EPENDYMIN MECHANISM OF ACTION:

FULL LENGTH EPN VS PEPTIDE CMX-8933

A THESIS

Submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Master of Science

in

Biology

by

Jennifer Kaska

May 28, 2003

APPROVED:

David S. Adams, Ph.D. Major Advisor WPI	Daniel Gibson, Ph.D. Committee Member WPI	Elizabeth Ryder, Ph.D. Committee Member WPI

ABSTRACT

Ependymin (EPN) is a goldfish neurotrophic factor (NTF) that is one of the most abundant secreted glycoprotein components of brain extracellular fluid (ECF) and cerebrospinal fluid. This protein was first discovered due to its enhanced turnover following learning events, but has since been found to function in other important cellular events such as long-term memory formation and optic nerve elongation (Shashoua, 1976; Shashoua, 1977; Shashoua, 1985). Goldfish EPN has several demonstrated effects on mammalian cells, and immuno-reactive EPN-like proteins have been observed in a variety of organisms ranging from invertebrates (*Limulus)* to mice.

Some NTFs have been shown to alleviate oxidative stress, one of the primary mediators of cell damage in neurodegenerative conditions. One mechanism by which they accomplish this is to increase cellular levels of anti-oxidative enzyme superoxide dismutase (SOD). In fact, our lab recently showed that a synthetic EPN fragment (CMX-8933) increases SOD mRNA and protein levels in rat primary cortical cultures (Parikh, 2003). Transgenic mice and rabbits that overexpress SOD are resistant to ischemia, while mice that lack SOD present with worse ischemic damage. Thus, due to this important SOD activating NTF-like feature of EPN may have potential therapeutic applications for treating neurodegenerative conditions such as Alzheimer's disease, Parkinson's or stroke.

Because full-length NTFs do not efficiently cross the blood brain barrier (BBB) when administered intravenously, our lab, in collaboration with Ceremedix, Inc. (Boston, MA), is interested in designing short peptides that mimic the action of full-length NTFs,

especially EPN. Due to proteases that naturally exist in ECF, EPN is partially cleaved to release the 8 aa peptide KKETLQFR. Other brain proteins, like encephalins and endorphins, also release similar short active components, so we hypothesized that this 8 aa peptide may represent an active component of EPN. Indeed, our lab demonstrated that administration of this sequence in synthetic form (termed CMX-8933) to rat primary cortical cells increases cellular titers of SOD (Parkih, 2003).

This thesis was divided into three parts. The first part investigated which signal transduction pathway is responsible for CMX-8933's ability to upregulate SOD. Because our lab also showed that CMX-8933 activates the MAPK pathway (Hasson, 1998; El-Khishin, 1999; Adams et al., 2003) we hypothesized that CMX-8933 may use this pathway to upregulate SOD. Inhibition experiments were performed to test three known components of the MAPK pathway, and a member of an unrelated pathway. Six independent SOD immunoblot experiments demonstrated that pre-treatment of rat primary cortical cultures with specific inhibitors for the protein kinase-C family (PKC), protein tyrosine kinases (PTKs), or MEK protein kinases (MEKK), completely blocked ($p = 0.0001$) CMX-8933's average 15-fold upregulation of SOD. Thus, these three critical components of the MAPK pathway appear to be involved in the CMX-8933-induced upregulation of SOD. An inhibitor of transcription factor NF-κB in an unrelated pathway had no significant effect ($p = 0.901$).

The second part of this thesis tested whether treatment of rat primary cortical cultures with CMX-8933 increases the cellular titers of mRNAs related to translation. Previous observations indicated that treatment of these cells with with CMX-8933 induces neurite sprouting (Shashoua, unpublished), and that EPN plays a role in optic

nerve elongation (Schmidt and Shashoua, 1988), two processes related to growth. So we hypothesized that EPN, or CMX-8933, may stimulate the transcriptioin of mRNAs related to growth. We tested mRNAs for translation factor EF-2, and ribosomal proteins S12 and L19 based on previous observations in our lab with hybridization arrays (Parikh, 2003). RT-PCR experiments indicated that treatment of rat primary cortical cultures with 10 ng/ml CMX-8933 for 5 hrs increased the mRNAs for S12 an average of 12-fold relative to untreated cultures ($N = 3$, $p = 0.02$), L19 an average of 9-fold ($N = 3$, $p = 0.048$), and EF-2 an average of 11-fold ($N = 3$, $p = 0.045$). Levels of housekeeper polyubiquitin remained unchanged. Thus 3 gene products related to growth are indeed upregulated by CMX-8933.

The third part of this thesis investigated the SOD stimulatory effects of full-length EPN versus its cleavage product CMX-8933. Previous studies showed that full-length NTFs BDNF and NGF upregulate SOD in neuronal cells. Because CMX-8933 upregulates SOD, maybe full-length EPN does too. We hypothesized that if CMX-8933 represents the receptor-binding domain of full-length EPN, that full-length EPN may show the same stimulatory effects as CMX-8933, and may upregulate SOD. Extracellular fluid (ECF) was prepared from goldfish brains, the traditional source for isolating EPN. Analysis of the ECF on protein gels demonstrated the presence of a complex protein pattern dominated by two bands at 37,000 and 31,000 daltons, the known sizes of EPN-β (glycosylated) and EPN-γ (non-glycosylated), respectively. Immunoblots performed with EPN antibody "Sheila" (directed against the C-terminal end of EPN, Shashoua and Moore, 1978) confirmed the identity of these two ECF bands as EPN. Cultured mouse Nb2a neuronal cells were treated in six independent experiments

with 12 μg/ml ECF protein for 5 hrs, and whole cell lysates were tested for levels of SOD by immunoblots. This ECF treatment of neuronal cells produced a mean 4-fold increase in SOD levels ($p = 0.007$), supporting our hypothesis.

However, since ECF is a complex mixture, this data did not show which ECF component was resonsible for the SOD signal. Since ECF is known to contain CMX-8933 EPN cleavage product, which by itself can upregulate SOD, it is possible CMX-8933 was responsible for the signal, not full-length EPN. To address this issue, microdialysis was performed using an 8,000 dalton MWCO membrane to remove low MW components from the ECF (including CMX-8933, MW = 1149), leaving EPN-β (MW 37,000) and EPN-γ (MW 31,000) present in the dialyzed ECF. Four independent experiments indicated no significant difference ($p = 0.116$) between dialyzed versus non-dialyzed ECF for activating SOD. Thus, CMX-8933 does not appear to be responsible for ECF's ability to increase SOD, but instead, high MW molecules (including full-length EPN) appear to be the active components. Altogether, the data from this thesis extends our knowledge of the mechanism of action of both full-length EPN and its cleavage product CMX-8933.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEGEMENTS

I would like to begin by expressing my greatest gratitude, appreciation and thanks to my major advisor, Dr. Dave Adams, for without his help and guidance this thesis would never have been possible. He helped me get off to an amazing start on a brand new project, and his patience, optimism and outstanding advice were my guide and savior throughout this entire project. So I owe unending thanks to him for taking me into his lab, and allowing me to learn and grow as a student with him; for the knowledge I learned in Salisbury Lab 406 will stay with me forever. I would also like to thank my committee members, Dr. Dan Gibson and Dr. Elizabeth Ryder for their valuable advice and guidance through this project, as well as their optimism. This thesis would also not have been possible without the support and sponsorship of Ceremedix, Inc. I wish to thank the members of Lab 406, especially my MQP partner Elizabeth Caswell, who originally got me involved, and was the best MQP partner I could have hoped for, as well as Suchi Parikh and Francis Saccoccio, for their help and company in the lab this past year. I wish them all the happiness and success the future holds for them. And finally I would like to thank my family, especially my mom and dad, who without their love and support, not only would this thesis not have been possible, but neither would WPI, so thank you Mom and Dad for making my hopes and dreams come true, and I hope I made you proud!

BACKGROUND

Neurotrophic Factors

Neurotrophic factors (NTFs), a subclass of growth factors, are naturally occurring brain glycoproteins that regulate both the survival and growth of various populations of neurons in the central and peripheral nervous system. Recent research shows that NTFs have an additional role in synaptic plasticity (Wu and Partridge, 1999; Nishi, 1994; Stahl, 2003).

NTFs can be divided into four categories: neurotrophins, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), the neuropoietic cytokines, the fibroblast growth factors (FGF), and the glial-derived growth factors (GDNF) (Jehan *et al.,* 1996; Cui *et al.,* 1998; Bear *et al.,* 2001; Stahl, 2003). The members of these NTF families demonstrate 50% or more homology, and have affinities for particular classes of receptors. Neurotrophins, in particular, act at specific cell surface receptors. Most of these receptors are neurotrophin-activated protein kinases, called *trk* receptors (Bear *et al.,* 2001). In addition to receptor interaction with ligand, neurons use many routes for internalization of these factors (Figure 1). They may acquire NTFs by target-derived acquisition (from their target tissue, whether neuronal or non-neuronal), by pancrine acquisition (neighboring neurons and other cells), or by autocrine acquisition.

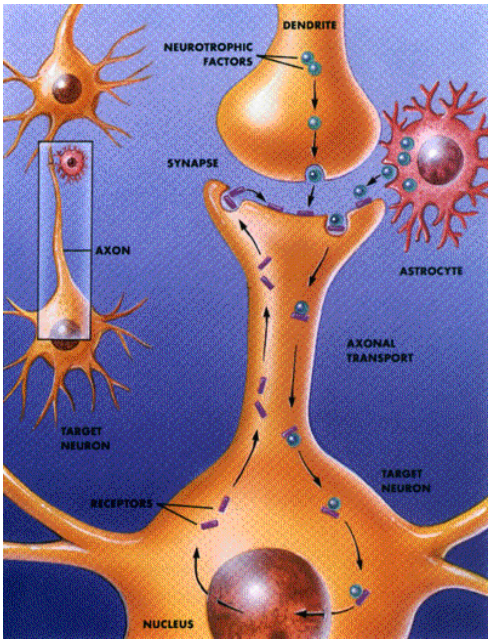


Figure 1: General Mechanism of Neurotrophic Factors (Kibiuk, 1994).

The survival and differentiation of neurons is mainly regulated by target-derived neurotrophic factors. In this cell survival model, developing neurons that fail to make correct connections will be deprived of necessary NTFs and die, whereas neurons that establish correct connections survive, differentiate and mature (Connor and Dragunow, 1998; Bear *et al.,* 2001; Stahl, 2003).

Role of NTFs in Stroke Therapy

One of the most important roles that NTFs have is their ability to help diminish the destructive neuronal oxidative stress associated with stroke. A variety of NTFs have been shown to converge on damaged tissue following stroke (Ferrer *et al.,* 1998) however, the NTFs that are best characterized relative to stroke are BDNF and GDNF. They have exhibited therapeutic effects in the brain, especially in the case of ischemic stroke. BDNF is known for promoting the survival and growth of various nerve cells, and has neuroprotective effects when administered *in vivo* (Wu and Partridge, 1999;

Ferrer *et al.,* 2001). GDNF is well known for its protection and rescue of dopaminergic neurons in Parkinson's Disease; however, it also provides protection for other neuronal motor neurons and peripheral ganglia in hypoxia-induced brain injury (Ikeda *et al.,* 2000).

Although the systemic administration of full-length NTFs has shown some therapeutic activities, the main problem lies with the ability to physically deliver these beneficial neuroprotective NTFs to the brain. The first obstacle is the brain capillary wall called the blood brain barrier (BBB), that the NTFs cannot efficiently penetrate. Therefore, NTFs need to be coupled with a BBB drug delivery system. The second obstacle is that NTFs have a rapid rate of removal from the blood, mainly by the liver (Wu and Partridge, 1999). These problems will be addressed later in more detail.

Ependymin

Ependymin (EPN) is a secreted glycoprotein component of the extracellular and cerebrospinal fluids of the goldfish brain. This protein was first discovered in goldfish brains due to its enhanced turnover rates after learning events (Shashoua, 1976; Shashoua 1977). At first, EPN was thought to be located only in the central nervous system, localized in the ependymal zone from which it derives its name (Benowitz and Shashoua, 1979), however later evidence found that it was also detected as a novel secretory protein in the brain extracellular fluid (Shashoua, 1985).

Ependymin exists in two forms, β and γ (MW 37,000 and 31,000, respectively). Ependymin β is glycosylated, and ependymin γ is non-glycosylated (Shashoua, 1976; Shashoua, 1985; Konigstorfer *et al.,* 1989; Shashoua *et al.,* 1990). The protein portion

consists of 216 amino acid residues, including two N-glycosylated sites. Both proteins can bind concanavalin A and can be isolated from the brain extracellular fluid by lectin affinity chromatography (Schmidt and Shashoua, 1983).

Preparations of ECF proteins from goldfish brains were found to contain proteases and esterases (Shashoua and Holmquist, 1986). Protease activity present in the ECF of goldfish brains (Schmidt and Shashoua, 1983; Shashoua, 1985; Shashoua, 1988) promotes the proteolysis of ependymin, releasing specific smaller peptides into the ECF (Shashoua, unpublished). This result indicates that ependymin might exert its biological activity *in vivo* via smaller polypeptide fragments. EPN β and γ were shown to constitute 14% of the total protein content of the brain extracellular fluid, and also a minor component of the serum proteins (0.3%) (Schmidt and Shashoua, 1981; Schmidt and Lapp, 1987).

Ependymin's Function

EPN is a multi-functional protein. A variety of cellular actions are common between EPN and other complex NTFs, such as involvement in synaptic biochemical changes associated with long-term memory consolidation, optic nerve elongation, and neuronal growth (Shashoua and Moore, 1978; Schmidt, 1987; Piront and Schmidt, 1988; Schmidt *et al.,* 1995; Schmidt, 1995). Injection of anti-EPN Ab can block memory formation (Schmidt and Shashoua, 1988; Adams and Shashoua, 1994). In addition, EPN has been linked with learning (Shashoua, 1985; Piront and Schmidt, 1988; Shashoua and Hesse, 1989; Rother *et al.,* 1995), acclimation to the cold (Tang *et al.,* 1999), optic nerve regeneration and neuroplasticity (Shashoua, 1985; Schmidt and Shashoua, 1988;

Shashoua, 1988; Shashoua, 1991), and neuronal growth (Schmidt and Shashoua, 1988; Schmidt *et al.,* 1991).

Ependymin Peptide Therapy: CMX-8933

Because full-length NTFs inefficiently cross the BBB, our laboratory in collaboration with Ceremedix Inc. (Boston) is currently investigating small peptide mimetics of EPN as potential therapeutics for stroke. Peptide CMX-8933 (KKETLQFR), corresponding to positions 78-85 in the mature EPN sequence (see Figure 2), was used as a potential mimetic of EPN (Shashoua *et al.,* 2001). This sequence was chosen because its 6-amino acid C-terminal portion (ETLQFR) is reproducibly generated when pure EPN is incubated with trypsin (Shashoua, 1991), or goldfish ECF (Shashoua, unpublished), and protease cleavage consensus sites exist nearby (Adams *et al.,* 1996).

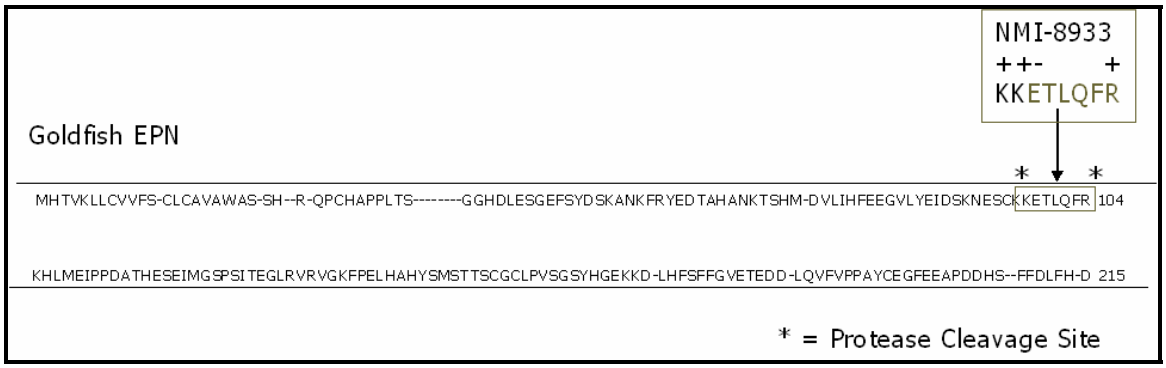


Figure 2: Full-length Ependymin Sequence and Rationale for Design of Therapy Peptide CMX-8933. The completely conserved 8 amino acid peptide drug sequence, represented in the box, is a proteolytic cleavage product of the 215 amino acid sequence of full-length EPN, both present in ECF. Charged amino acids are indicated by '+' or '-'above sequence (Adapted from Adams *et al.,* 1995; Adams *et al.,* 2003).

Also, the sequence is completely conserved among the six deduced protein sequences of the only true (highly homologous)-*epn* genes sequenced to date from the order *Cypriniformes* (Adams and Shashoua, 1994; Adams *et al.,* 1996). Our working hypothesis is that peptide CMX-8933 may be a biologically active proteolytic cleavage

product of EPN in the ECF (where EPN is present as well) possibly comprising EPN's receptor-binding domain. Encephalins and endorphins serve as a precedent for the proteolytic removal of functional byproduct fragments (Goldstein, 1976).

DHA as a BBB-Carrier: Protarga, Inc.

Protarga, Inc, a clinical stage pharmaceutical company located outside of Philadelphia, Pennsylvania, focuses on linking well characterized drugs to BBB carrier DHA to facilitate delivery into the brain, and other tissues. The principal areas of interest are: oncology, central nervous system disorders, and infectious diseases. The company, co-founded by Victor Shashoua, utilizes fatty acid technology to develop *Targaceutical*® compounds that consist of known therapeutic agents attached to fatty acids (Protarga Homepage, 2003). This technology is based on previous findings that the fatty acid docosahexaenoic acid (DHA) has hydrophobic properties that allow it to cross the Blood Brain Barrier (BBB). DHA is a naturally occurring fatty acid that is 22 carbons long with six double bonds, and is a primary fatty acid component of synaptic endings, which is necessary for brain development (Shashoua and Hesse, 1996). Protarga uses DHA as the carrier molecule for several therapeutic drugs they want to deliver to the brain. The resulting *Targaceutical*® drugs are designed to improve pharmacological performance by delivering more drug to a specific location for an extensive period of time, while maintaining the overall safety profile of the original well characterized product (Protarga Homepage, 2003).

Protarga has developed three main drugs, Taxoprexin, Doprexin, and Clozaprexin. All are currently being tested in both animal and human trials. It's lead

Targaceutical® product is *Taxoprexin*®, taxol (paclitaxel) linked to DHA, which is used in the treatment of certain cancers. DHA-paclitaxel, the active ingredient in *Taxoprexin*®, has already advanced through Phase I human clinical trials, and is now in Phase II human clinical trials (Protarga Homepage, 2003). Doprexin, or NMI-8739, consists of dopamine linked to DHA, and is used to transport dopamine to the brain to treat Parkinson's disease patients (Shashoua and Hesse, 1996). NMI-8739 has been found to increase dopamine uptake in the brain by more than 10-fold in animal models (Shashoua *et al.,* 2001). Clozaprexin is a combination of clozapine, one of the most potent anti-psychotic substances known, and DHA. It is used to treat a variety of psychotic diseases, such as schizophrenia, by transporting clozapine across the BBB using DHA as the carrier. Clozaprexin has been found to be ten times more potent and longer lasting than clozapine alone (Baldessarini *et al.,* 2001).

Adams Lab Contributions to NTF Research to Date

In collaboration with Ceremedix Inc., numerous WPI students have contributed to the research involving EPN. The earliest work began in 1994 with the isolation of goldfish *epn* genes (Adams and Shashoua, 1994), with the ultimate goal of cloning full-length human *epn*. After that, in an effort to walk up the evolutionary ladder, two more species were cloned in 1996 (Adams *et al.,* 1996).

Research related to small peptide mimetics of EPN started in 1998, when a Master's student demonstrated that ependymin-like therapy peptides NMI-9236 and NMI-8933 activate AP-1 transcription factor in mouse neuroblastoma NB2a cells (Hasson, 1998). Sleeper (1999) engineered C-127 cells, an alternative cell culture based

in vitro production system, to secrete 8933 and 9236. Transfection experiments by the late Adam El-Khishin demonstrated that the AP-1 activated by NMI-9236 and NMI-8933 administered to NB2a cells is indeed a functional particle (El-Khishin, 1999). In 2000, further research confirmed El-Khishin's findings, and demonstrated that anti-oxidative enzymes are upregulated by 9236 and 8933 therapy peptides (LeBlanc, 2000).

Then in 2001, our lab demonstrated that peptide conjugated to DHA carrier more efficiently upregulated SOD in the brain (Pfeiffer *et al.,* 2001). Later that year, CMX-204-36 was also determined to upregulate SOD, GPX, and CAT in the brain of a rat (Armistead, 2001).

More recently, studies in 2002 found that peptides CMX-204-16, when compared to CMX-115-2, increased the cellular titer of SOD protein, as well as peptides CMX-204-75, CMX-207-07, CMX-115-9 (Luiz *et al.,* 2002). In addition, in 2003 the data indicated that peptides 115-2, 115-9, 2115 and 8933 are able to increase the total cellular levels of SOD by as much as 14.0 fold, that SOD is a part of the cell's natural response to anoxia, and that kinases PKC, PTK and MAPKK are required for the 8933-induced up-regulation of SOD (Duffy and Smith, 2003). Eventually, one peptide 9236 was found to decrease infarct volume in a rat model for stroke (Shashoua *et al.,* 2003). Future experiments may include the identification and cloning of EPN in other species, characterization of the putative EPN receptor, and synthesis of second-generation EPNs.

Superoxide Dismutase

Superoxide dismutase (SOD) is an anti-oxidative enzyme that catalyzes the dismutation of two superoxide anions to hydrogen peroxide and molecular oxygen. The toxic hydrogen peroxide is further rapidly reduced by Catalase or Glutathionine Peroxidase (GPX) into water and molecular oxygen, as shown in Figure 3. SOD is the rate limiting enzyme in this pathway (Sun and Chen, 1998; Allen and Tresini, 2000); thus, its upregulation is of high therapeutic interest. Superoxide radicals play a detrimental role in a variety of neurodegenerative diseases (Venarucci *et al.,* 1999), including stroke, and their accumulation ultimately leads to oxidative stress, causing cellular damage and death. The rapid breakdown of superoxide anions is needed to minimize overall tissue damage. Over-expression of SOD in transgenic mice decreases infarct volume following ischemia (Sheng *et al.,* 1999a; Sampei *et al.,* 2000) and increases resistance to neurotoxin MPTP (Przedborski *et al.,* 1992; Klivenyi *et al.,* 1998). Over-expression of SOD in transgenic rabbits provides cardioprotection from myocardial infarction (Li *et al.,* 2001). Levels of c-Jun and c-Fos proteins increased in transgenic animals over-expressing SOD, indicating AP-1 may be involved in SOD's neuroprotection against oxidative stress (Huang *et al.,* 2001). SOD deficiency worsens cerebral ischemia in mice (Sheng *et al.,* 1999b). Therefore, the application of a peptide able to increase SOD to levels seen in the transgenic animals might be therapeutic following heart attacks or strokes.

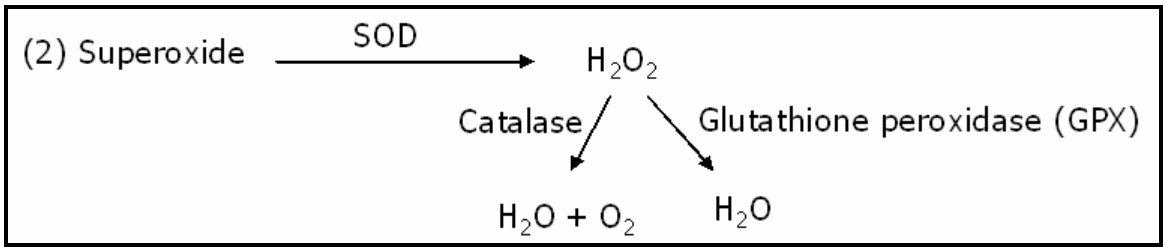


Figure 3. Biological Function of Superoxide Dismutase.

It is also important to keep in mind that hydrogen peroxide can act as a second messenger within the cell (Allen and Tresini, 2000). Thus some researchers have worried that altering its concentration by manipulating SOD will be detrimental to the animal. However, in the transgenic models that over-express SOD by as much as 30-fold in all tissues, not only are these animal models healthy, but they also resist ischemia (Przedborski *et al.,* 1992; Chan *et al.,* 1993; Chan and Kawase, 1998; Sheng *et al.,* 1999 Li *et al*, 2001). Therefore, even if SOD is highly over-expressed, the concentration of hydrogen peroxide is regulated closely enough to allow the cell to survive.

In addition, SOD has been found to have a potential role in deterring the aging process. Cellular oxidative stress increases with age, and is one of the major factors contributing to the aging process. For example, Parkes *et al.* (1998) performed studies on *Drosophila melanogaster* and found that by over-expressing human SOD-1 in the fly's motor neurons, the fly's lifespan dramatically increased. Therefore, the studies performed so far on the over-expression of SOD in transgenic models have not shown any detrimental effects on these models, but quite the opposite.

Mechanism of Action of Full-Length EPN and EPN-NTF Peptides

Little is currently known about the exact molecular mechanism *in vivo* by which the brain utilizes full-length EPN or the EPN peptides. Certain EPN mechanistic aspects resemble intra-cellular events activated by other NTFs. AP-1 is a ubiquitous transcription factor (Shaulian and Karin, 2002) that is activated by other NTFs, such as nerve growth factor (NGF) (Ip *et al.,* 1993; Tong and Perez-Polo, 1996) and brain-derived neurotrophic factor (BDNF) (Gaiddon *et al.,* 1996). CMX-8933, a synthetic peptide created to mimic a peptide naturally cleaved from EPN, has been shown to activate AP-1. CMX-8933 increased the DNA-binding affinity of two AP-1 nuclear factors (a homodimer and a heterodimer) containing c-Jun and c-Fos proteins (Shashoua *et al.,* 2001). It also increased the activity of c-Jun N-terminal kinase (JNK) (an enzyme that directly activates AP-1), increased the phosphorylation state of c-Jun protein (a component of AP-1), and increased the transactivation of synthetic and natural AP-1 dependent promoters (Adams *et al.,* 2002). Because AP-1 has been shown to be a master switch that controls long-term memory consolidation (Sanyal *et al.,* 2002), and because EPN also functions in long-term memory consolidation (Shashoua and Moore, 1978; Schmidt, 1987; Piront and Schmidt, 1988; Schmidt *et al.,* 1995) the activation of AP-1 may be a key component of EPN's mechanism of action.

Based on cumulative data from the Adams and Shashoua labs, we have proposed a tentative model for how CMX-8933 activates AP-1 (Figure 4). Since all known growth factors and NTFs act via cell surface receptors, EPN may too (Adams *et al.,* 2002). EPN, or its proteolytic cleavage products present in the ECF, may interact with an EPN receptor present on the surface of neuronal cells, which initiates a signal transduction

cascade involving the MAP kinase pathway and AP-1 as depicted in Figure 4. Activation on protein kinase C (PKC), protein tyrosone kinases (PTKs), and MEK kinase (MEKK) (Hasson, 1998) lead to the activation of JNK kinase. JNK phosphorylates the c-Jun component of AP-1, inducing AP-1's translocation into the nucleus. Nuclear AP-1 then presumably activates AP-1 dependent therapeutic genes, including those for anti-oxidative enzymes SOD (Parikh, 2003), catalase (CAT), and glutathione peroxidase (GPX) (Armitstead, 2001). Part of the purpose of this thesis is to determine whether some of these key MAPK events are necessary for the activation of SOD. Based on information published from other labs about the kind of genes activated by AP-1, this information could be used to predict genes other than c-Jun, c-Fos, SOD, CAT, and GPX that may be activated by treatment of nerve cells with peptide (Adams *et al.,* 2002).

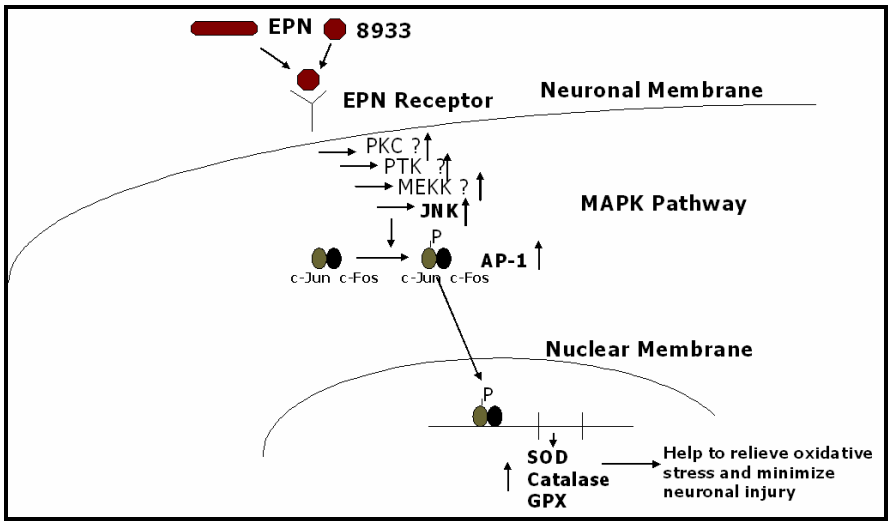


Figure 4. Working Hypothesis for the Mechanism of Action of Full-length EPN and Peptide Mimetic 8933. The full-length EPN sequence and/or the synthetic mimetic 8933 is thought to interact with a receptor on the neuronal cell surface activating the MAP kinase pathway, and JNK which phosphorylates the c-Jun component of AP-1 which translocates into the nucleus and transactivates genes for the antioxidative enzymes, and others. This is thought to ultimately lead to an increase of antioxidative enzymes within the cell.

THESIS PURPOSE

The purpose of this thesis was to test a three-part hypothesis on full-length EPN and CMX-8933's mechanism of action. It is well established that increases in oxidative stress result from neurodegenerative disorders and ischemia. Recent work in our lab has shown that CMX-8933, a synthetic small peptide EPN mimetic, can alleviate ischemic damage in a rat model for stroke (Shashoua, unpublished). Therefore, we hypothesized that both full-length EPN, as well as CMX-8933, may help alleviate oxidative stress in neuronal cells. Previous work in our lab showed that CMX-8933 activates the MAPK pathway, transcription factor AP-1, and increases cellular levels of SOD protein. We hypothesize that 1) CMX-8933 uses the MAPK pathway to activate SOD. This pathway is also utilized by other common neurotrophic factors. 2) Treatment of rat primary cortical cultures with CMX-8933 should up-regulate mRNAs involved in translation, as expected for any NTF that functions in neuronal growth. 3) If CMX-8933 represents the receptor binding domain of EPN, full-length EPN, isolated from the extracellular fluid of goldfish brains, may have the same effect of alleviating neuronal oxidative stress associated with stroke in NB2a cells, by up-regulating superoxide dismutase (SOD), as does the naturally occurring synthetic peptide mimetic CMX-8933.

MATERIALS AND METHODS

Murine Neuroblastoma Cell Culture Conditions

The neuronal cell line grown and maintained for this project was neuroblastoma (Neuro-2A). It was originally derived from mouse brain and neuroblasts. These cells were purchased frozen from American Type Culture Collection (ATCC) (catalog #CCL-131). This cell line is an adherent cell line (ATCC, 2003). Other materials used for cell culture included Dulbecco's Modified Eagle's Medium (DMEM; with glutamine and sodium pyruvate), Trypsin-EDTA solution (0.25% Trypsin; 1 mM EDTA), Fetal Bovine Serum (FBS), MEM Non-Essential Amino Acids Solution (100x, 10 mM) and Gentamycin (10 mg/ml). All were purchased from Life Technologies, Inc.

Complete Medium Preparation

A 500 ml bottle of DMEM culture medium was supplemented with the following in order to make it complete medium (final concentrations are in parentheses): Non-essential amino acids (0.1 mM), Fetal Calf Serum (10%), and gentamicin (5 μg/ml). The contents were filtered aseptically through a pre-sterilized 500 ml filter using a vacuum pump, then mixed briefly. All components used for cell culture were pre-warmed before use in a 37°C water bath.

Subculturing

The first step was to thaw the frozen ATCC stock of NB2a cells by rapid (<1 min) agitation in a 37°C water bath, and then transfer them to a vented T-25 culture flask

diluted with 6 ml of complete medium. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2 overnight, then the toxic freezing medium containing DMSO was replaced with normal complete medium. When the cells reached confluency, the medium was removed, and the cells were briefly rinsed with a small volume of outdated DMEM stock stored at 4°C. This step was done to remove the FCS proteins. The wash was immediately removed, and a small volume (refer to Table 1 for exact amounts) of Trypsin-EDTA solution (0.25% Trypsin) was added to just cover the cell monolayer. The flask was then incubated at room temp (or 37° C in the incubator) until the cells detached, approximately 2 minutes. The cells would come off in white sheets after the incubation. An equal amount of fresh complete culture medium was then added to the flask so that the FCS in the medium occupies the remaining trypsin. Then the cell suspension was transferred to a 15ml plastic tube and centrifuged in a clinical centrifuge (25°C, 5 min, 500 x g) to pellet the cells. The supernatant was then removed, and the cell pellet was re-suspended in fresh culture medium, and the cells were dispensed into new sterile flasks.

Table 1: Overview of Some Specific Culture Amounts Used

Flask Type	Amt. Used For Trypsinizing	Amt. Used For Washes	Normal Total ml in Flask During Culture
T-25	2 ml	3 ml	6 ml
T-75	5 ml	10 ml	40 ml
T-150	7 ml	20 ml	60 ml

The normal split ratio specified by the ATCC was 1:3 to 1:6, and this is what was used for the most part. Feeding the flasks was normally done twice or three times a week. When aliquots were to be frozen, the freezing medium was normal complete culture medium plus 5% filtered DMSO. A total of 2 ml of cells from two flasks were frozen at

-80°C, then moved to liquid nitrogen. One T-25 flask was maintained between experiments.

Drug Treatment

For whole cell lysate experiments, confluent T-25 flasks were used, and treated with various amounts of ECF or the dialyzed ECF.

For the inhibition experiments, Calphostin-C (250 nM), Genistein (15 μM), PD-98059 (10 μM) or PDTC (500 μM) were added to the medium at 5X IC_{50} concentrations 5 minutes prior to the addition of CMX-8933.

RT-PCR Experiments with Translation Factors

At Ceremedix Inc., CMX-8933 was synthesized by the Merrifield process (Shashoua *et al.,* 2001) and purified by HPLC to give a 99.5% pure white solid. The structure was confirmed by amino acid analysis and mass spectroscopy. Mass spectroscopy was also used to verify the absence of trifluroacetic acid. The peptide was converted to its acetate salt, and lyophilized to yield a product that was stable for at least three months at 4°C. Peptide was dissolved in cell culture medium just prior to use. Rat primary cortical culture flasks obtained from Ceremedix Inc. were treated with 10 ng/ml of peptide CMX-8933 for 5 hours, followed by RNA isolation.

Isolation of Total Cellular RNA

Total cellular RNA was isolated from the rat primary cortical cultures discussed above using the Clontech Atlas Pure Total RNA Labeling System (# K1038-1).

Cell Collection

The first step in the RNA isolation process was to use a plastic cell scraper to dislodge the cells from the bottom of the flask. The cell suspension was then poured into a 15ml or 50ml plastic tube, and centrifuged in a clinical centrifuge for 5 minutes at about 2,000 rpm (500 x g, medium setting) to pellet the cells. The supernatant was then discarded, leaving a small volume of medium for cell transfer. The pellet was resuspended in a small volume of the supernatant, and transferred into 2 ml sterile eppendorf tubes. Typically 2 ml eppendorf tubes were used for experiments using cell counts ranging from 10^6 to 10^7 total cells. The tubes were then microfuged for 5 minutes at about 2,000 rpm at 4ºC to pellet the cells. The supernatant was then thoroughly removed, sometimes spinning the tubes twice to remove extra supernatant.

Cell Lysis

For a total cell count of 10^7 cells split between 2 epp tubes, 0.25 ml of denaturing solution (1 M NaOH, 10 mM EDTA) was added to each cell pellet. The cells were re-suspended completely by pipetting up and down vigorously, and vortexing. The solution was then incubated on ice for 5-10 min, and then re-vortexed. The tubes were microfuged at 12,000 rpm for 5 minutes at 4ºC in order to pellet cellular debris. Following centrifugation, the supernatant (a volume of approximately 0.5 ml) was removed and transferred to new sterile 2 ml eppendorf tubes.

Phenol Extraction

A volume of 0.5 ml of TE saturated phenol (an equal volume) was added to each tube. Tubes were securely closed and vortexed vigorously for one minute, then placed on ice for five minutes. 0.15 ml of chloroform was then added to each tube to make a total volume of 1.15 ml. The tubes were vortexed vigorously for one to two minutes, assuring that the tubes did not leak, and then placed on ice for five more minutes. After incubation on ice, the tubes were centrifuged at 12,000 rpm for ten minutes at 4ºC to separate aqueous and phenolic phases. Avoiding the white interphase and lower organic phase of the sample, the upper aqueous phase containing RNA (approximately 0.5 ml) was then transferred to a new sterile eppendorf tube. For samples expected to contain low amounts of RNA 1 µl of 20 mg/ml, glycogen was added to the sample to act as a carrier.

Next, a second round of saturated phenol and chloroform extraction was performed on the aqueous phase. Note that this second extraction was skipped for samples containing low amounts of RNA to which glycogen was added. First, 0.4 ml of saturated phenol was added per aqueous phase from one tube; the sample was mixed for one minute, and incubated on ice for 5 min. Then 0.15 ml of chloroform was added, the sample was vortexed for 1-2 min, incubated on ice for 5 min, and then centrifuged at 12,000 rpm for 10 min at 4ºC. Once again, the upper aqueous phase was then transferred to new eppendorf tubes.

Isopropanol Precipitation

A volume of 0.5 ml of isopropanol was slowly added to the 0.5 ml of supernatant in each tube and mixed. The tubes were incubated on ice for 10 min, and then centrifuged at 12,000 rpm for 15 min at 4ºC to the pellet the RNA. Once centrifuged, the supernatant was carefully removed and discarded, without disrupting the RNA pellet.

Pellet Wash

Then 0.25 ml of 80% ethanol was added to the pellet, and the tubes were once again centrifuged at 12,000 rpm for 5 min at 4ºC. Being very cautious to not disturb the loose RNA pellet now present, the supernatant was removed and discarded from each tube. The tubes were left on the bench allowing the pellets to air dry, or the vacuum drying apparatus was used to expedite the drying process.

Pellet Dissolution

Once the RNA pellet had completely dried, 40 µl of RNase-free dH_20 water was added, producing an RNA concentration of 1-2 µg/µl. The tube was then heated at 50ºC for 5 minutes, occasionally flipping the tube to dissolve the RNA. It was then briefly microfuged. For OD 260 analysis, 1 µl of the final solution was aliquoted and added to 1 ml dH_2O prior to UV spectrophotometry. The rest of the RNA was aliquoted into several epp tubes (about 10 µl per tube) to avoid repeated freeze/thaw. The samples were labeled and stored at -80ºC for later use.

Assessing the Integrity of Total RNA

Before the RT-PCR was performed, the integrity of the total cellular RNA samples was assessed. This was done by preparing and running a denaturing agarose gel as described in the Clontech Atlas™ Pure Total RNA manual. Before the gel was made, a mini-gel box, gel tray and combs were washed with deionized water. For the preparation of the gel, a 250 ml beaker with a magnetic stir bar was obtained, and 1 gram of agarose was weighed into the beaker and mixed with 82.5 ml of deionized water. The solution was microwaved for 2 min, then placed on a magnetic stir-plate under the hood and stirred slowly for 2 min to cool. While the solution was stirring, 10 ml of 10X MOPS buffer (0.4 M MOPS pH 7.0 , 0.1 M NaOAc pH 7.0, and 10 mM EDTA pH 7.0) and 7.5 ml of formaldehyde were added, and stirring was continued for 1 min. The solution was taken from the hood and poured into the gel tray. The gel was left to solidify at room temperature for one hour. The gel comb was then removed and the gel was submerged in the gel box with 1X MOPS buffer.

The RNA loading solution was made immediately before the gel was to be run. The solution was made to accommodate 6-10 samples and consisted of: 45 µl of formaldehyde, 45 µl deionized formamide, 10 µl 10X MOPS buffer, 3.5 µl EtBr (10 mg/ml), 1.5 µl 0.1 M EDTA (pH 7.5), and 8 µl bromophenol blue dye (in 50% glycerol to make a total of 113 µl). A volume of 15 µl of RNA loading solution was added to 2 µg of total RNA (usually about 2 µl) and mixed well. The solution was heated in the thermal cycler at 70ºC for 15 min, and then cooled on ice for 1 min. The sample solutions were then loaded into the wells of the gel, and electrophoresed at 60-70V until the blue dye reached the midpoint of the gel. The RNA was visualized on a UV transilluminator.

Strong discrete 18S and 28S rRNA bands indicated intact RNA for that sample. The 28S band should predomintae at a ratio of 1.5-2.5 :1.0.

Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

To test whether the primary rat cortical RNA samples expressed genes of particular interest, RT-PCR was performed using Ambion's RETROscript, First Strand Synthesis Kit for RT-PCR Protocol (Catalog #1710). The procedure followed the RETROscript Protocol (Two-Step RT-PCR) and all the reagents came with the kit. In addition, some Ready-To-Go RT-PCR beads in 0.5ml tubes were used from Amersham Pharmacia Biotech (www.apbiotech.com). These tubes already contained all of the reagents described below but needed the addition of the primers, RNA and dH_2O. Then they followed the same procedure as the above two-step RT-PCR. All primers used were purchased through Fisher Sigma Genosys and were designed for the rat sequence using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Reverse Transcription of the RNA

The following items were added to 0.5 µl eppendorf tubes: a total of 2 µg RNA, 2 µl of the anti-sense oligo (dT) (50 µM) and enough nuclease-free dH_2O to total 12 µl. This was mixed, spun briefly in a microcentrifuge at room temperature, and heated for 3 min at 70-85°C in the thermal cycler. The tubes were then removed, spun again briefly and placed on ice. The remaining RT components were then added: 2 µl of 10x RT buffer (500 mM Tris pH 8.3, 750 mM KCl, 30 mM $MgCl_2$, 50 mM DTT), 4 µl dNTP mix, 1 µl RNase inhibitor, and 1 µl MMLV reverse transcriptase to make a total volume of 20 µl. The reaction was mixed and spun again, and then incubated at 42-44°C for one

hour in the thermal cycler. Upon completion of the reverse transcription, the reactions were then incubated at 92°C for 10 min to inactivate the reverse transcriptase. After this point, the samples could be stored at -20°C, or the procedure could continue to PCR.

PCR

The following components were added to 0.5 ml eppendorf tubes: 5 µl RT reaction (containing the antisense primer and cDNA), 5 µl 10x PCR buffer, 2.5 µl dNTP mix, 37.5 µl nuclease-free dH_2O, 2.5 µl PCR sense primers (50 µM stock) and 0.4 µl thermostable Taq DNA polymerase (5 units/µl). The samples were mixed, spun briefly and then placed in the thermal cycler for the following program: 94-95°C for 2-4 min initial denaturation, 30 cycles of 94°C for 20-30 seconds, annealing for 20-30 seconds (usually at 55°C unless otherwise stated), 72°C for 40 seconds to 1 min, and finally a polishing step at 72°C for 5 min once. The samples were then stored at 4°C overnight in the thermal cycler, or used immediately.

Analysis of RT-PCR Reactions

To analyze the RT-PCR reactions, aliquots of the reaction mixes were loaded onto a non-denaturing agarose gel in the presence of ethidium bromide, and then visualized under UV light. A total of 2 µl of the high-resolution gel loading solution included in the Ambion kit was mixed with 10 µl of the RT-PCR mix, and 10 µl of this 12 µl mixture was loaded into the wells of the gel. Since the TBE-based loading dye that came with the kit was used, a 1X TBE (Tris-borate-EDTA) buffer was used to make the gel solution and electrode buffer. A 2.5% agarose gel containing about 0.5 µg/ml of ethidium bromide

was typically used. The remaining RT-PCR products and PCR reaction mixes were stored at -20°C.

Western Blots Analysis

Western blots were used to analyze the levels of SOD protein in NB2a neuronal cell lysates, and the levels of full-length EPN in goldfish ECF.

Whole Cell Extracts

Whole cell extracts were prepared from *in vitro* cultured NB2a cells in T-25 flasks, in order to obtain protein for immunoblots.

Cell Harvesting

The flasks were collected and the media was poured off and 10 ml of ice-cold PBS-EDTA (1X PBS, 20 mM EDTA) was added to each flask. Using a plastic scraper, the cells were scraped into the PBS and then removed with a pipette. The suspension was placed in 15ml conical tubes and pelleted at 500 x g for 5 minutes. The PBS was then discarded, and the cell pellet was washed once with ice cold PBS-EDTA, and centrifuged again as previously described. Once again the wash was discarded, then the tube was briefly inverted onto a towel to get rid of as much of the PBS as possible. Then the tube was placed on ice.

Cell Lysis

Once all the PBS wash was removed, 200 µl (for a small cell pellet from a T-25 flask) of Complete Lysis Buffer (20 mM HEPES pH 7.9, 10 mM KCl, 300 mM NaCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 20% Glycerol, and freshly added 0.5 mM DTT and 0.5 mM PMSF) was added to each pellet from one T-25 cultured flask. Using a blue-tip, the cell pellet was re-suspended in the buffer and then transferred to 1.5 ml epp tubes on ice. The suspension was incubated on ice for at least 10 min to thoroughly lyse the cells.

Lysate Clarification

The pellet was then microfuged at 4°C for 5 min to pellet cell debris. The supernatant was put into 0.5 ml epp tubes (about 40 µl per tube) and 1 µl of the supertatant was put into 0.5 ml of dH_2O for protein determination (1:500) using Coomassie Reagent (Pierce). The tubes were stored at –80°C.

Protein Determination

BSA protein standards were prepared (1.25, 2.50, 5.00, 10.00, 20.00, 40.00 µg/ml) and 0.5 ml of each were added to 1.5 ml epp tubes. A blank was prepared with 0.5 ml of dH_2O. Next, 10 µl or 5 µl of the sample plus 0.5 ml of dH_2O were put into 1.5 ml epp tubes. All tubes were then put into a water bath for 10 min to ensure that all were at the same temperature. The tubes were then microfuged, and 0.5 ml of Coomassie Reagent were added to all tubes. The tubes were then vortexed to mix. The spectrophotometer was set at OD 595 nm, and zeroed on the dH_2O blank. All samples

were read, then a BSA standard curve was created and used to determine protein concentration in the whole cell lysate.

Protein Gels and Immunoblots

Protein gels were used to test goldfish brain extracellular fluid for the presence of EPN (refer to Appendix 1-6 for exact whole cell lysate information used to assess ECF protein content used for the western blots). The samples were run with the kaleidoscope marker. Also, Western blots were performed to measure SOD levels in whole cell lysates prepared from in vitro cultured mouse neuroblastoma (NB2a) cells.

Gel Polymerization

A BRL V-16 glass plate apparatus was assembled using two non-siliconized glass plates (the front plate being 6 x 8 inches, the back plate 6 x 6 inches), and 0.8 mm thick spacers, held secure with clamps around the edges of the plates. The first gel, a lower resolving gel, was poured first as the base layer. It was composed of 7.6 ml of distilled H_2O, 6.7 ml of 30% acrylamide (2.7% crosslinking), 5.1 ml of resolving gel buffer (1.5 M), 200 µl of 10% SDS, 400 µl of 5% ammonium persulfate, and 10 µl of TEMED (added just prior to pouring the gel as a catalyst which initiates gel polymerization). The solution was poured between the two plates to about 1.5 cm from the top. A thin layer of dH_2O was put on the top in order to level off the top of the gel, and to make sure the lower gel was in a straight line. The gel was then allowed to polymerize for 20 minutes.

After the lower resolving gel had fully polymerized, the water was poured off and the upper stacking gel was poured on. The upper stacking gel was composed of 5.52 ml

of distilled H2O, 1.67 ml of 30% acrylamide (2.7% crosslinking), 2.5 ml of stacking gel buffer (0.5 M), 100 µl of 10% SDS, 200 µl of 5% ammonium persulfate, and 10 µl of TEMED (added just prior to pouring the gel as a catalyst which initiates gel polymerization). The upper stacking gel was poured on top of the lower resolving gel until it reached the top of the smaller back glass plate. A 20-stall comb was then carefully placed between the two plates, with about 0.5 cm into the upper stacking gel, making sure no air bubbles were in the gel). The gel was then allowed to polymerize for another 20 minutes. If the gel was left overnight, the top of the plates was covered with plastic wrap so that the gel would not dry out.

Gel Electrophoresis

When the gel was completely polymerized, the clamps and the lower spacers were removed. After making sure that the black gaskets on the side spacers were pushed down, with no space between them and the top of the smaller plate, the plates were then mounted into the V-16 vertical electrophoresis unit, secured in place by clamps on both sides. Protein electrode buffer (25 mM Trizma Base, 0.192 M Glycine, 0.1% SDS) was then poured in the upper reservoir to check for leaks, if no leaks were present, then it was also poured in the lower reservoir until level with the electrodes. The comb was then removed, the wells were flushed out, and the gel was pre-run for one hour at 150 volts. After the pre-run, the samples were prepared for electrophoresis by inserting them in a boiling water bath for two minutes, and then microfuging briefly. Samples were then loaded into each well (3 µl loads were used per lane corresponding to 5 µg of protein

unless otherwise noted), and electrophoresed for about 3 hours at 150 volts so that the dye traversed down ¾ of the gel.

Protein Staining of Gel

Visualization of ECF proteins was achieved by Coomassie blue staining. When the gel was finished running, it was removed from the electrophoresis apparatus and the area containing the samples was trimmed. This area was then placed in a Tupperware container with 100 ml of staining solution (0.25% Coomassie Blue, 40% Methanol, 9% Acetic acid) and stained until it turned blue. Then, the staining solution was discarded and 100 ml of de-staining solution (5% Methanol, 7% Acetic Acid) was added to the Tupperware container and left for between 1-2 days. The gel was photographed using a white light transilluminator.

Western Protocol - Gel Transfer

After the 3 hours of electrophoresis, the side spacers were removed, and one of the glass plates was carefully taken off. A notch was made in the original right corner of the gel, as an orientation aid, so the bands could be viewed in the correct order. The gel was trimmed to contain only the sample area, and then a nitrocellulose membrane was cut to cover the gel (notched on the same side to match the notch in the gel). It was then surrounded on both sides by two pieces of 3 MM paper, all presoaked in transfer buffer (48 mM Trizma base, 39 mM Glycine, 0.037% SDS, and 20% Methanol) from the electroblotter. The gel/membrane sandwich was then placed in the plastic electroblot holder (with sponges on either side), fastened shut, and placed in the electroblot unit,

submerged in chilled transfer buffer. The nitrocellulose membrane side must face the positive anode to ensure proper protein transfer from the gel to the membrane. The membrane was then transblotted in the refrigerator at 4°C, on a slow speed stir, for 2 hours at 50 volts.

Membrane Blocking/Antibody Incubations

Once the membrane had been transblotted, it was removed from the unit and placed in a Tupperware hybridization tray for blocking. The side that originally was in contact with the gel was marked with a felt pen, and remained face up at all times. The membrane was blocked with 50 ml of freshly made chilled blocker (1X PBS, 1% Casein powder, and 0.2% Tween-20) for one hour, mixing at low speed on the red rocker shaker. After the block, if the sample was to be tested for SOD, the blocker was replaced with fresh blocker, and 50 µl of the primary antibody (rabbit anti-SOD, Rockland [Penn], catalog #100-4191, main stock: 90 mg/ml) was added to the liquid, not directly onto the membrane. If EPN protein was being detected, 500 µl of 1:50 antibody Sheila (rabbit anti-goldfish C-terminal end of EPN) was added to the 50 ml blocker. The membrane was then left to mix again, as previously described for two hours. After the incubation with the primary antibody, the solution was removed, and the membrane was washed twice in PBS-Tween, 5 minutes each time, at a vigorous rate on a gyratory shaker (the volume of liquid was just enough to cover the entire membrane). Next, the membrane was incubated with 50 ml of fresh blocker supplemented with 50 µl of the secondary antibody (goat anti-rabbit-HRP, Pierce, catalog #31460, final concentration: 0.4 µl/ml) for two hours on the red rocker shaker. After the two-hour incubation with the secondary

antibody, the used solution was removed, and the membrane was washed four times (3 times in PBS-Tween, once in 1X PBS, each for 5 minutes on the gyratory shaker at vigorous speed) before x-ray development.

Chemiluminescent Detection/X-ray Film Development

After the last washes, the membrane was moved from the hybridization tray to a piece of foil on the bench top, protein side up. In a 15 ml conical tube, the detection solution (5 ml Luminol/Enhancer Solution plus 5 ml Stable Peroxide Solution) was mixed. The freshly prepared solution was then poured on the membrane, and spread evenly with a glass pipette and allowed to incubate for 5 minutes. After 5 minutes, the membrane was picked up using tweezers, and the corner was dabbed with a Kimwipe to remove excess detection solution. The membrane was placed between two sheets of clear plastic Photogene Development Folders (Gibco BRL #18195-016), and any air bubbles were removed by rubbing a Kimwipe over the plastic surface. The folder was then placed in a film cassette without a screen for transportation to the darkroom for development.

Before developing, the development tank was filled with water at a constant 25°C maintained throughout development. Kodax X-Omat AR film was exposed to the membrane for ~1-10 seconds for SOD, or for 20 sec for EPN). The film was then placed in the GBX developer, agitating initially to remove air bubbles, and left for 5 minutes. It was then removed, dipped briefly in the water to remove the developer, and submerged into the fixative solution for three minutes, with intermittent agitation over the entire 3

minutes. Finally, the film was placed in the water bath for 5 minutes to remove excess fixative, and hung to air dry. Bands were quantitated using Scionimage Software (NIH).

Microdialysis

Microdialysis was performed to remove low molecular weight components from the ECF, such as any naturally occurring proteolytic cleavage products of full-length EPN, retaining the higher molecular weight components. The Pierce Microdializer System 500 (Catalog # 66350) with a 5 x 500 µl sample capacity was used, along with Pierce framed dialysis Membranes with a MW cutoff of 8,000 (Catalog # 66310).

First, the framed membrane was put into place, then a syringe was used to deliver 1X PBS Buffer into the bottom of the dializer. A volume of 200 µl of ECF was added to each sample well. The apparatus was then covered and placed in the 4°C refrigerator, and left overnight with a spinning magnet on low speed. After the overnight incubation, the buffer was removed and fresh ice-cold 1X PBS buffer was replaced. The unit was incubated for another 2 hours, and then the dialyzed ECF in the well (approximately 200 µl) was put into 1.5ml epp tubes and stored in the 4°C refrigerator.

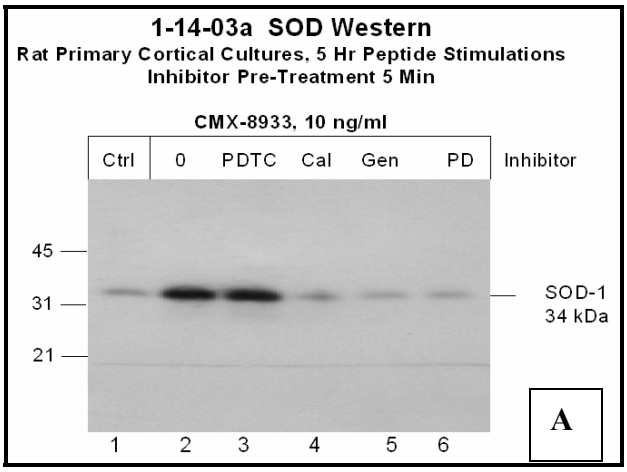
RESULTS

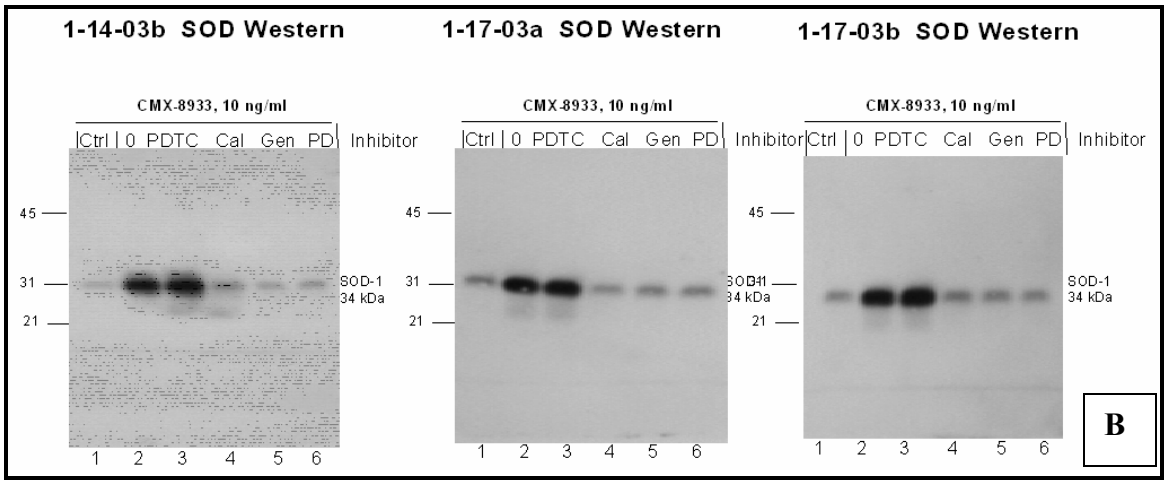
The goal of this thesis project was to test three hypotheses involved in investigating the mechanism of action of both full-length EPN and CMX-8933. The first part of the thesis investigated which signal transduction pathway is responsible for CMX-8933's upregulation of SOD found previously in our lab (Parikh, 2003). Because our lab previously showed that CMX-8933 activates the MAPK pathway (Hasson, 1998; Adams *et al.,* 2003) we hypothesized that CMX-8933 may use this pathway to upregulate SOD.

CMX-8933 Uses the MAPK Pathway to Upregulate SOD in Rat Primary Cortical Cultured Cells

Inhibition experiments were performed to test three known components of the MAPK pathway, as well as one member of an unrelated pathway from the NF-κB family. Calphostin-C (CAL) inhibits members of the protein kinase C family (PKC) (for a review, see: Tamaoki and Nakano, 1990). Genistein (GEN) inhibits members of the protein tyrosine kinase family (PTK) (Akiyama *et al.*, 1987; Platanias and Colamonici, 1992). PD98059 (PD) inhibits MEK members of the MAP kinase kinase (MAPKK) family (Dudley *et al*., 1995). All these kinases have previously been shown to be critical components the MAPK pathway (Akiyama *et al.,* 1987; Plantanias and Colamonici, 1992; Dudley *et al.,* 1995). Pyrrolidine dithiocarbonate (PDTC) inhibits anti-oxidative members of the NF-κB family, an unrelated pathway (Schreck *et al*., 1992; Tsai *et al*., 1996).

Six independent SOD Western experiments were performed (Figure 5) using whole cell lysates prepared from *in vitro* cultured rat primary cortical cells treated for 5 minutes with an inhibitor before a 5 hour treatment with CMX-8933. SOD protein levels were compared to a control with no treatment of peptide (lane "ctrl") and to cultures treated with 8933 but no inhibitor (lane "0"). The 10 ng/ml of CMX-8933 treated culture without any inhibitor (lane "0") showed a dark SOD band at the expected size of SOD protein (34 kDa), as expected (Parikh, 2003). A strong SOD signal was also seen in the sample treated with inhibitor PDTC, indicating NK-kB plays no role in CMX-8933's activation of SOD. However, levels of SOD showed no increase above untreated cuoltures when inhibitors CAL, GEN or PD were pre-incubated with the peptide treated flasks. The bands were quantified using Scionimage Software. The histobars shown below each panel indicate the fold increase relative to untreated sample. The results of 5 additional independent experiments (panels B and C) show identical data to trial-1.





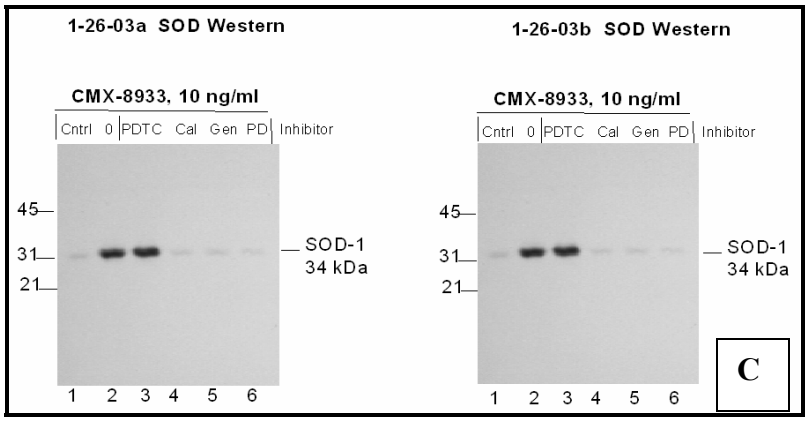


Figure 5. Inhibitors CAL, GEN, and PD Inhibit CMX-8933-Induced Increases in SOD Levels in Rat Primary Cortical Cells. Rat primary cortical cultures were pre-incubated with an inhibitor at its IC_{50} concentration (see Methods) for 5 min, then 10 ng/ml of CMX-8933 was added for a period of 5 hours. Panel A represents Trial-1, while Panels B and C represent five additional trials. Arrows to the right denote the positions of the 34 kDa SOD-1. Arrows to the left denote positions of the biotinylated marker.

The means of Trials 1 through 6 for the *in vitro* inhibition experiment are illustrated in Figure 6. Treatment of the rat primary cells with 10 ng/ml CMX-8933 for 5 hr increased SOD an average 14-fold relative to untreated, as expected (Parikh, 2003). No significant difference (p = 0.901) occurred between lanes "0" and "PDTC", indicating no involvement of NF-kB. In the presence of inhibitors CAL, GEN and PD, the cellular titers of SOD showed no observable difference from the control sample. The data implicate the involvement of components of the MAPK pathway in CMX-8933's upregulation of SOD.

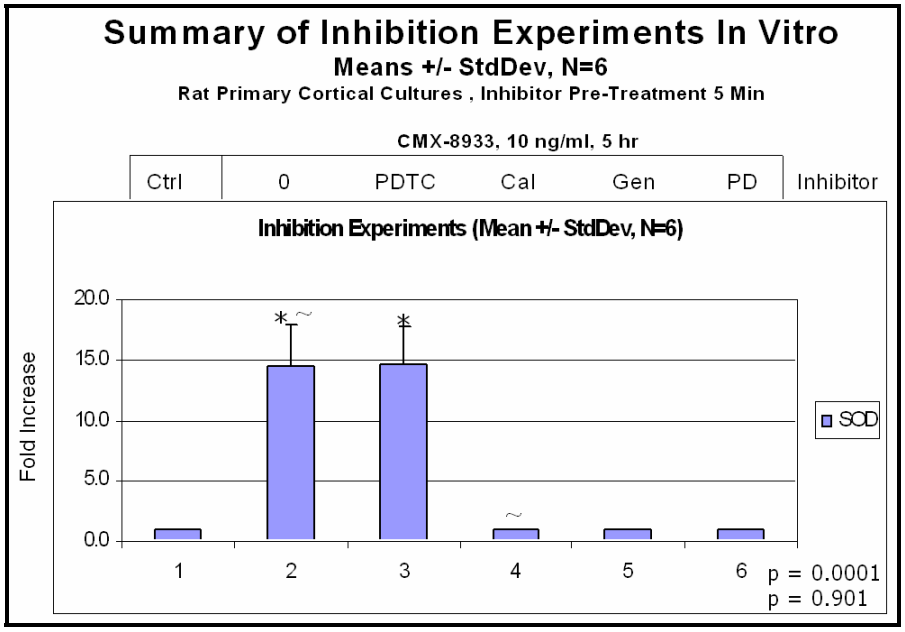


Figure 6: Statistical Analysis of Six *in Vitro* Inhibition Experiments. Error bars denote standard deviation calculated in Excel. P values were calculated using a student's two-tailed T-test in Excel. * denotes histobars 2 and 3, compared for p value 0.901. ~ denotes histobars 2 and 4, compared for p value 0.0001.

CMX-8933 Upregulates mRNAs Related to Translation

The second part of this thesis was designed to determine whether treatment of rat primary cortical cultures with CMX-8933 increases the cellular titers of mRNAs related to translation. Previous observations indicated treatment of rat primary cortical cultures

or mouse NB2a cultures with CMX-8933 induced neurite sprouting (Shashoua, unpublished), and that EPN plays a role in optic nerve elongation (Schmidt and Shashoua, 1988), so we hypothesized that EPN or its derived peptides may stimulate the transcription of mRNAs related to growth. We decided to test mRNAs for translation factors and ribosomal proteins based on previous observations in our lab with hybridization arrays (Parikh, 2003).

Isolation of Total Cellular RNA from Rat Primary Cortical Cultures Cells

Cultured rat primary cortical cells at confluence were treated with 10 ng/ml of CMX-8933 for a period of 5 hours prior to total cellular RNA isolation. Table 2 summarizes some of the neuronal cell samples tested in this thesis, along with their corresponding RNA yields after the isolation procedure.

Table 2. Rat Primary Cortical Cell Sample Size and Corresponding RNA Yields

Date	Sample	Flasks	RNA Yield μg	Conc. μg/μl
12/12/02	Control 1	2, T-75	35.3	0.84
12/12/02	Drug 2	2, T-75	38.6	0.42
12/12/02	Control 3	2, T-75	17.6	0.92
12/12/02	Drug 4	2, T-75	17.6	0.42

In order to verify that the isolated RNA was intact, denaturing formaldehyde agarose gel electrophoresis was performed. Discrete 28S and 18S ribosomal RNA bands were used as indicators of intact RNA. Figure 7 shows the RNA integrity gel for the above samples in Table 2. The 28S and 18S ribosomal RNA bands at 4.5 kb and 1.9 kb can be clearly seen in each sample indicating that the RNA was intact.

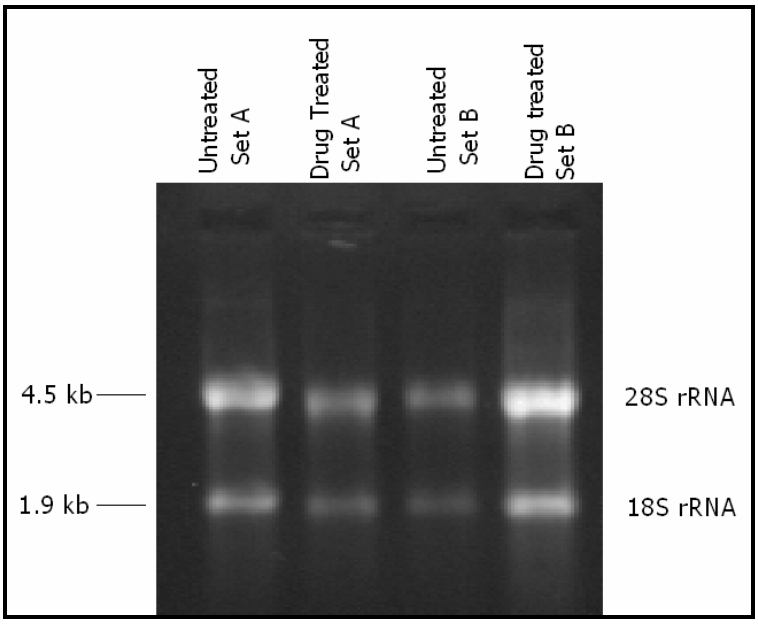


Figure 7: RNA Integrity Gel by Denaturing Formaldehyde Agarose Gel Electrophoresis. Analysis of total cellular RNA isolated from rat primary cortical cells.

Previous multiple gene profiling, using Clontech's Atlas Nylon Rat cDNA Arrays, indicated mRNAs for several ribosomal proteins increased in rat primary cortical cells treated with 8933 (Table 3).

Table 3. Hybridization Array Summary Table for Ribosomal Proteins and EF-2 (Parikh, 2003). Bold text indicates candidate primers chosen for investigation in this thesis. +++ on the array signal is strong, ++ is moderate, and + is a weak signal.

ARRAY COORDINATE	GENBANK ACCESSION	GENE/PROTEIN	CLASSIFICATION	ARRAY SIGNAL CTRL	8933
C11e	**M18547**	**Ribosomal Protein S12**	**Ribosomal Protein**	++	+++
C11g	X62184	Ribosomal Protein L11	Ribosomal Protein	+	++
C11h	X78327	Ribosomal Protein L13	Ribosomal Protein	+	++
C11j	X53504	Ribosomal Protein L12	Ribosomal Protein	+	++
C11k	X51707	Ribosomal Protein S19	Ribosomal Protein	+	++
C11L	M27905	Ribosomal Protein L21	Ribosomal Protein	+	++
C11m	**J02650**	**Ribosomal Protein L19**	**Ribosomal Protein**	++	+++
C12d	**K03502**	**Elongation Factor-2**	**Translation Factor**	++	+++

RT-PCR: CMX-8933 Increases the Titer of mRNAs Involved in Translation

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was performed to verify the preliminary array results. Translation candidates ribosomal protein R-S12, ribosomal protein R-L19 and elongation factor-2 were chosen for investigation by RT-PCR because of their strong signals on the array. Figure 8 shows the RT-PCR result for ribosomal protein R-S12 performed in triplicate on independent samples. As predicted, the levels of the housekeeper Polyubiquitin (amplicon size 231 bp) were equal between treated and untreated samples, while levels of R-S12 mRNA (amplicon size 286 bp) increased in all 3 drug-treated samples.

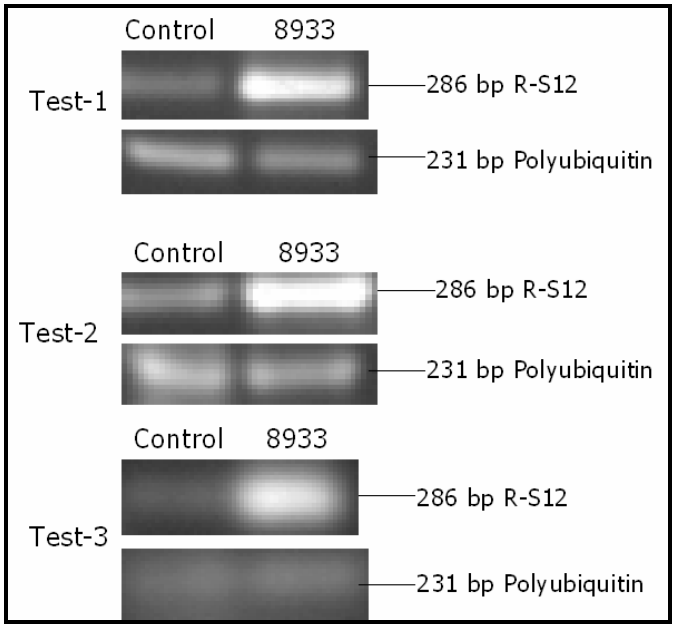


Figure 8. CMX-8933 Elevates R-S12 mRNA Levels in Rat Primary Cortical Cells. Polyubiquitin (amplicon size 231 bp) was used as a housekeeper, shown in the bottom panel in each test. The results of 3 independent trials indicated that the R-S12 bands (amplicon size 286 bp) were stronger in all 3 samples treated with 10 ng/ml of CMX-8933 for 5 hours. This data correlates with the preliminary results obtained from the hybridization array.

The means +/- standard deviation for the 3 trials shown in Figure 8 are shown in Figure 9. The histobars show the mean fold increase quantitated using Scion image in a triplicate determination. Error bars denote standard deviations within triplicate set. This figure shows an average 12 fold increase in R-S12 mRNA levels for the 8933 treated samples compared to the control. A statistical analysis was performed using the two-tailed student T-test in Microsoft Excel. It indicated that R-S12 up-regulation was significant ($p = 0.02$).

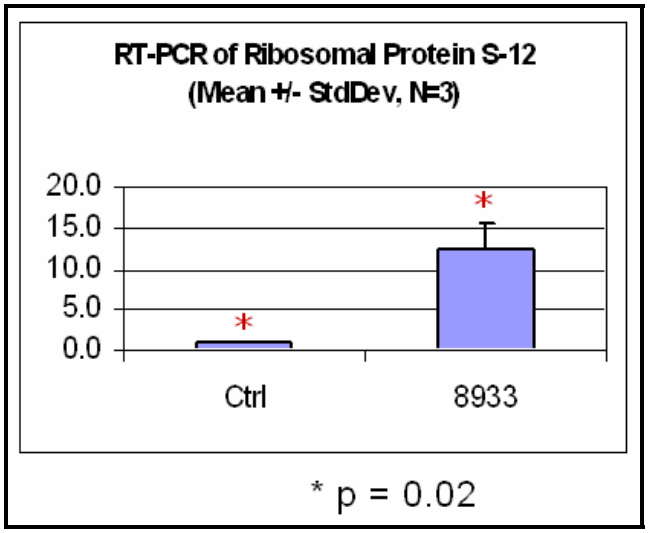


Figure 9. Quantitation Data for R-S12 RT-PCR. Histobars indicate means for 3 independent trials. Error bars denote standard deviations in the triplicate set. Asterisks denote the two histobars compared for the p value.

The second candidate tested by RT-PCR was ribosomal protein R-L19. Figure 10 illustrates the RT-PCR results performed in triplicate. As predicted, the levels of the housekeeper Polyubiquitin (amplicon size 231 bp) were equal between treated and untreated samples, but R-L19 mRNA (amplicon size 257 bp) increased in all 3 drug treated samples. The mean 9-fold upregulation was significant ($p = 0.048$) (figure 11).

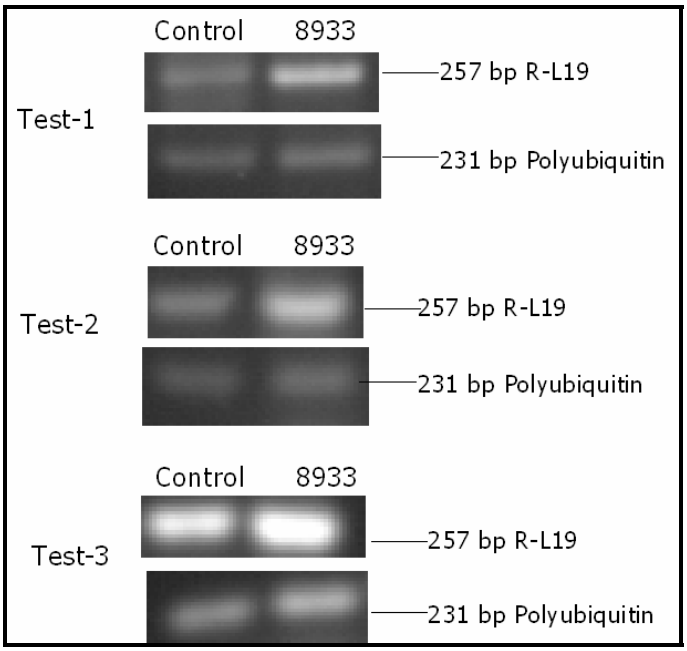


Figure 10. CMX-8933 Elevates R-L19 mRNA Levels in Rat Primary Cortical Cells. Polyubiquitin (amplicon size 231 bp) was used as a housekeeper, shown in the bottom panel in each test. The results of 3 independent trials indicated that the R-L19 bands (amplicon size 257 bp) were stronger in samples treated with 10 ng/ml of CMX-8933 for 5 hours. This data correlates with the results obtained from the hybridization array.

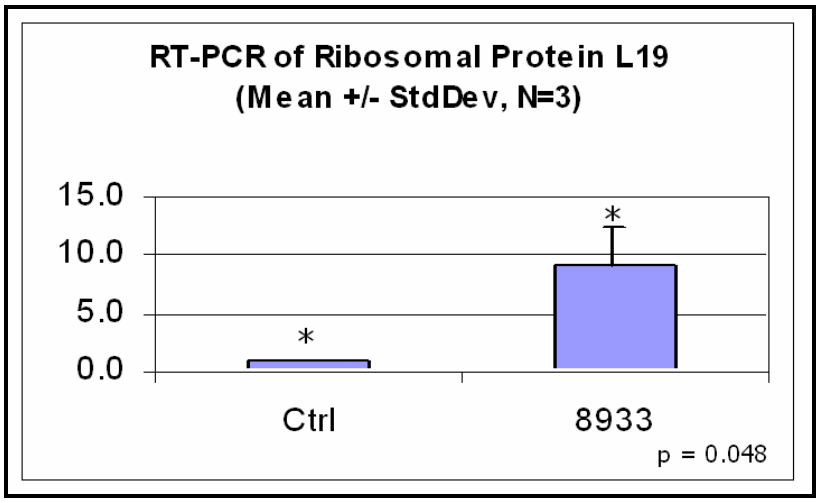


Figure 11. Quantitation Data for R-L19 RT-PCR. Histobars indicate means for 3 independent trials. Error bars denote standard deviations in triplicate set. Asterisks denote the two histobars compared for the p value calculated by the two-tailed T-test.

The third candidate tested was elongation factor 2 (EF-2). Figure 12 illustrates the EF-2 RT-PCR results performed in triplicate. As predicted, the levels of the housekeeper Polyubiquitin (amplicon size 231 bp) were equal between treated and

untreated samples, but EF-2 mRNA (amplicon size 227 bp) increased in all 3 drug-treated samples. The mean 11-fold upregulation was significant (p = 0.045) (figure 13).

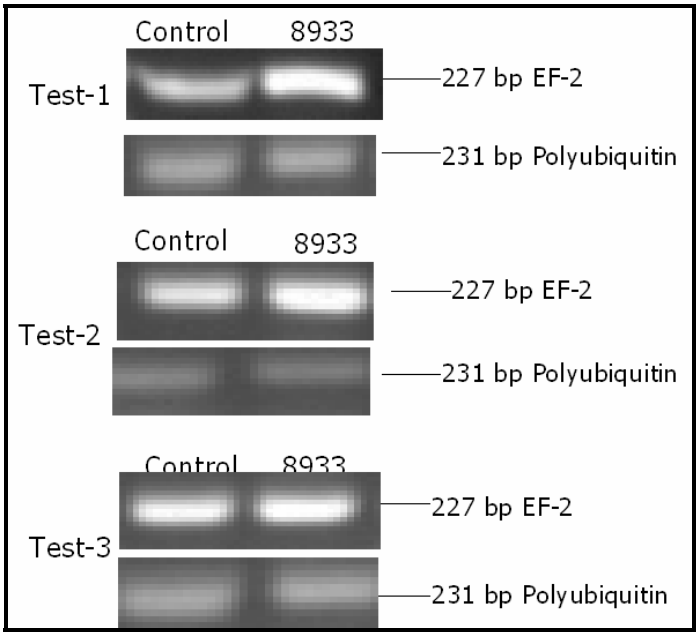


Figure 12. CMX-8933 Elevates EF-2 mRNA Levels in Rat Primary Cortical Cells. Polyubiquitin (amplicon size 231 bp) was used as a housekeeper, shown in the bottom panel in each test. The results of 3 independent trials indicated that the EF-2 bands (amplicon size 227 bp) were stronger in samples treated with 10 ng/ml of CMX-8933 for 5 hours. This data correlates with the results obtained from the hybridization array.

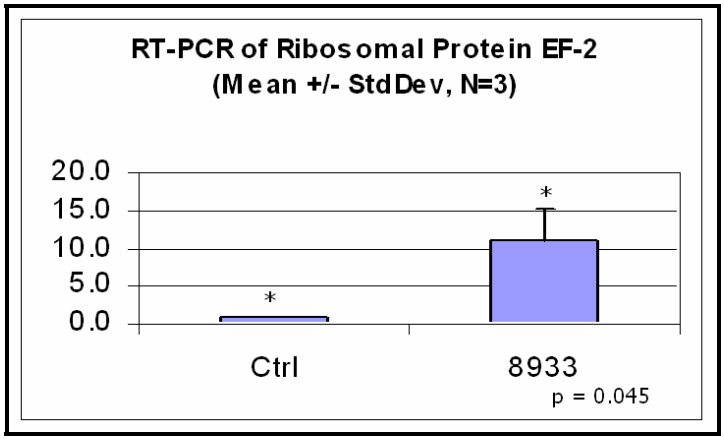


Figure 13. Quantitation Data for EF-2 RT-PCR. Histobars indicate means for 3 independent trials. Error bars denote standard deviations in the triplicate set. Asterisks denote the two histobars compared for the p value calculated by the two-tailed T-test.

Full-Length Ependymin Increases SOD Levels in Mouse NB2a Cells

The third part of this thesis investigated the stimulatory effects of full-length EPN versus its cleavage product 8933. Previous research showed that true neurotrophic factors BDNF and NGF can upregulate SOD in neuronal cells. CMX-8933 can also upregulate SOD in neuronal cells (Parikh, 2003). We hypothesized that if 8933 represents the receptor-binding domain of EPN, that full-length EPN may show the same stimulating effects as 8933. Therefore full-length EPN may also upregulate SOD in neuronal cells.

The first step in testing thesis hypothesis 3 was to isolate goldfish brain extracellular fluid, and to verify that it does indeed contain full-length ependymin (Figure 14). Goldfish ECF is the normal source for isolating EPN (for a review, see Shashoua, 1991).

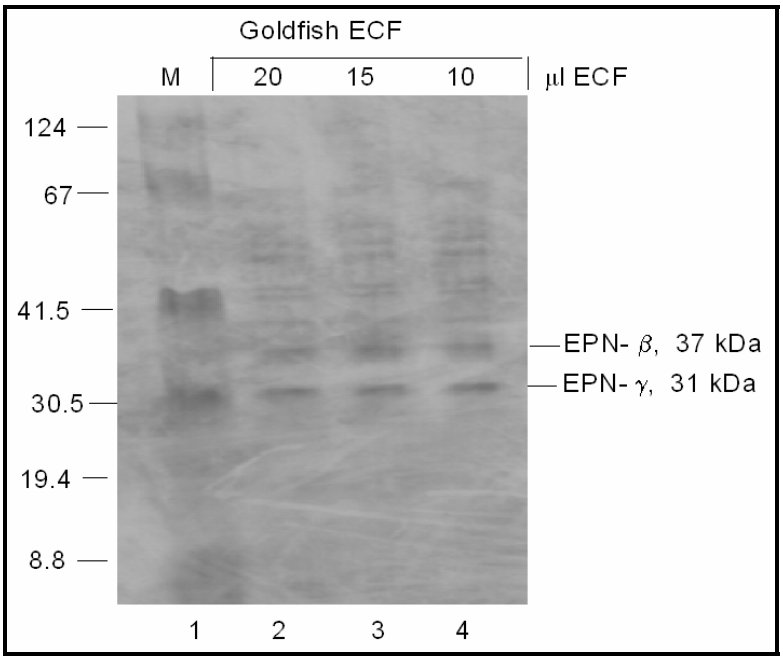


Figure 14: Coomassie Protein Gel Analysis of Goldfish Extracelluar Fluid. Arrows to the right denote the 37 and 31 kDa EPN bands. Arrows to the left denote the positions of the biotinylated markers.

Figure 14 shows the presence of numerous proteins in the extracellular fluid, and the most prominent/abundant of these proteins are at 31 and 37 kDa. These results are exactly as expected since the two forms of EPN have previously been shown to predominate in the extracellular fluid of goldfish at exactly these sizes (Shashoua, 1991).

However, to make certain that these bands are indeed full-length ependymin, the extracellular fluid sample was tested in an EPN immunoblot using rabbit anti-EPN antibody induced in rabbits with a synthetic peptide representing the C-terminal end of goldfish EPN (Shashoua and Moore, 1978) (termed antibody Sheila, graciously donated by Dr. Dan Gibson, WPI). Figure 15 illustrates the results of the EPN western blot analysis.

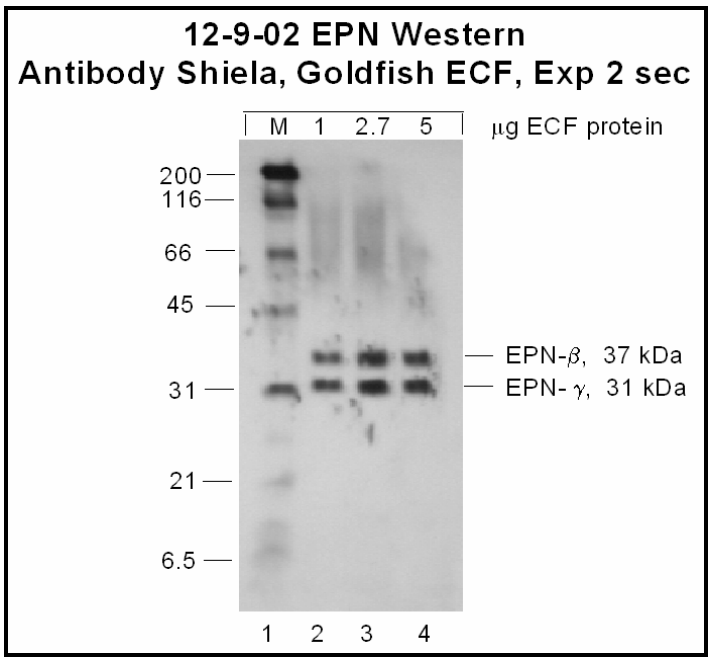


Figure 15: Analysis of EPN Protein in Goldfish Extracellular Fluid by EPN Immunoblot. Lane 1 is the kaleidoscope marker. Lanes 2-4 show increasing amounts of goldfish extracelluar protein in each lane. Arrows on the right denote the 37 and 31 kDa expected size bands for full-length ependymin. Arrows to the left denote the positions of the biotinylated markers.

Together, Figures 14 and 15 show that full-length EPN resides in our ECF preparation.

Next, NB2a cells were treated with 200 µl (12 µg/ml ECF protein) of the goldfish ECF for 5 hours to see if SOD levels increased (Figure 16). The two ECF-treated samples showed 3-4 fold increases in SOD relative to untreated samples.

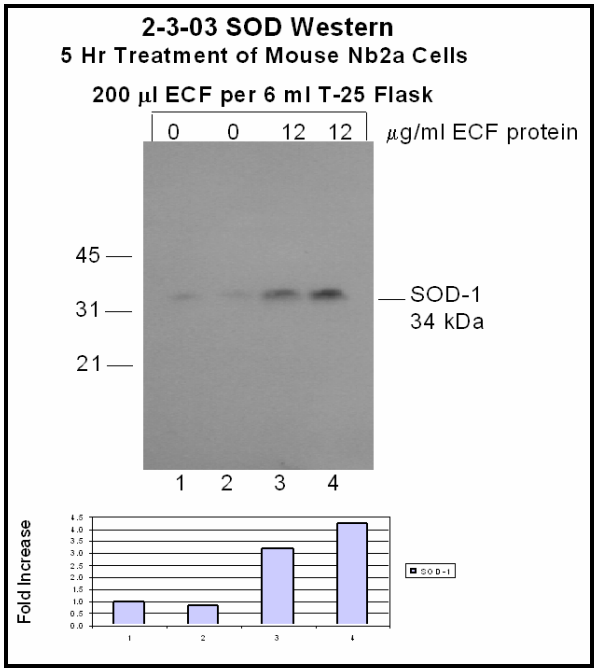


Figure 16: SOD western blot analysis of total cellular proteins isolated from mouse NB2a cells treated with a 5 hour stimulation of 200 µl (12 µg/ml) goldfish extracellular fluid. Lanes 1-2 are controls, not treated with ECF. Lanes 3 and 4 are treated with ECF. Arrows on the left denote the positions of biotinylated markers in kDa. The arrow on the right denotes the position of the 34 kDa SOD-1.

Figure 17 illustrates two randomly chosen duplicates of six total independent experiments done with the same batch of ECF.

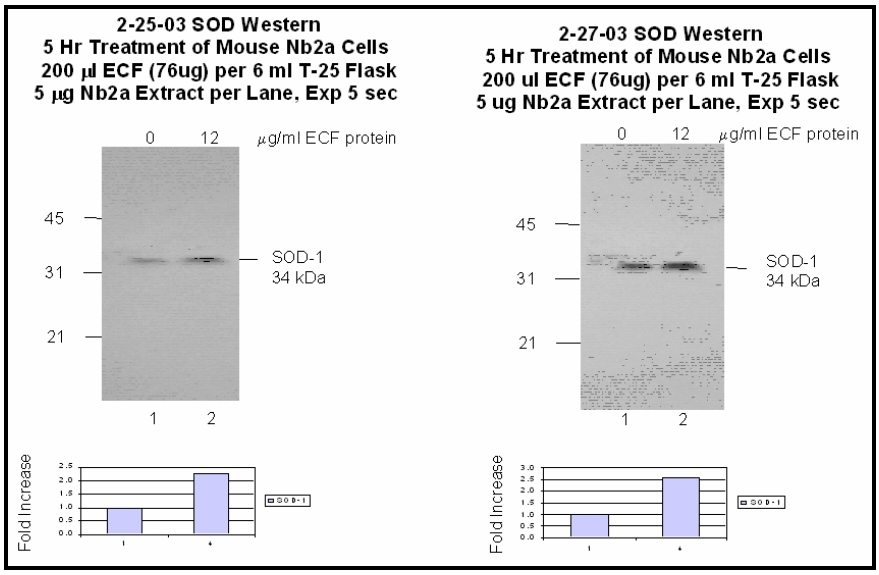


Figure 17: Example Duplicates of the Experiment Shown in Figure 16.

The means of trials 1-6 for the *in vitro* ECF experiments are illustrated in Figure 18. The figure shows that there is about a 4-fold increase in the cellular titers of SOD protein induced by the 5 hour incubation with ECF. Error bars denote standard deviation, and a statistical analysis was also performed using the two-tailed student T-test in Microsoft Excel. This analysis indicated that the ability of goldfish ECF to upregulate SOD in mouse NB2a cells was significant ($p = 0.007$).

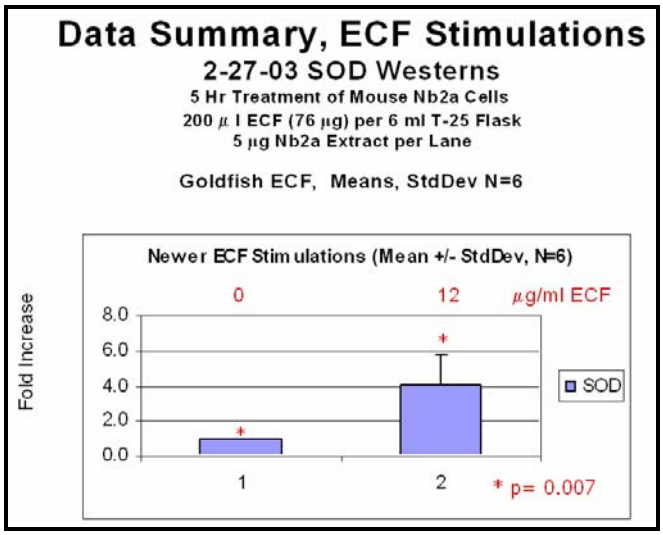


Figure 18: Statistical Analysis of Six *In Vitro* ECF EPN Experiments. Error bars denote standard deviation. Asterisks denote the two histobars to obtain the p value.

Since ECF contains a variety of components, this preliminary data did not directly show that the EPN component of ECF was responsible for our observed SOD upregulation. Since ECF contains EPN cleavage products, it is possible that the presence of 8933 in the ECF was responsible for our observed upregulation. To address this issue, microdialysis was performed on ECF to remove all components less than 8,000 daltons. The MW of 8933 is 1149 daltons (Shashoua *et al.,* 2001). Therefore, 200 µl of ECF was put in a microdialyzer system with a membrane pore size of 8,000 daltons. This would remove any small peptides and molecules present in the ECF, while retaining the higher molecular weight components, such as full-length EPN at 37,000 and 31,000 daltons.

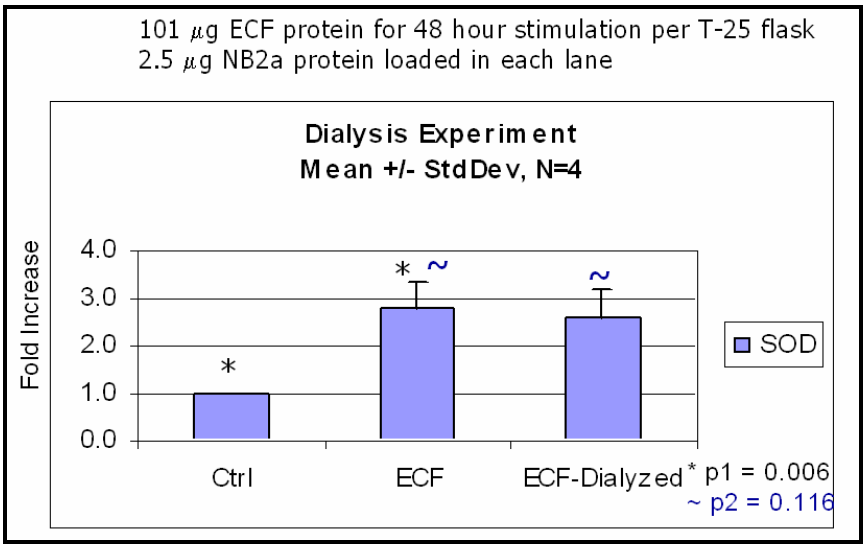


Figure 19: Statistical Analysis of Four *In Vitro* Microdialysis Experiments. Error bars denote standard deviation.

Figure 19 illustrates the results of the 4 trials of the microdialysis experiment. There is only a slight decrease (not significantly different, $p = 0.116$) in SOD protein levels in the dialyzed ECF (right histobars) compared to the non-dialyzed ECF (middle histobars). Thus, peptide 8933 does not appear to be responsible for this activity because manufacturer's data indicates sample clearance is complete by 20 minutes in this microdialyzer unit, and we dialyzed overnight. This analysis indicated that dialyzed ECF was as active as non-dialyzed. Therefore, it appears that the higher molecular weight components of ECF (including full-length EPN) do indeed contribute to the up-regulation of SOD seen previously in this section of the thesis.

DISCUSSION

The data from this thesis demonstrate several components of ependymin's mechanism of action. The first part of this thesis tested whether the mitogen-activated protein kinase (MAPK) pathway was involved in CMX-8933's role in upregulating SOD. Previous inhibition experiments in our lab showed that CMX-8933 activates transcription factor AP-1 using this MAPK pathway (Hasson, 1998; Adams *et al.,* 2003), so we hypothesized that this pathway may upregulate SOD. We chose three components of this pathway to test. Calphostin-C is a cell-permeable highly specific inhibitor of the PKC family of proteins. For PKC, its 50% inhibition concentration (IC_{50}) is 50 nM (for a review, see: Tamaoki and Nakano, 1990). GEN is a highly specific inhibitor of the PTK, with an IC_{50} of 2.6 μM (Akiyama *et al.,* 1987; Platanias and Colamonici, 1992). PD selectively blocks the activity of MEK-type mitogen-activated protein kinase kinase (MAPKK) in vitro and in intact cells (Dudley *et al.,* 1995). The IC_{50} for PD is 2 μM. Finally, the last inhibitor used in this thesis, PDTC, is a potent inhibitor of NF-κB activation, and has an IC_{50} = 100 μM (Schreck *et al*., 1992; Tsai *et al.,* 1996). The inhibition experiments in Figure 5 illustrate that inhibition of pathway members PKC, PTK and MEKK, completely blocks CMX-8933's induced SOD signal, while inhibition of NF-κB had no effect on SOD signal. So indeed the MAPK pathway appears to be involved in SOD's upregulation. Future experiments to be done to further these pathway findings are to test whether the CMX-8933/MAPK upregulation of SOD is dependent on the simultaneous upregulation of AP-1. This could be done by using a new AP-1 inhibitor to "knock out" AP-1 (Palanki *et al.,* 2000). In addition, one could utilize

specific kinase/substrate assays to determine which specific components of the PKC, PTK and MEKK families are involved in SOD upregulation.

The second portion of this thesis used RT-PCR to test whether rat primary cortical cells treated with CMX-8933 increase the cellular mRNA titers of known translational factors. Such a finding would help to solidify evidence of EPN's role in neuronal growth. Figures 8-13 illustrate that treatment of rat primary cortical cultures with 10 ng/ml CMX-8933 for 12 hours, increases the mRNA's for ribosomal proteins R-S12 (12 fold average, $p = 0.02$), R-L19 (9 fold average, $p = 0.048$), as well one elongation factor, EF –2 (11 fold average, $p = 0.045$), relative to the control. Future experiments on this theme could continue RT-PCR for other translation candidates observed from the array (Parikh, 2003), such as L-11 and S-19, which all gave moderately increased signals on the array. In addition, through inhibition experiments, one could test whether the MAPK pathway is also responsible for these ribosomal protein upregulations. Immunoblots could determine whether ribosomal proteins are upregulated coordinately with their mRNAs. And finally, since all known growth factors increase protein synthesis, a comparison study could be done to compare the types of protein synthesis factors up-regulated by NGF for example versus EPN, to test the uniqueness of EPN as a NTF.

The last portion of this thesis explored the mechanism of action of full-length EPN to compare it to our previous data on the mechanism of action of EPN protease cleavage product CMX-8933. CMX-8933 represents a naturally cleaved product of full-length EPN. It exists extra-cellularly and may interact with a specific cell surface receptor to initiate a cascade of biochemical steps leading to AP-1 up-regulation, and ultimately to SOD up-regulation. Figures 14 and 15 demonstrate that our method for

extracting the ECF of goldfish brains leaves significant amounts of full-length EPN present. Figures 16-18 show that treatment of mouse NB2a neuronal cells with a 200 μl dose of ECF for 5 hours induces an average 4 fold ($p = 0.007$) upregulation of SOD. However, ECF is a complex fluid, also known to contain EPN cleavage product 8933, which by itself can upregulate SOD. To determine whether the ECF-induced upregulation of SOD resulted from low MW ECF components (including 8933), microdialysis was performed using a membrane with a MWCO of 8,000 daltons to eliminate smaller components of the ECF. Figure 19 illustrates that the higher molecular weight components, including full-length EPN, are responsible for nearly the entire SOD signal. Therefore, from these initial findings it is hypothesized that full length EPN can interact with EPN receptor, like CMX-8933, to elicit a signal transduction response that upregulates SOD. Future experiments could include immuno-purification of EPN to determine if that component was responsible for the observed high MW ECF result, or the injection of a variety of protease inhibitors into goldfish brain ECF to try to diminish the formation of CMX-8933 and see if the same results occur with the up-regulation of SOD.

Overall, the study presented here provides evidence to further understand the mechanism of action of both full-length ependymin as well as its cleavage peptide CMX-8933. Future experiments will need to be done to extend this understanding, including experiments to isolate and characterize the specific cell surface receptor(s), and investigating other signal trasduction pathways. Even though this particular study focused only on the upregulation of SOD, this enzyme alone is not enough to alleviate the destructive neuronal death following such occurrences as stroke. At least two other

important enzymes play a key role in keeping oxidative stress at safe levels in the cells. Catalase and GPX breakdown the H_2O_2 generated by SOD. However, because this latter H_2O_2 breakdown reaction is so quick, it is not clear yet whether their titers must be increased to maintain safe oxidative stress levels. Previous experiments in our lab have shown that CMX-8933 increases CAT and GPX (Armistead, 2001), however more studies on full-length EPN could further strengthen this finding.

In conclusion, the data in this thesis are consistent with our hypothesized mechanism of action of full-length EPN and CMX-8933. Both increase the cellular titers of the antioxidant enzyme SOD. CMX-8933 increases the mRNAs that participate in neuronal growth, as part of EPN's known role as a NTF. EPN and the peptide mimetic CMX-8933 may potentially be used for future treatments of neuronal degenerative diseases to help reduce the harmful damage caused by dangerous oxiradicals.

BIBLIOGRAPHY

Adams DS and Shashoua VE (1994) Cloning and sequencing the genes encoding goldfish and carp ependymin. *Gene* **141**: 237-241.

Adams DS, Kiyokawa M, Getman M, Shashoua VE (1996) Genes encoding giant danio and golden shiner ependymin. *Neurochemical Research* **21(3)**: 377-384.

Adams DS, Boyer-Boiteau A, Hasson B, El-Khisin, Shashoua VE (2003) A peptide fragment of ependymin neurotrophic factor uses PKC and the MAPK pathway to activate JNK and a functional AP-1 containing c-Jun and c-Fos proteins in mouse NB2a cells. *Journal of Neuroscience Research* **72**: 405-416.

Allen RG, Tresini M (2000) Oxidative stress and gene regulation. *Free Radical Biology and Medicine* **28:** 463-499.

Akiyama T, Ischida J, Nakagawa S, Ogawara H, Watanabe S, Itoh N, Shibuya M, Fukami Y (1987) Genistein, a specific inhibitor of tyrosine-specific protein kinases. *Journal of Biological Chemistry* **77**: 221-233.

Armistead S (2001) Conjugating a neurotrophic peptide to blood-brain barrier carrier DHA increases its activity for upregulating therapeutic enzymes SOD, catalase, and GPX. WPI MQP, December, 2001.

Baldessarini RJ, Campbell A, Webb N, Swindell CS, Flood JG, Shashoua VE, Kula NS, Hemamalini S, Bradley MO (2001) Fatty acid derivitives of clozapine: prolonged antidopaminergic activity of docosahexaenoylclozapine in the rat. *Neuropsychopharmacology* **24**: 55-65.

Bear M, Connors B, Paradiso M (2001) Neuroscience: Exploring the Brain. Baltimore, Maryland: Lippincott Williams & Willins.

Benowitz LI and Shashoua VE (1979) Immunoreactive sites for nerve growth factor (NTF) in the goldfish brain. *Brain Research Review* **172**: 561-565.

Chan PH, KinouchiC, Epstein E, Carlson S, Chen S (1993) Role of Superoxide dismutase in ischemic brain injury: reduction of edema and infarction in transgenic mice following focal cerebral ischemia. *Progress in Brain Research* **96:** 97-104.

Chan PH and Kawase M (1998) Overexpression of SOD1 in transgenic rats protects vulnerable neurons against ischemic damage after cerebral ischemia and reperfusion. *Journal of Neuroscience* **20**: 8292-8299.

Connor B, Dragunow M (1998) The role of neuronal growth factors in

neurodegenerative disorders of the human brain. *Brain Research Review* **27**:1-39.

Cui Q, So KF, Yip HK (1998) Major biological effects of neurotrophic factors on retinal ganglion cells in mammals. *Biological Signals Recep* **7**: 220-226.

Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proceedings of the National Academy of Science USA* **92**: 7686-7689.

Duffy J and Smith A (2003) Small peptide substitutes for full-length ependymin neurotrophic factor up-regulate SOD, which is part of the cell's natural response to anoxia. WPI MQP, January 2003.

El-Khishin A (1999) The activator protein-1 upregulated by neurotrophic peptides NMI-9236 and NMI-8933 is capable of transcriptional *trans*-activation. WPI MQP, May 1999.

Ferrer I, Lopez E, Pozas E, Ballabriza J, Marti E (1998) Multiple neurotrophic signals converge in surviving CA1 neurons of the gerbil hippocampus following transient forebrain ischemia. *Journal of Computational Neurobiology* **394**: 416-430.

Ferrer I, Krupinski J, Goutan E, Marti E, Ambrosio S, Arenas E (2001) Brain-derived neurotrophic factor reduces cortical cell death by ischemia after middle cerebral artery occlusion in the rat. *Acta Neuropathologica* **101:** 229-238.

Gaiddon C, Loeffler JP, Larmet Y (1996) Brain-derived neurotrophic factor stimulates AP-1 and cyclic AMP-responsive element dependent transcriptional activity in central nervous system neurons. *Journal of Neurochemistry* **66**: 2279-2289.

Goldstein A (1976) Opioid peptide endorphins in pituitary and brain. *Science* **193**: 1081-1086.

Hasson BG (1998) Calcium-chelating neuroprotective peptides NMI-8933 and NMI-9236 activate transcription factor AP-1 in neuronal cells. WPI Master's Thesis, December, 1998.

Ikeda T, Xia XY, Xia YX, Ikenoue T, Han B, Choi BH (2000) Glial cell line-derived neurotrophic factor protects against ischemia/hypoxia-induced brain injury in neonatal rat. *Acta Neuropathologica* **1000**: 161-167.

Ip NY, Stitt TN, Tapley P, Klein R, Glass DJ, Fandl J, Greene LA, Barbacid M, Yancopoulos GD (1993) Similarities and differences in the way neurotrophins

interact with the Trk receptors in neuronal and non neuronal cells. *Neuron* **10**: 137-149.

Jehan F, Naveilhan P, Neveu I, Harvie D, Dicou E, Brachet P, Wion D (1996) Regulation of NGF, BDNF and LNGFR gene expression in ROS 17/2.8 cells *Molecular and Cellular Endocrinology* **116**: 149-156.

Kibiuk, Lydia (1994) Brain briefings; Society for Neuroscience.

Konigstorfer A, Sterrer S, Hoffmen W (1989) Biosynthesis of ependymins from goldfish brain. *Journal of Biological Chemistry* **264(23):** 13689-13692.

LeBlanc J (2000) Neurotrophic peptides that are therapeutic for strokes in a rat model upregulate enzymes which reduce oxidative stress in various tissues. WPI MQP, May, 2000.

Li Q, Bolli R, Qiu Y, Tang XL, Guo Y, French BA (2001) Gene therapy with extracellular superoxide dismutase protects conscious rabbits against myocardial infarction. *Circulation* **103:** 1893-1898.

Luiz LA, Peranelli AC, Peters TL (2002) Small peptide substitutes for full-length neurotrophic factors. WPI MQP, April 2002.

Nichi R (1994) Neurotrophic factors: Two are better than one. *Science* **265**:1050-1053.

Palanki MS, Erdman PE, Manning AM, Ow A, Ranson LJ, Spooner D, Suto C, Suto M (2000) Novel inhibitors of AP-1 and NF-kB mediated gene expression: Structure-activity relationship studies of ethyl 4-[(3-methyl-2,5-dioxo(3-pyrrolinyl))amino]-2-(trifluoromethyl) pyrimidine-5-carboxylate. *Bioorganic Medicicinal Chemistry Letters* **10**: 1645-1648.

Parikh S (2003) Ependymin peptide mimetics that assuage ischemic damage increase gene expression of the anti-oxidative enzymes SOD. WPI Master's Thesis, April, 2003.

Pfeiffer TJ, Reynolds LGG, Tremblay C (2001) Peptides mimicking neurotrophic factors effective in treating neurodegenerative disorders upregulate anti-oxidative enzymes. WPI MQP, April 2001.

Piront ML and Schmidt R (1988) Inhibition of long-term memory formation by anti-ependymin antisera after shock-avoidance learning in goldfish. *Brain Research* **442(1):** 53-62.

Plantanias LC and Colamonici OR (1992) Interferon-α induces rapid tyrosine phosphorylation of the α subunit of its receptor. *Journal of Biological Chemistry*

267: 24053-24057.

Protarga Homepage (2003) Protarga: Improving Medicine with Targaceutical Products. URL: Http://www.protarga.com.

Przedborski S, Kostic V, Jackson-Lewis V, Naini AB, Simonetti S, Fahn S, Carlson E, Epstein CJ, Cadet JL (1992) Transgenic mice with increased Cu/Zn superoxide dismutase activity are resistant to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *Journal of Neuroscience* **12**: 1658-1667.

Rother S, Schmidt R, Brysch W, Schlingensiepen KH (1995) Learning induced expression of meningeal ependymin mRNA and demonstration of ependymin in neurons and glial cells. *Journal of Neurochemistry* **65(4):** 1456-1464.

Sanyal S, Sandstrom DT, Hoeffer CA, Remaswami M (2002) AP-1 functions upstream of CREB to control synaptic plasticity in Drosophila. *Nature* **416**: 870-874.

Schmidt R and Shashoua VE (1981) A radioimmunoassay for ependymins beta and gamma: two goldfish brain proteins involved in behavioral plasticity. *Journal of Neurochemistry* **36(4):** 1368-1377.

Schmidt R and Shashoua VE (1983) Structural and metabolic relationships between goldfish brain glycoproteins participating in functional plasticity of the central nervous system. *Journal of Neurochemistry* **40(3):** 652-660.

Schmidt R (1987) Changes in subcellular distribution of ependymins in goldfish brain induced by learning. *Journal of Neurochemistry* **48(6):** 1870-1878.

Schmidt R and Lapp H (1987) Regional distribution of ependymins in goldfish brain measured by radioimmunoassay. *Neurochemistry International* **10**: 383-390.

Schmidt JT and Shashoua VE (1988) Antibodies to ependymin block the sharpening of the regenerating retinotectal projection on goldfish. *Brain Research* **446(2):** 269-284.

Schmidt JT, Schmidt R, Lin WC, Jian XY, Stuermer CA (1991) Ependymin as a substrate for outgrowth of axons from cultured explants of goldfish retina. *Journal of Neurobiology* **22(1**): 40-54.

Schmidt R (1995) Cell-adhesion molecules in memory formation. *Behavioral Brain Research* **66(1-2):** 65-72.

Schmidt R, Brysch W, Rother S, Schlingensiepen KH (1995) Inhibition of memory consolidation after active avoidance conditioning by antisense intervention with ependymin gene expression. *Journal of Neurochemistry* **65(4):** 1465-1471.

Schreck R, Meier B, Mannel DN, Droge W, Baeuerle PA (1992) Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. *Journal of Experimental Medicine* **175**: 1181-1194.

Shashoua VE (1976) Identification of specific changes on the pattern of brain protein synthesis after training. *Science* **193**: 1264-1266.

Shashoua VE (1977) Brain protein metabolism and the acquisition of new patterns of behavior. *Proceedings of the National Academy of Science USA* **74**: 1743-1747.

Shashoua VE and Moore ME (1978) Effect of antisera to and goldfish brain proteins on the retention of a newly acquired behavior. *Brain Research* **148**: 441-449.

Shashoua VE (1982) Molecular and cell biological aspects of learning: toward a theory of memory. *Advances in Cell Biology* **3**: 97-141.

Shashoua VE (1985) The role of brain extracellular proteins in neuroplasticity and learning. *Cell Molecular Neurobiology* **5(1-2):** 183-207.

Shashoua VE and Homquist B (1986) Extracellular fluid proteins of goldfish brain: evidence for the presence of proteases and esterases. *Journal of Neurochemistry* **47(3):** 738-743.

Shashoua VE (1988) Monomeric and polymeric forms of ependymin: a brain extracellular glycoprotein implicated in memory consolidation processes. *Neurochemical Research* **13(7):** 649-655.

Shashoua VE and Hesse GW (1989) Classical conditioning leads to changes in extracellular concentrations of ependymin in goldfish brain. *Brain Research* **484(1-2)**: 333-339.

Shashoua VE, Hesse GW, Milinazzo B (1990) Evidence for the in vivo polymerization of ependymin: a brain extracellular glycoprotein. *Brain Research* **522(2):** 181-190.

Shashoua VE (1991) Ependymin, a brain extracellular glycoprotein, and CNS plasticity. *Annals NY Academy of Science* **627**: 94-114.

Shashoua VE, Nolan PM, Shea TB, Milinazzo B (1992) Dibutyryl cyclic AMP stimulates expression of ependymin mRNA and the synthesis and release of the protein into the culture medium by neuroblastoma cells (NB2a/d1*)*. *Journal of Neuroscience Research* **32(2):** 239-244.

Shashoua VE and Hesse GW (1996) N-docosahexaenoyl, 3 hydroxytyramine: a dopaminergic compound that penetrates the blood-brain barrier and supresses

appetite. *Life Sciences* **58**: 1347-1357.

Shashoua VE, Adams DS, Boyer-Boiteau A (2001) CMX-8933, a peptide fragment of the glycoprotein ependymin promotes activation of AP-1 transcription factor in mouse neuroblastoma and rat cortical cultures. *Neuroscience Letters* **312**: 103-107.

Shashoua VE, Adans DS, Boyer-Boiteau A (2003) Neuroprotective effects of a new synthetic peptide, CMX-9236, in *in vitro* and *in vivo* models of cerebral ischemia. *Brain Research* **936**: 214-223.

Shaulian E and Karin M (2002) AP-1 as a regulator of cell life and death. *Nature Cell Biology* **4(5)**:E131-6

Sheng H, Bart RD, Oury TD, Pearlstein RD, Crapo JD, Warner DS (1999a) Mice overexpressing extracellular superoxide dismutase have increased resistance to focal cerebral ischemia. *Neuroscience* **88**: 185-191.

Sheng H, Brady T, Pearlstein R, Crapo J, Warner D (1999b) Extracellular superoxide dismutase deficiency worsens outcome from fetal focal cerebral ischemia in the mouse. *Neuroscience Letters* **267:** 13-16.

Sleeper AA (1999) Development of an *in vitro* production system for neurotrophic peptides NMI-8933 and NMI-9236. WPI Master's Thesis, May 1999.

Stahl S (2003) Brain tonics for brain sprouts: how neurotrophic factors fertilize neurons. URL: www.psychiatrist.com.

Sun AY and Chen Y (1998) Oxidative stress and neurodegenerative disorders. *Journal of Biomedical Scienc*e **5**: 401-414.

Tamaoki T and Nakano H (1990) Potent and specific inhibitors of PKC of microbial origin. *Biotechnology* **8:** 732-735.

Tang S, Sun K, Sun G, Lin G, Lin W, Chuang M (1999) Cold-induced ependymin expression in zebrafish and carp brain: implications for cold acclimation. *FEBS Letters* **459**: 95-99.

Tong L and Perez-Polo JR (1996) Effect of nerve growth factor on AP-1, NF-kB and Oct DNA binding activity in apoptotic PC12 cells: extrinsic and intrinsic elements. *Journal of Neuroscience Research* **45:** 1-12.

Tsai JC, Jain M, Hsieh CM, Lee WS, Yoshizumi M, Patterson C, Perella MA, Cooke C, Wang H, Haber E, Schlegel R, Lee ME (1996) Induction of apoptosis by pyrrolidinedithiocarbamate and N-acetylcysteine in vascular smooth muscle cells. *Journal of Biological Chemistry* **271**: 3667-3670.

Venarucci D, Venarucci V, Vallese A, Battila L, Casado A, De la Torre R, Lopez-Fernandez ME (1999) Free radicals: important cause of pathologies related to ageing. *Panminerva Medicine* **4:** 335-339.

Wu D and Partridge WM (1999) Neuroprotection with noninvasive neurotrophin delivery to the brain. *Neurobiology* **96:** 254-259.