival of Cultured Müller Cells

Our examination of intrinsic cell signaling pathways in Müller cells continued with an examination of the PI3K pathway. Cultured Müller cells were treated with inhibitors of the PI3K pathway to determine the role of this pathway to Müller cell survival. Table 4 (p. 76) summarizes the experimental design of Specific Aim 2.

Effect of PI3K inhibition on Müller cell survival

In order to determine if the PI3K inhibitor LY294002 reagent was active, we examined its effect on pERK expression. Although pERK is not directly downstream of PI3K, it is downstream of Raf – and the activity of Raf is blocked by Akt. Presumably if Akt is blocked by LY294002 then the block provided by Akt would be removed and pERK could be expected to increase. To examine the effect of LY294002 on the expression of pERK, an immunoblot with an antibody to pERK was done on cell lysate collected from cultured Müller cells treated with varying concentrations of LY294002 (10 and 50 μM) which showed appropriately sized bands for pERK1/2 at 44 and 42 kDa. Treatment with LY294002 resulted in a marked (~ 150%) increase in pERK1/2 expression at the higher concentration of LY294002 (Fig 5a).

Having established that the LY294002 inhibitor was active, Müller cells were treated with LY294002 at various concentrations (10 – 50 μM) for 24 hours and then assayed for cell survival with an MTS/PMS survival assay in order to investigate the effect of PI3K inhibition on Müller cells. In this experiment, and proliferation assays to follow, since DMSO was the diluent used in the inhibitor reagents, DMSO controls at the most concentrated levels of DMSO used in the experiment (1:1000) were run to show that DMSO did not have a significant (($p < 0.05$) effect on Müller cell survival. Treatment with LY294002 (1- 50 μM) resulted in a marked (up to 60%) dose dependent decrease in Müller cell survival (Fig 5b). The graph shows treatment at 10-50 mM LY294002 but earlier data shows that there was a significant ($p < 0.05$) decrease in cell survival at 1 μM LY294002 (data not shown) as well. The IC_{50} of LY294002 is 1.4 – 6 μM (Product specification, product # 440202, CalBiochem, La Jolla, CA) so a significant loss of cell survival was seen near the IC_{50} of LY294002.

Effect of PI3K inhibitor on Bcl2 and NFkB transfected Müller cell survival

To further elucidate the role of PI3K signaling in Müller cells we used Bcl2 and NFκB transfected cells in survival assays once again (this time with PI3K inhibitor) because these two transcription factors are downstream effector proteins in the PI3K pathway (58, 22) as well as in the MAPK(ERK) pathway. Müller cells were transfected with Bcl2 or NFκB plasmid DNA and grown in culture. After selection with geneticine the success of the transfection was verified by probing cell lysate with antibodies to Bcl2 and NFκB (Figs 4e,f). Cultures of successfully transfected cells were treated with

various concentrations of LY294002 (10 –50 μM) and then analyzed for survival by MTS/PMS assay. Neither the Bcl2 gene, nor the NFkB gene were able to provide protection from the PI3K inhibitor since cell survival was markedly decreased (~ 50 % in both cell lines) with inhibitor treatment (Figs 5c,d). Treatment of untransfected Müller cells, Bcl2 transfected cells and NFκB transfected cells with 10, 20 and 50 μM LY294002 resulted in no significant ($p < 0.05$) changes cell survival at each treatment concentration, showing that NFκB and Bcl2 provide Müller cells with no protection from LY294002 treatment.

Effect of Akt inhibition on Müller cell survival

Next, inhibition of a downstream protein in the PI3K pathway – Akt was examined to further elucidate the role of the PI3K signaling pathway in Müller cell survival. The effectiveness of the Akt inhibitor was shown indirectly with an immunoblot of increased pERK expression by Müller cells treated with Akt inhibitor (data not shown). Cultured Müller cells were treated with various concentrations of Akt Inhibitor (1 – 10 μM) for 24 h and then the cells were assayed for survival with an MTS/PMS proliferation assay. Surprisingly even though a PI3K inhibitor decreased Müller cell survival significantly, treatment Akt Inhibitor did not result in significant ($p < 0.05$) decreases in cell survival at any of the concentrations used in the experiment (Fig 5e). The IC_{50} of Akt Inhibitor is 5 μM (Product specification, product # 124005, CalBiochem, La Jolla CA).

Summary/Concluding Remarks

The results of the experiments done for Specific Aim 2 show that the PI3K signaling pathway is essential for Müller cell survival. Inhibition of PI3K with LY294002 clearly showed a decrease in cell survival when this kinase is blocked. Surprisingly, the PI3K mediated mechanism of Müller cell survival appears to be Akt independent since there was no significant decrease in cell survival at any of the concentrations of Akt Inhibitor tested. As opposed to the MAPK(ERK) pathway, neither Bcl2 nor NFκB appear to be downstream mechanisms of PI3K mediated survival, since cells transfected with these proteins exhibited as much sensitivity to inhibition of PI3K as the untransfected cells. Finally since LY294002 treatment results in increased pERK and causes decreases in Müller cell survival it appears that expression of pERK is not related to Müller cell survival unless the levels drop below a crucial level (as seen in the Specific Aim 1 results).

CHAPTER V TABLES AND FIGURES

Inhibitor	Cell type	Target	IC_{50}	Concentration used in study	Sig. decrease in survival seen at:
LY294002	Müller	PI3K	1.4 - 6 μM	1 – 50 μM	1 μM
LY294002	Bcl2 X	PI3K	1.4 - 6 μM	1 – 50 μM	1 μM
LY294002	NFκB X	PI3K	1.4 - 6 μM	1 – 50 μM	1 μM
Akt Inhibitor	Müller	Akt	5 μM	1 – 10 μM	-------

Table 4. Table summarizing PI3K inhibitor treatment and effect.

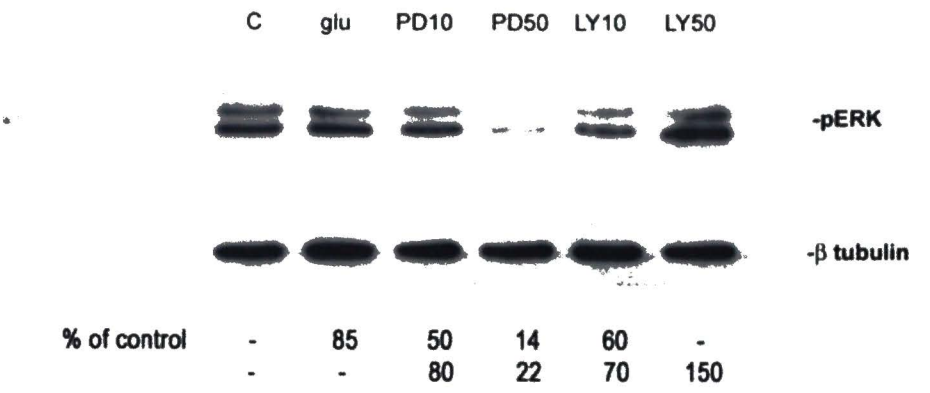


Figure 5a Effect of LY294002 on pERK expression.

Cell lysate was collected from Müller cells treated with varying concentrations of LY294002 (10, 20 and 50 μM) for 2 h. Cell lysate was separated by SDS-PAGE followed by immunoblot with an antibody to pERK (1:500). Treatment with 50 μM LY294002 resulted in a marked (150%) increase in pERK expression. Figure shows a representative blot, immunoblot was done 3 times to confirm results.

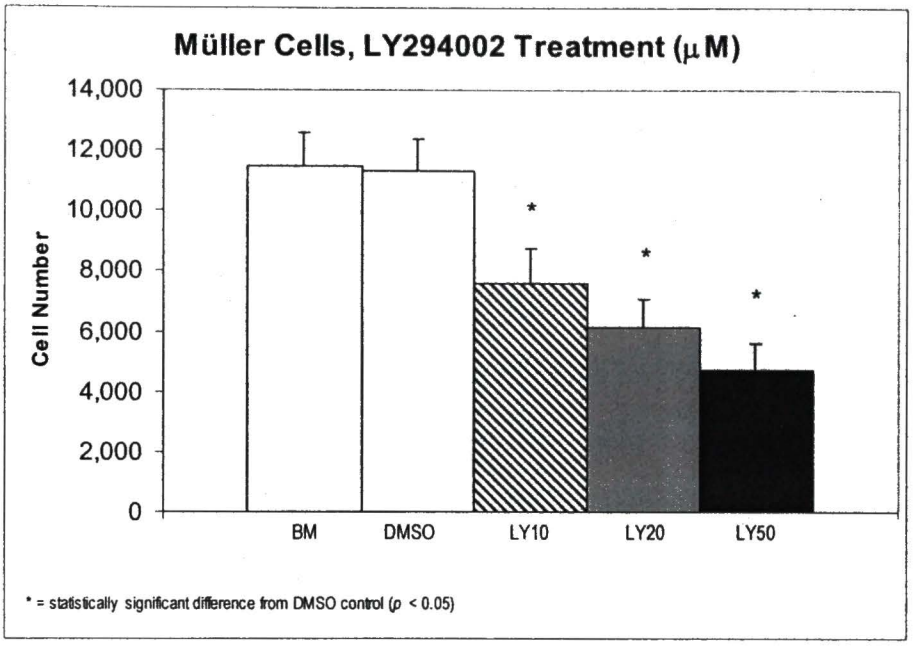


Figure 5b. Effect of LY294002 on Müller cell survival.

Müller cells were treated with 10, 20 or 50 μM LY294002 for 24 h then assayed for survival using MTS/PMS assay. DMSO (1:1000) was used as diluent, and treated cultures were compared to a DMSO control (1:1000). Treatment with LY294002 resulted in a significant ($p < 0.05$) dose dependent decrease in Müller cell survival. Bars represent mean $\pm$ SD. * = statistically significant difference from DMSO control ($p < 0.05$). LY10, LY20, and LY50 = treatment with 10, 20, and 50 μM LY294002 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

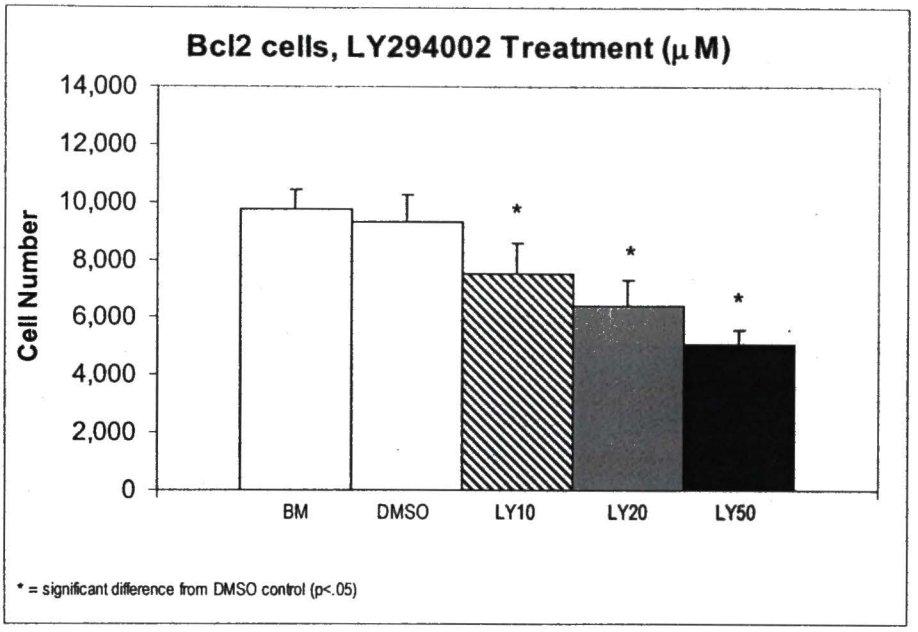


Figure 5c. Effect of LY294002 on Bcl2-transfected Müller cells.

Müller cells were transfected with Bcl2 plasmid DNA and then treated with 10, 20 or 50 μM LY294002 for 24 h prior to being assayed for survival with MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine the number of surviving cells in treated Müller cell cultures. Treatment with LY294002 resulted in a significant ($p < 0.05$) dose dependent decrease in Müller cell survival. Bars represent mean $\pm$ SD. * = statistically significant difference from DMSO control ($p < 0.05$). LY10, LY20, and LY50 = treatment with 10, 20, and 50 μM LY294002 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

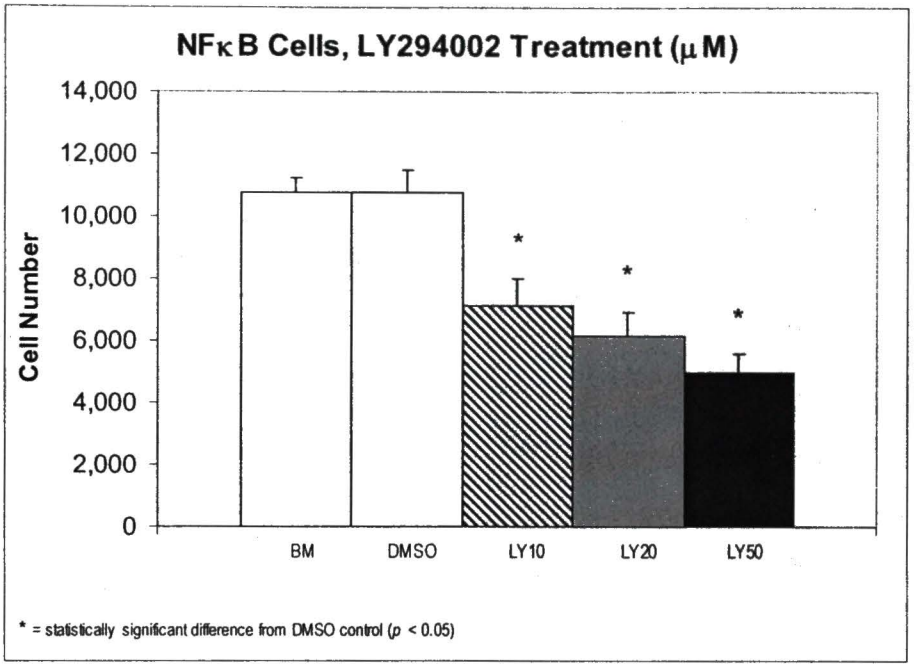


Figure 5d. Effect of LY294002 on NFκB transfected Müller cells.

Müller cells stably transfected with NFκB plasmid DNA were treated with 10, 20 or 50 μM LY294002 for 24 h then assayed for survival using MTS/PMS assay. DMSO (1:1000) was used as diluent, and treated cultures were compared to a DMSO control (1:1000) to determine the number of surviving cells. Treatment with LY294002 resulted in a significant ($p < 0.05$) dose dependent decrease in Müller cell survival. Bars represent mean $\pm$ SD. * = statistically significant difference from DMSO control ($p < 0.05$). LY10, LY20, and LY50 = treatment with 10, 20, and 50 μM LY294002 respectively. Proliferation assays done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

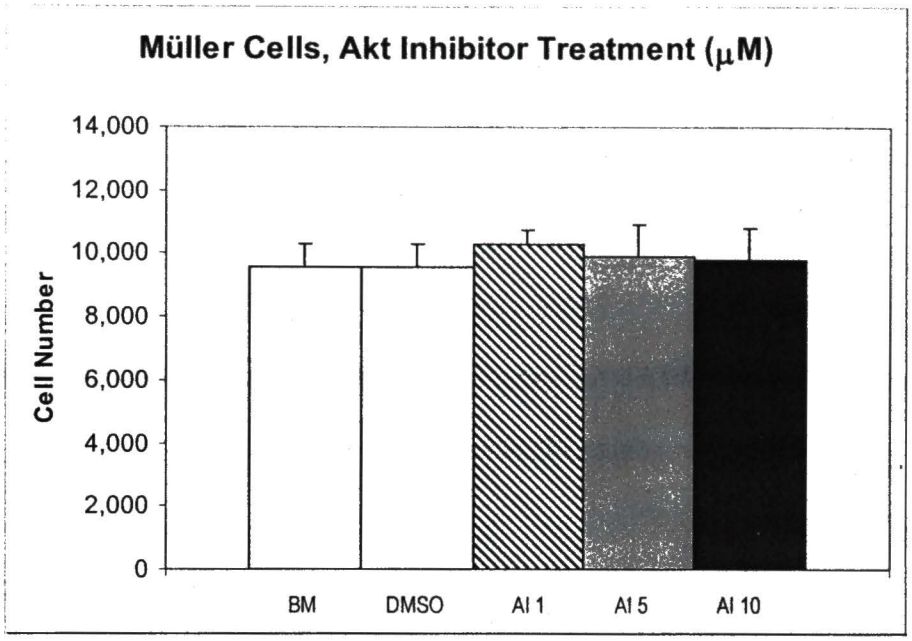


Figure 5e. Effect of Akt Inhibitor on Müller cell survival.

Proliferation Assay. Müller cells were treated with 1, 5 and 10 μM Akt Inhibitor for 24 h and then assayed for survival with a non-radioactive MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. Treatment with Akt Inhibitor did not cause a significant ($p < 0.05$) decrease in Müller cell survival. Bars represent mean $\pm$ SD. * = statistically significant difference from DMSO control ($p < 0.05$). AI1, AI5, and AI10 = treatment with 1, 5, and 10 μM Akt Inhibitor respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

CHAPTER VI

RESULTS-SPECIFIC AIM 3:

The Effects of Brain-Derived Neurotrophic Factor in MAPK and PI3K Signaling Pathways in Cultured Müller Cells

The final Specific Aim of this project was to examine the effects of BDNF on the key signaling pathways in Müller cells. Although all the neurotrophins were increased by glutamate treatment (Preliminary Data), we felt that the focus should be on the effect of BDNF on these signaling pathways since BDNF was the major neurotrophin expressed in Müller cells both in the presence and absence of glutamate treatment. While it is true that a major portion of the BDNF expressed may be a proform rather than a mature form, that is work that remains to be elucidated and the smaller form of BDNF was also expressed in Müller cells.

Effect of BDNF on Raf inhibited Müller cell survial

Our study of the effects of BDNF on the MAPK(ERK) and PI3K signaling pathways began with examination of the effect of BDNF treatment on GW5074 treated Müller cells. Cultured Müller cells were treated with BDNF (50 ng/ml) administered along with Raf inhibitor GW5074 at various concentrations (5-20 nM) for 24 hour prior to being

assayed by MTS/PMS proliferation assay. In this experiment, and in proliferation assays to follow, since DMSO was the diluent used in the inhibitor reagents, DMSO controls at the most concentrated levels of DMSO used in the experiment (1:1000) were run to show that DMSO did not have a significant (($p < 0.05$) effect on Müller cell survival. BDNF and K252a controls were also included in the assay to show that neither BDNF nor K252a at concentrations used in the experiment (50 ng/ml and 100 nM respectively) had a significant ($p < 0.05$) effect on Müller cell survival. Co-administration of BDNF and GW5074 together resulted in a significant ($p < 0.05$) 15 –30% increase in cell survival over cells treated with the inhibitor only (Fig 6a). In other cultures, Müller cells were treated with various concentrations of GW5074 (5, 10, and 20 nM) in the presence of BDNF (50 ng/ml) and K252a (100 nM) – a tyrosine kinase inhibitor. Although K252a is not a specific TrkB inhibitor, the decreases in cell survival with this treatment ressembled treatment with inhibitors alone.

Effect of BDNF on MEK inhibited Müller cell survival

Having examined the effect of BDNF on Raf inhibited Müller cells, we next looked at the effect of BDNF on MEK1/2 inhibited Müller cells. Cultured Müller cells were treated with various concentrations of a MEK1/2 inhibitor - U0126 (50 – 500 nM) in the presence of BDNF (50 ng/ml) for 24 h and then assayed for survival by MTS/PMS proliferation assay. Interestingly there was no significant ($p < 0.05$) difference in cell survival between U0126 only treated cells and those cells treated with U0126 in the presence of BDNF (Fig 6b).

Müller cell cultures treated with various concentrations of PD98059 (10 – 50 μM) in the presence of BDNF (50 ng/ml) did not result in significant ($p < 0.05$) increases in cell survival compared to cell cultures treated with the inhibitor alone (Fig 6c).

Effect of MEK inhibitors on Bcl2 and NFkB transfected Müller cell survival

To further substantiate the effect of BDNF on the Ras-Raf-MEK-ERK-Bcl2 signaling pathway, we examined the effect of neurotropins on pERK and Bcl2 levels in Müller cells. An immunoblot done with an antibody to pERK was done on cell lysate collected from Müller cells treated with BDNF, NGF, NT3, and NT4 (50 ng/ml) for 2 h showed appropriately sized bands for pERK1/2 of 44 and 42 kDa (Fig 6d). The immunoblot showed that all neurotrophins including BDNF upregulated pERK expression by 1.4 fold in the case of NT4, and by 2 fold in the case of the other neurotrophins (Fig 18a). In addition, an immunoblot done with an antibody to Bcl2 done on cell lysate collected from cultured Müller cells treated with BDNF (50 ng/ml) for 48 h showed appropriately sized bands for Bcl2 of ~ 29kDa, so Bcl2 expression increased by 2 fold in the presence of BDNF (Fig 6e).

Proliferation assay data done in Specific Aim 1 showed previously that that Bcl2 and NFκB transfected cells were able to withstand treatment with PD98059 (Figs 4g,h) so as expected, the addition of BDNF to these cultures did not result in significant increases in cell survival (Figs 6f,g).

Effect of BDNF on PI3K inhibited Müller cell survival

Turning our attention to the effect of BDNF on PI3K inhibited Müller cells, Müller cell cultures were treated with various concentrations of LY294002 (10 – 50 μM) in the presence of BDNF (50 ng/ml) for 24 h, in other cultures, Müller cells were treated with various concentrations of LY294002 (10 – 50 μM) in the presence of BDNF (50 ng/ml) and K252a (100 nM) for 24 h. All cultures were then assayed for survival by MTS/PMS proliferation assay. BDNF treatment was able to significantly ($p < 0.05$) increase Müller cell survival of LY294002 treatment with cultures receiving neurotrophin co-treatment showing a 35 – 50% increase in cell survival over inhibitor only treated cells (Fig 6h).

Effect of BDNF on PI3K inibited, Bcl2 or NFkB transfected Müller cell survival

In the next set of experiments, we wanted to determine what kind of effect BDNF would have on Bcl2 or NFκB transfected cells that were PI3K inhibited. Transfected cells were treated with various concentrations of LY294002 (10 –50 μM) in the presence of BDNF (50 ng/ml) for 24 h, in other cultures, Müller cells were treated with various concentrations of LY294002 (10 – 50 μM) in the presence of BDNF (50 ng/ml) and K252a (100 nM). All cultures were then assayed for survival by MTS/PMS proliferation assay. BDNF was able to rescue both the Bcl2 and the NFκB transfected cells from LY294002 inhibitor treatment, with inhibitor treated cells in the presence of BDNF showing a significant ($p < 0.05$) increase in cell survival ranging from a 17 – 28% over inhibitor only treated cells (Figs 6i,j).

Summary/ Concluding Remarks

The results of the experiments done for Specific Aim 3 show that although BDNF is able to increase the expression of both pERK and Bcl2 in Müller cells, it is not able to rescue Müller cells from inhibition by MEK inhibitors. BDNF did not increase cell survival in cells treated with a level of PD98059 great enough to cause a significant decrease in cell survival (50 μM) nor was it able to rescue cells that could be inhibited by treatment with U0126. Interestingly, BDNF did reverse the decrease in cell survival caused by the Raf inhibitor GW5074 although Raf is an upstream signaling molecule in the MAPK(ERK) pathway. In addition, BDNF was able to rescue any of the cells that showed decreased survival in the presence of PI3K inhibitor. BDNF increased cell survival in all cells treated with LY294002 – untransfected as well as Bcl2 and NFκB transfected cells.

CHAPTER VI TABLES AND FIGURES

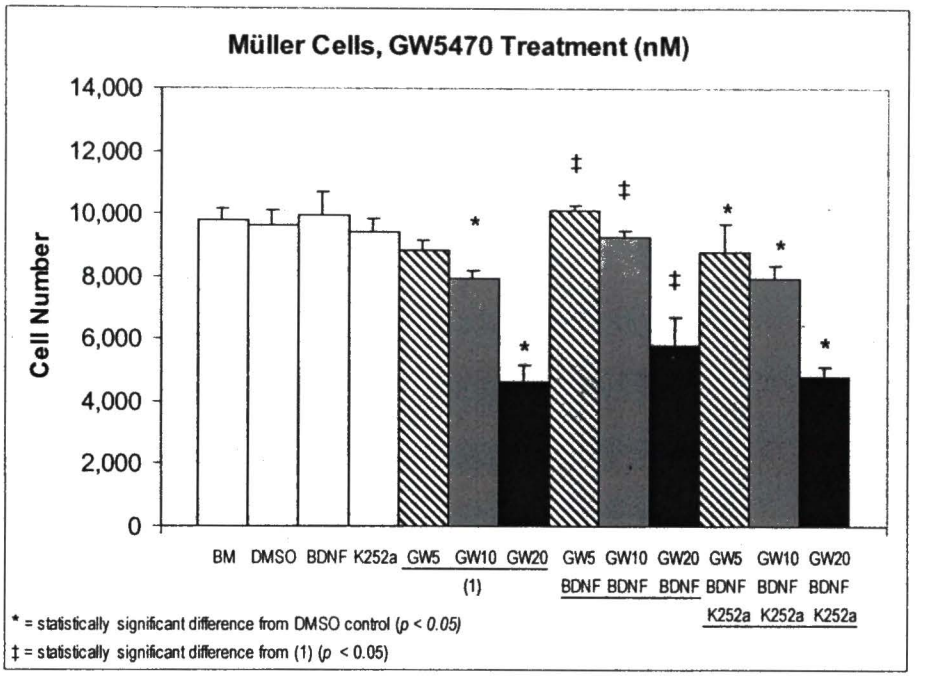


Figure 6a. The effect of BDNF on GW5074 treated Müller cell survival.

Müller cells were treated with 5, 10 or 20 nM GW5074 in the presence or absence of BDNF (50 ng/ml) for 24 h. Additionally some cells were treated with GW5074 in the presence of BDNF (50 ng/ml) and K252a (100nM) for 24 h and then assayed for survival using MTS/PMS assay. DMSO (1:1000) was used as diluent, so treated cultures were compared to a DMSO control (1:1000). GW5074 caused a dose dependent decrease in Müller cell survival as compared to DMSO treated cells, which BDNF treatment was able to rescue. Bars represent mean $\pm$ SD. * = significant difference from DMSO control; ‡ = statistically significant difference from inhibitor treatment only ($p < 0.05$). GW5, GW10, and GW20 = treatment with 5, 10, and 20 nM GW5074 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 software package.

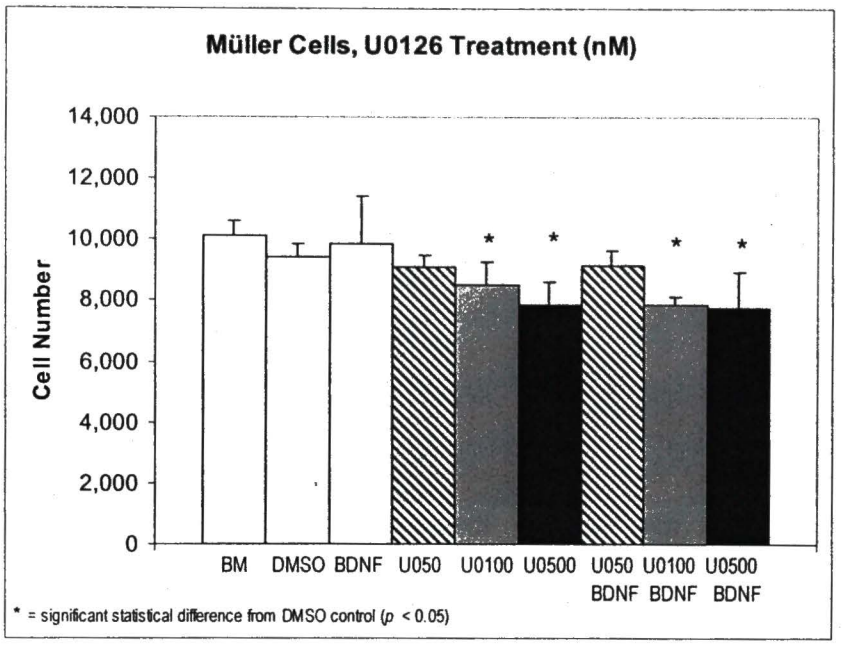


Figure 6b. The effect of BDNF on U0126 treated Müller cell survival.

Müller cells were treated with 50, 100 and 500 nM U0126 only for 24 h or treated with inhibitor in the presence of BDNF (50 ng/ml) for 24 h. Cell cultures were then assayed for survival using MTS/PMS assay. DMSO (1:1000) was used as diluent, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. U0126 caused a significant ($p < 0.05$) decrease in Müller cell survival at 100 and 500 nM as compared to DMSO treated cells, which BDNF was not able to rescue. Bars represent mean $\pm$ SD. U050, * = statistically significant difference from DMSO control ($p < 0.05$). U0100, and U0500 = treatment with 50, 100, and 500 nM U0126 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

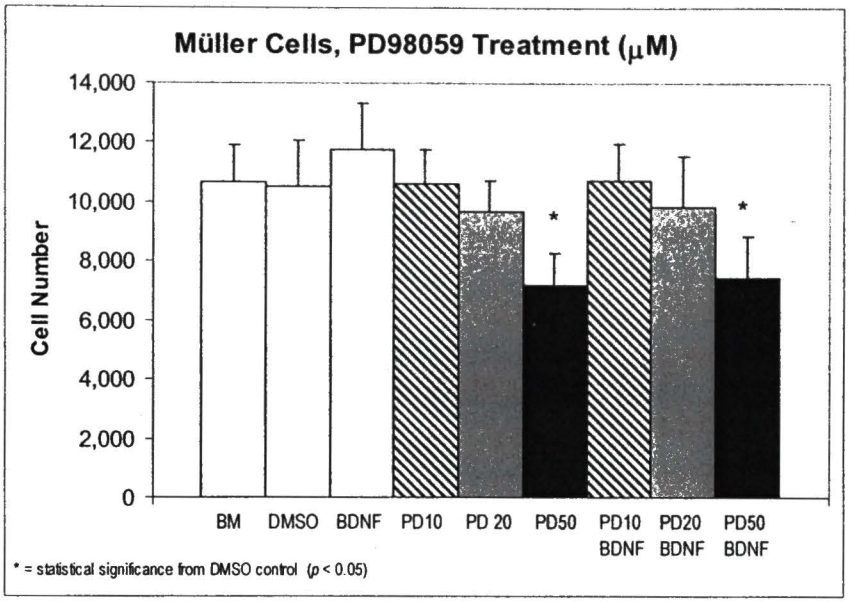


Figure 6c. Effect of BDNF on PD98059 treated Müller cell survival.

Müller cells were treated with 10, 20 or 50 μM PD98059 for 24 h or treated with inhibitor in the presence of BDNF (50 ng/ml) for 24 h. Cell cultures were then assayed for survival with a non-radioactive MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. PD98059 did not decrease Müller cell survival significantly ($p < 0.05$) until a concentration of 50 μM PD98059 was used. BDNF did not rescue Müller cells treated with 50 μM PD98059. Bars represent mean $\pm$ SD. * = statistically significant difference from DMSO control ($p < 0.05$). PD10, PD20, and PD50 = treatment with 10 mM, 20 mM, and 50 μM PD98059 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

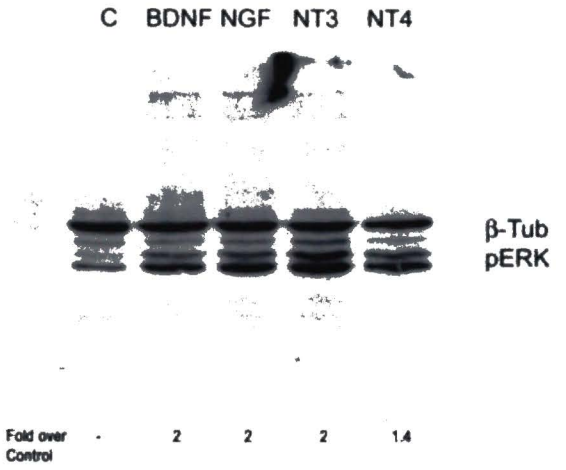


Figure 6d. Effect of neurotrophins on pERK expression in Müller cells.

Cell lysate was collected from cultured Müller cells treated with BDNF, NGF, NT3, and NT4 (50 ng/ml) for 2 hours followed by separation by SDS-PAGE and immunoblot analysis with an antibody to pERK (1:500). All of the neurotrophins produced an increase in pERK expression in cultured Müller cells, up to 2 fold. Figure shows a representative blot, immunoblot was done 3 times to confirm results.

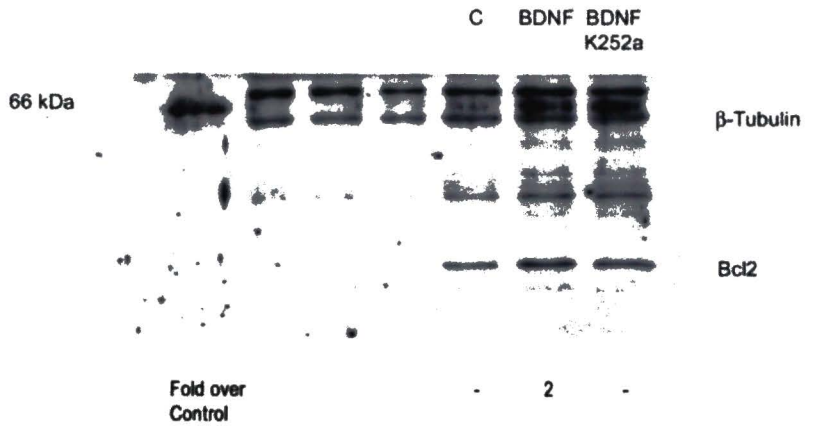


Figure 6e. Effect of BDNF on Bcl2 expression in Müller cells.

Cell lysate was collected from Müller cells treated with BDNF (50 ng/ml) and BDNF (50 ng/ml) in the presence of K252a (100 nM) for 48 h followed by separation by SDS-PAGE and immunoblot analysis with an antibody to Bcl2 (1:200). Treatment with BDNF resulted in a 2 fold increase in Bcl2 expression which was blocked in the presence of K252a. Figure shows a representative blot, immunoblot was done 2 times to confirm results.

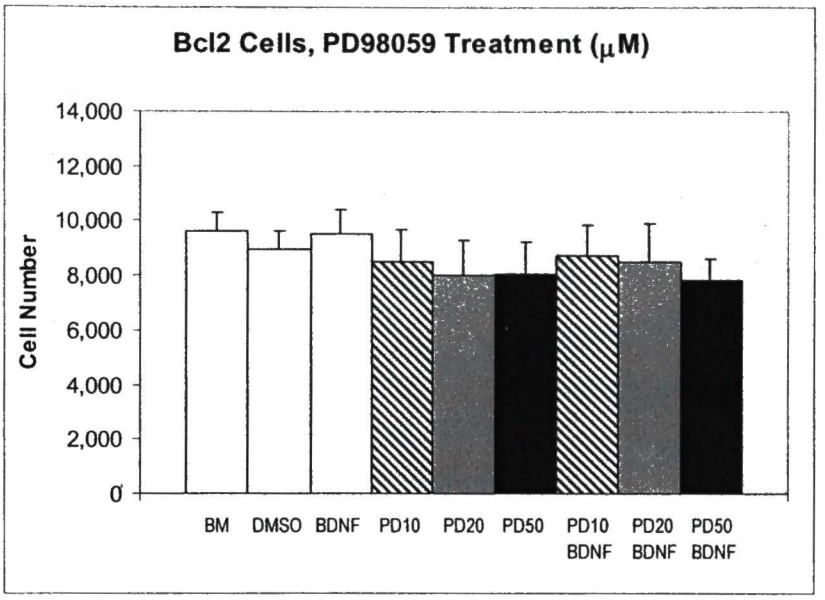


Figure 6f. Effect of BDNF on PD98059-treated Bcl2-transfected Müller cells.

Müller cells transfected with Bcl2 plasmid were treated with 10, 20 or 50 μM PD98059 for 24 h. Additionally some cells were treated with PD98059 in the presence of BDNF (50 ng/ml) and K252a (100nM) for 24 h, and then all cell cultures were assayed for survival using MTS/PMS assay. DMSO (1:1000) was used as diluent, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. PD98059 did not decrease Müller cell survival significantly ($p < 0.05$) even at a concentration of 50 μM PD98059. BDNF did not significantly ($p < 0.05$) increase or decrease cell survival in treated cells. Bars represent mean $\pm$ SD. PD10, PD20, and PD50 = treatment with 10 mM, 20 mM, and 50 mM PD98059 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

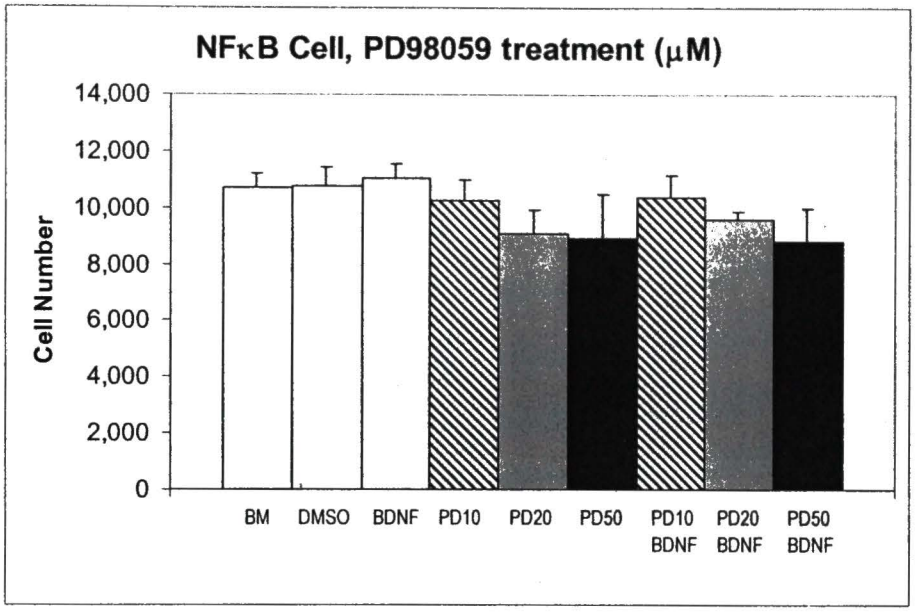


Figure 6g. Effect of BDNF on PD98059-treated NFκB-transfected Müller cells.

Müller cells transfected with NFκB plasmid were treated with 10, 20 or 50 μM PD98059 for 24 h or treated with inhibitor in the presence of BDNF (50 ng/ml) for 24 h. Cell cultures were then assayed for survival with a non-radioactive MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. PD98059 did not decrease NFκB transfected Müller cell survival significantly ($p < 0.05$) at any of the concentrations of PD98059 used. BDNF did not significantly ($p < 0.05$) increase or decrease cell survival in treated cells. Bars represent mean $\pm$ SD. PD10, PD20, and PD50 = treatment with 10 mM, 20 mM, and 50 μM PD98059 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

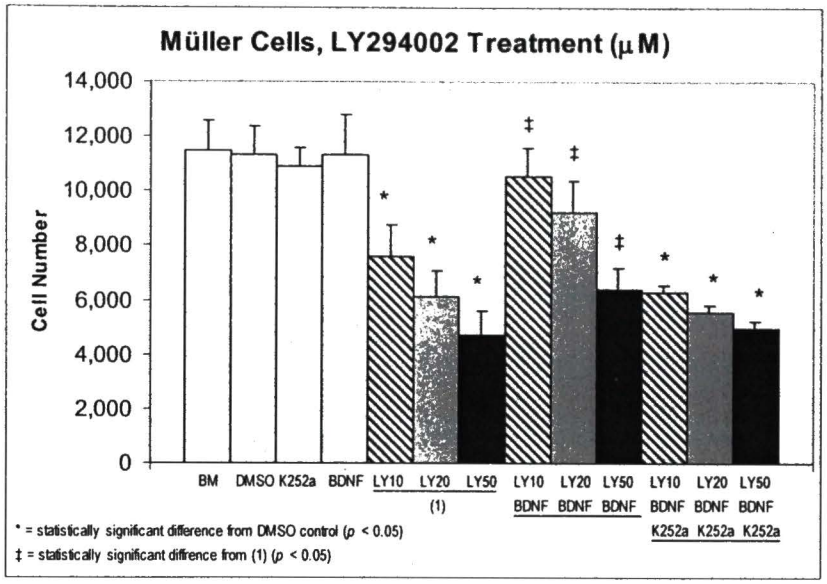


Figure 6h. Effect of BDNF on LY294002-treated Müller cells.

Müller cells were treated with 10, 20 or 50 μM LY294002 for 24 h or treated with inhibitor in the presence of BDNF (50 ng/ml) for 24 h. Additionally some cells were treated with inhibitor in the presence of BDNF (50 ng/ml) and K252a (100nM) for 24 h and then all cell cultures were assayed for survival with a non-radioactive MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. LY294002 caused a significant ($p < 0.05$) dose dependent decrease in Müller cell survival which BDNF was able to rescue. Bars represent mean $\pm$ SD. * = statistically significant difference from DMSO control; ‡ = statistically significant difference from inhibitor treatment only ($p < 0.05$). PD10, PD20, and PD50 = treatment with 10 mM, 20 mM, and 50 mM PD98059 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 software package.

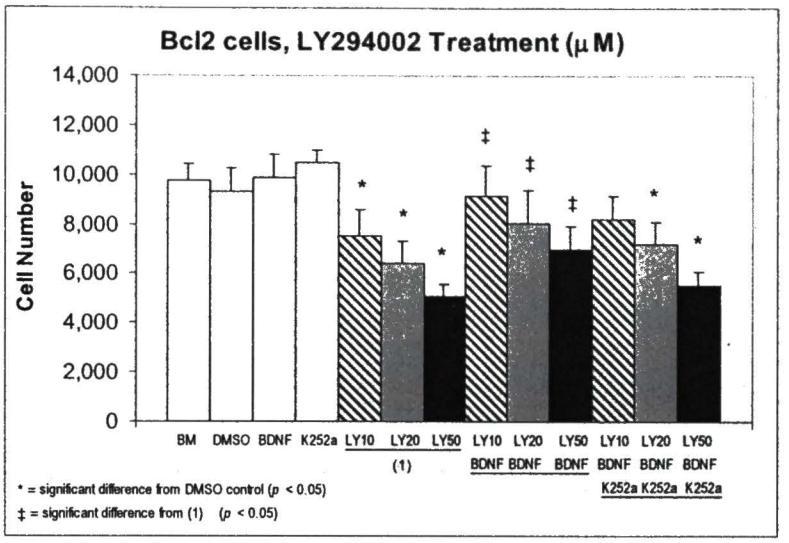


Figure 6i. Effect of BDNF on LY294002-treated Bcl2-transfected Müller cells.

Müller cells were treated with 10, 20 or 50 μM LY294002 for 24 h or treated with inhibitor in the presence of BDNF (50 ng/ml) for 24 h. Additionally some cells were treated with inhibitor in the presence of BDNF (50 ng/ml) and K252a (100 nM) for 24 h and then all cell cultures were assayed for survival with a non-radioactive MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. LY294002 caused a significant ($p < 0.05$) dose dependent decrease in Müller cell survival, which could be rescued with BDNF. Bars represent mean $\pm$ SD. * = statistically significant difference from DMSO; ‡ = statistically significant difference from inhibitor treatment only ($p < 0.05$). PD10, PD20, and PD50 = treatment with 10 mM, 20 mM, and 50 mM PD98059 respectively. Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 software package.

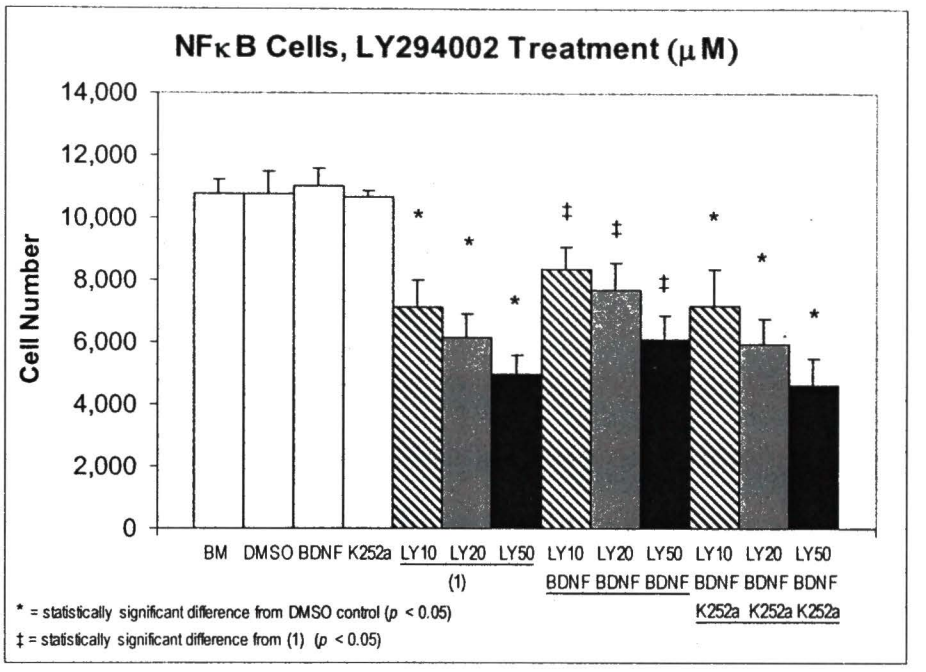


Figure 6j. Effect of BDNF on LY294002 treated, NFκB-transfected Müller cells.

Müller cells were treated with 10, 20 or 50 μM LY294002 for 24 h and then assayed for survival with a non-radioactive MTS/PMS assay. DMSO (1:1000) was the diluent used in reagent preparation, so treated cultures were compared to a DMSO control (1:1000) to determine significant decreases ($p < 0.05$) in treated Müller cell cultures. LY294002 caused a significant ($p < 0.05$) dose dependent decrease in Müller cell survival that could be rescued with BDNF treatment. Bars represent mean $\pm$ SD. * = statistically significant difference from DMSO; ‡ = statistically significant difference from inhibitor treatment only ($p < 0.05$). PD10, PD20, and PD50 = treatment with 10 mM, 20 mM, and 50 mM PD98059 respectively Proliferation assays were done in triplicate at least 3 times to confirm results. Statistical analysis was done by ANOVA with Bonferoni's correction using Systat Version 7.0 statistical software package.

CHAPTER VII

DISCUSSION

Numerous studies have suggested that glutamate is involved in many of the pathologies of the CNS including diseases of the retina. Although the exact nature of the role of glutamate in CNS neurodegeneration remains to be elucidated, indirect evidence links glutamate to neurodegeneration. Because glutamate has been implicated in retinal disease and has been shown to be lethal to RGCs (55, 66); and because the effect of glutamate on Müller cell survival has not been well studied, we decided to begin our research project with an examination of the effects of glutamate on the major retinal glial cell – the Müller cell.

The preliminary data collected for this project included immunoblots and ELISA of conditioned media collected from Müller cells treated with glutamate. The results of the immunoblot revealed that Müller cells express two different sizes of BDNF neurotrophin. One has the expected size of 14 kDa, but the other is much larger protein – about 36 kDa.

Recently various cells of the CNS such as cortical neurons and the RPE have been shown to produce an early precursor form of neurotrophins called proneurotrophins. These proforms are processed (cleaved) intracellularly by proteases after synthesis to produce the smaller mature forms (35). Since ELISA does not differentiate between

mature and proforms of BDNF, the large amount of BDNF that is seen in ELISA probably reflects the large amounts of the slow migrating 36 kDa band seen in the BDNF immunoblot. While future work in our laboratory will involve characterization of these larger bands, it is possible that they are proforms of BDNF. The appearance of putative proforms of BDNF during glutamate treatment raises some interesting questions as to the actual role of Müller cells during glutamate stress. While binding of the mature form of neurotrophins to their high affinity Trk receptors results in differentiation, neurite outgrowth, gene regulation, and survival in neurons, (35) showed that proforms of NGF and BDNF bound $p75^{NTR}$ with greater affinity than did the mature forms and resulted in apoptosis in sympathetic neurons. If neurons in the retina express $p75^{NTR}$, it is possible that increased secretion of proBDNF by the Müller cells could prove to be detrimental to these cells, belying the protective role usually assigned to Müller cells.

We also found that cell lysate from Müller cells treated with glutamate showed increased expression of EAAT1 receptors and decreased expression of NMDAR receptors. These findings are somewhat contrary to those by (38) in which concurrent downregulation of EAAT1 and NMDARs was seen in glaucoma, but our findings support the hypothesis that Müller cells may have protective mechanisms that enable them to survive glutamate stress. Since excitotoxic neurodegeneration is believed to be mediated in part by NMDARs (19, 24, 48), downregulation of this ion channel associated receptor would be expected to have a protective effect for the cell. The marked upregulation of EAAT1 transporter may have a protective function to the entire retinal environment. It stands to reason that if high concentrations of glutamate are harmful to neurons (and glia)

then increased removal and conversion of extracellular glutamate to non-toxic glutamine is likely to be beneficial to the cells of the retina.

Finally our preliminary data showed that Müller cells are not susceptible to glutamate toxicity either during prolonged exposure (up to 10 days) or at high concentrations (up to 20 mM). Although the mechanisms that are involved in Müller cell resistance to glutamate toxicity need to be elucidated, our data suggests that Müller cells maintain their expression of TrkB and BDNF binding to TrkB which may activate survival pathways in Müller cells that have been described in other cell types (31, 9). The persistent expression of TrkB and the unablated binding of BDNF to TrkB suggested to us that BDNF is important to Müller cells and perhaps maintains an intrinsic signaling mechanism that is essential for Müller cell survival. Thus the objective in this research project was to examine signaling pathways in Müller cells that may contribute to Müller cell survival during such stresses as glutamate treatment.

The Müller cells were first treated with an inhibitor upstream of MEK and ERK in the MAPK pathway, the Raf inhibitor GW5470. Treatment with this inhibitor resulted in a marked dose dependent loss of cell survival, however the concentrations used in this experiment were based on IC_{50} values for GW5074 of 9 nM. The IC_{50} of GW5470 is 9 nM but it does not inhibit the Raf/MEK/ERK cascade in cell culture until concentrations of 5 μM are used (34). Although Raf is an upstream signaling molecule in the MAPK(ERK) pathway, it seems likely that the death caused by the concentrations of GW5074 used in our experiments is due to inhibition of a pathway other than the MAPK(ERK) pathway.

Although Raf inhibition may not have been decreasing Müller cell survival by inhibiting the MEK/ERK portion of the pathway, treatment with a MEK inhibitor (U0126) reduced Müller cell survival in a dose dependent manner with significant loss of cell survival seen near the IC_{50} of U0126. Treatment of cultured Müller cells with the MEK1/2 inhibitor PD98059 resulted in no significant loss of cell survival until the PD98059 concentration reached 50 μM. The IC_{50} of MEK1 is 5–10 μM but the IC_{50} of MEK 2 is 50 μM so this result suggests MEK2 is crucial for survival that in Müller cells. Immunoblots of Müller cell lysate treated with PD98059 and then probed with pERK antibody showed that a low concentration of PD98059 (10 μM) resulted in levels of pERK2 that were 80% of the control treatment, but that the concentration of PD98059 that resulted in significant decrease in Müller cell survival in the proliferation assays (50 μM), downregulated pERK2 expression by more than 75%. (20) refers to U0126 as an ERK2 inhibitor which is supportive of the hypothesis that ERK2 is an element of the MAPK signaling pathway that is crucial for cell survival. When taken together, the results of the immunoblot analysis and the results of the survival assays supports the hypothesis that pERK2 is essential to MAPK(ERK) mediated survival of cultured Müller cells. This doesn't rule out the significance of pERK1 to Müller cell survival, but it does suggest that pERK2 is a critical component of the MAPK(ERK) signaling cascade. Our proliferation assays done with Bcl2 and NFκB transfected cells suggest that the MAPK(ERK) signaling cascade is mediated by these proteins.

Next we turned our attention to the role of the PI3K pathway in Müller cell survival. Treatment with the PI3K inhibitor LY294002 resulted in a dose dependent decrease in

cell survival. We used Bcl2 and NFκB transfected cells in survival assays once again (this time with PI3K inhibitor) because these two transcription factors are downstream effector proteins in the PI3K pathway (58, 20) as well as in the MAPK(ERK) pathway. Bcl2 transfected cells showed a pattern of sensitivity similar to untransfected Müller cells when treated with LY294002 as did NFκB transfected Müller cells. Thus it appears that PI3K does not act through either Bcl2 or NFκB to effect Müller cell survival, PI3K must contribute to Müller cell survival through another pathway. Interestingly, treatment with an inhibitor of a signaling molecule downstream of PI3K signaling (Akt Inhibitor) resulted in no significant loss of cell survival even at high concentrations of Akt inhibitor. The susceptibility of Müller cells to treatment with LY294002 coupled with their resistance to treatment with Akt Inhibitor suggests that there is an Akt independent - PI3K dependent cell signal that is crucial to Müller cell survival.

Immunoblot data shows that LY294002 treatment at low concentrations (10 μM) lowered pERK expression but high concentrations (50 μM) increased pERK expression over control by 150%. Thus, high concentrations of PD98059 and LY294002 both decrease Müller cell survival and yet they have opposite effects on pERK expression. We conclude from this that the PI3K pathway is able to modulate pERK but that it acts through another mechanism to promote Müller cell survival. The likely mechanism by which PI3K modulates pERK expression is via the activation of Akt which acts as an inhibitor of Raf (16, 36). LY294002 inhibits Akt, removing the block on Raf, resulting in an increase in pERK. The decreases in pERK at low concentrations of LY294002 present a conundrum, a possible explanation is pharmacological – that intense modulation

of the Akt signaling molecule has a different effect on its targets than less forceful control.

After establishing that both the MAPK(ERK) and the PI3K pathways contribute to cell survival in Müller cells, we began our examination of the effect of BDNF on these two signaling pathways. Interestingly, the GW5470 induced decrease in cell survival could be rescued with BDNF treatment, while loss of cell survival induced by either of the MEK inhibitors PD98059 or U0126 could not be rescued with BDNF treatment. Since the concentrations of the MEK inhibitors that were great enough to decrease Müller cell survival were concentrations that were great enough to decrease the activity of pERK2, this suggests that once pERK2 is compromised, BDNF is not able to rescue the cells. It seems counterintuitive that BDNF should rescue GW5074 cells since it cannot rescue PD98059 or U0126 treated cells, since Raf is upstream of MEK in the MAPK(ERK) pathway. One possible explanation may be that Raf is able to activate PI3K as well as MEK in Müller cells. If this is true, it would explain why treatment with GW5074 can be rescued with BDNF as can treatment with LY294002. Another explanation is that Raf and PI3K both activate a common downstream mechanism that responds to BDNF and contributes to Müller cell survival. The IC_{50} of GW5470 is 9 nM but it does not inhibit the Raf/MEK/ERK cascade in cell culture until concentrations of 5 μM are used so although Raf is an upstream signaling molecule in the MAPK(ERK) pathway, it seems likely that the death caused by the concentrations of GW5074 used in our experiments is due to inhibition of a pathway other than the MAPK(ERK) pathway.

It's possible that the alternative pathway is PI3K mediated – hampered when the Raf inhibitor is applied.

Immunoblots done on Müller cell lysate treated with BDNF, NGF, NT3 and NT4 and probed with pERK antibody showed that all these neurotrophins upregulate pERK expression and we showed that BDNF treatment upregulates the expression of Bcl2 protein in Müller cells as well. This data suggests that BDNF may contribute to the increase in expression of anti-apoptotic proteins. Bcl2 and NFκB transfected Müller cells were not inhibited by PD98059 treatment, and taken together these results suggest that constitutive BDNF stimulation of the MAPK pathway works through Bcl2 and NFκB to contribute to Müller cell survival.

In the PI3K pathway, BDNF was able to rescue LY294002 treated cells and the susceptibility of Müller cells to treatment with LY294002 coupled with their resistance to treatment with Akt Inhibitor suggests that there is an Akt independent - PI3K dependent cell signal that is crucial to Müller cell survival and is responsive to BDNF. Whether or not cells were transfected with Bcl2, NFκB or left untransfected, BDNF was able to rescue cells from LY294002 treatment. Since Bcl2 and NFκB are generally believed to be downstream of Akt (36, 16, 63), it follows that the Bcl2 and NFκB transfected cells should not be able to provide protection from inhibition of a PI3K mediated mechanism of cell survival that appears to be Akt independent.

It's interesting to speculate what this PI3K dependent – Akt independent mechanism of cell survival might be. A possible alternative Akt independent mechanism through which PI3K might be able to effect cell survival is via the activation of the JNK pathway.

Recently it has been reported that ablating PI3K with LY294002 decreases the phosphorylation and activiation of pAkt while increasing the activity of the JNK pathway (53). Alternatively, PI3K might function to keep levels of nitric oxide synthase (NOS) low in the cell. Indeed, activated PI3K has been shown to be essential in keeping NOS levels low in macrophages (11).

Our results suggest that while Müller cells do not die when subjected to glutamate treatment, they seem to exhibit signs of acitvation. Recently it has been reported that there is immunocytochemical evidence of retinal astrocyte and Müller cell activation in glaucoma. Immunocytochemistry of retinas from glaucomatous versus normal donors showed profound increases in density and GFAP staining in the glaucomatous donors as compared to normal donors that is localized to astrocytes and Müller cells (67). Furthermore in the CNS, glial cells exhibiting the hallmark identifier of activation (increased GFAP expression) have been correlated to changes in expression of ion channels, glutamate receptors, and neurotrophins (51, 18, 60). It's entirely possible that increased glutamate levels in the retina can activate Müller cells, but at present it is unknown whether or not the effects of activated Müller cells are beneficial or deleterious to the other cells in the retina. It will be one of the challenges facing those who work in this area to continue to clarify the intricate interaction of the cells of the retina.

In conclusion, our study suggests that although the role of glutamate in neurodegeneration remains an enigma, it does not induce Müller cell death and the Müller cells appear to maintain constitutive signaling pathways that contribute to their survival.

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