

**AP-1 IS REQUIRED FOR CMX-8933-INDUCED SOD  
UPREGULATION AND IS TRANSLOCATED IN RESPONSE  
TO A HUMAN EPN MIMETIC**

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## ABSTRACT

Ependymin (EPN) is a neurotrophic factor (NTF) that functions in goldfish long-term memory formation and optic nerve elongation (Shashoua, 1976; Shashoua, 1977; Shashoua, 1985). Goldfish EPN, or CMX-8933 (a short goldfish EPN mimetic studied by our lab), surprisingly have several demonstrated effects on mammalian cells, including neuroregenerative effects in a rat stroke model (Shashoua *et al*, 2003), and the activation of therapeutic superoxide dismutase (SOD) (Parikh, 2003) and transcription factor AP-1 (Adams *et al*, 2003) in mouse neuroblastoma cells or rat primary cortical neuronal cultures. Among its various functions, AP-1 can function as a master switch in long-term memory consolidation (Sanyal *et al*, 2002), so it may be a key event in EPN's mechanism of action. AP-1 activation is also a characteristic associated with other full-sized neurotrophic factors, including nerve growth factor and brain-derived nerve growth factor.

This thesis was divided into three parts. The purpose of part I was to determine whether our previously observed upregulation of SOD by CMX-8933 is dependent upon (or merely concurrent with) AP-1 activation. Four independent SOD immunoblot experiments demonstrated that pre-treatment of rat primary cortical cultures with trifluoromethyl pyrimidine carboxylate (TFPC), a specific inhibitor of AP-1, significantly ( $p = 0.0004$ ) decreased cellular levels of SOD by 67% at its  $IC_{50}$  concentration of 1  $\mu$ M, and completely inhibited the upregulation at 10 and 100  $\mu$ M concentrations. Thus, the CMX-8933-induced upregulation of SOD appears to depend (directly or indirectly) on AP-1 activation.

Part II of this thesis included the use of bioinformatics to re-verify exciting recent observations that EPN-like proteins exist in mammals, termed mammalian-ependymin-related proteins or MERPs (Apostolopoulos *et al*, 2001). If our analyses were convincing, human EPN mimetics would then be designed and tested for AP-1 activation. Computer alignments and hydropathy plots performed with EPN amino acid sequences deduced from gene entries in GenBank verified the existence of mammalian homologs containing highly conserved domains with fish EPN's, suggesting the possibilities of similar protein conformation and function. Two human EPN mimetics were designed, hEPN-1 (8 aa long, corresponding to the same region as CMX-8933) and hEPN-2 (14 aa long, containing CMX-8933 and 6 upstream aa). Several mimetic doses were tested on mouse Neuro-2a cultures for nuclear translocation of c-Jun and c-Fos proteins (comprising the AP-1 particle upregulated by fish CMX-8933). Seven independent c-Jun immunoblot experiments, and five c-Fos experiments, demonstrated a strong (as high as 25-fold) dose-dependent increase in the nuclear titers of the AP-1 proteins. Both peptides had statistically equivalent effects. Thus, human EPN appears to exist, and two mimetics derived from its sequence appear to be biologically active against mouse neuroblastoma cells. Since hEPN-1 and -2 have only a few residues in common with CMX-8933, we hypothesize that the mimetic shape rather than sequence may be important for biological activity.

In part III of this thesis, the biological effects of hEPN-1 and hEPN-2 on mouse Neuro-2a cells were studied further using RT-PCR to analyze potential increases in specific mRNAs. mRNAs related to growth, energy production, and protein translation were tested since previous data in our lab (Kaska, 2003) indicated mRNAs for translational elongation factor-2 (EF-2), and ribosomal proteins L19 and S12 were upregulated in rat primary

cortical cultures by fish mimetic CMX-8933 (Kaska, 2003). Treatment of Neuro-2a cells with 1.0 µg/ml hEPN-1 (the highest dose tested for the AP-1 translocation experiments) for 24 hrs appeared to increase (N = 1) mRNAs for ATP Synthase-C, ribosomal protein L19, and translational EF-2, relative to the levels of housekeeper polyubiquitin. Thus hEPN-1 may be involved in processes related to growth.

Altogether, the data from this thesis extends our knowledge of fish EPN mimetic CMX-8933 (showing that its induction of SOD requires AP-1), demonstrates that human EPN may exist (bioinformatics), shows that two human EPN mimetics are biologically active (induce AP-1 translocation), and shows that one mimetic hEPN-1 may activate several mRNAs related to growth in mouse Neuro-2a cells.

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## BACKGROUND

### Neurotrophic Factors

Neurotrophic factors or neurotrophins (NTFs) are naturally occurring proteins that promote and control the development, survival, and maintenance of neuronal and glial cells (Abe, 2000). NTFs play a key role in the development of both the central and peripheral nervous systems (Ikeda *et al*, 2000). Because of the ability of some NTFs to regenerate brain tissue, they are implicated to have potential restorative effects in neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease and stroke (Sariola *et al*, 1994).

The survival and differentiation of neurons is mainly regulated by target-derived neurotrophic factors. 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).

NTFs have been shown to alleviate oxidative stress, one of the primary mediators of cell damage in neurodegenerative conditions, but the mechanism of how they achieve this is unknown. One mechanism by which they accomplish this is to increase cellular levels of the anti-oxidative enzyme superoxide dismutase (SOD). EPN (synthetic CMX-8933) has been shown by our lab to increase the anti-oxidative enzyme superoxide dismutase SOD mRNA and protein levels in rat primary cortical cultures (Parikh, 2003). Short peptides like CMX-8933, CMX-9236, NMX-8933 and NMX-9236, that have a combination of properties which include the activation of a specific transcription factor AP-1 (Shashuoa *et*

*al*, 2001) that up-regulates genes that promote synaptic plasticity (Sanyal *et al*, 2002) and the inhibition of intracellular  $\text{Ca}^{++}$  accumulation, have been designed by our lab in collaboration with Ceremedix Inc., that mimic the action of full-length NTFs, especially EPN. EPN is partially cleaved by the proteases that naturally exist in ECF to release an active component of 8 amino acid peptide KKETLQFR, like other brain proteins. Our lab demonstrated that administration of this sequence in synthetic form (termed CMX-8933) to rat primary cortical cells and mouse neuroblastoma cells increases cellular titers of SOD (Parikh, 2003).

### **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 goldfish central nervous system, localized in the *zona ependyma* from which it derives its name (Shashoua and Benowitz, 1977; Benowitz and Shashoua, 1979), however later evidence found that it was also detected as a novel secretory protein in brain extracellular fluid (ECF), in the cerebrospinal fluid (CSF) (Shashoua, 1985) and in the meninges of goldfish and zebrafish brains surrounding the ventricles (Hoffmann *et al*, 1990).

Ependymin exists in two forms,  $\beta$  and  $\gamma$  (MW 37,000 and 31,000, respectively). Ependymin  $\beta$  is glycosylated, and ependymin  $\gamma$  is non-glycosylated (Shashoua, 1976; Shashoua, 1985; Konigstorfer *et al*, 1989; Shashoua *et al*, 1990). In goldfish, the protein portion consists of 216 amino acid residues, including two N-glycosylated sites (Adams

and Shashoua, 1994). Both  $\beta$  and  $\gamma$  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 (Schmidt and Shashoua, 1983; Shashoua and Holmquist, 1986; Shashoua, 1985; Shashoua, 1988) that promote 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  $\beta$  and  $\gamma$  were shown to constitute 14% of the total protein content of the brain extracellular fluid, and are also a minor component (0.3%) of serum proteins (Schmidt and Shashoua, 1981; Schmidt and Lapp, 1987).

### *Ependymin's Function*

EPN is a multi-functional protein. Although the precise molecular mechanism by which the brain uses EPN remains unknown, certain functional aspects of EPN closely resemble the properties of other 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). Addition of goldfish EPN to mouse neuroblastoma NB2a cell cultures enhances neurite sprouting and extension (Shashoua *et al*, 1992), a phenomenon also observed with NGF (Conner and Varon, 1995) and BDNF (Patel and McNamara, 1995). Injection of anti-EPN Ab can block memory formation (Schmidt and Shashoua, 1988). In addition, EPN has been linked with learning (Shashoua, 1985; Piront



The CMX-8933 sequence is completely conserved among the six deduced protein sequences of 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 full-length EPN is present as well) possibly comprising EPN's receptor-binding domain. Enkephalins and endorphins serve as a precedent for the proteolytic removal of functional receptor-binding byproduct fragments (Goldstein, 1976).

### **Ependymin Bioinformatics and Mammalian Ependymin-Related Proteins (MERPs)**

Although EPN was originally discovered in goldfish, and that species remains the only one in which functional studies have been performed to date, recent bioinformatic approaches argue for EPN's existence outside that species. The rate and pattern of EPN DNA evolution was studied (Orti and Meyer, 1996) in other teleost fish including Cypriniforms, Siluriforms, Gymnotiforms and Characiforms. It showed that among 25 different fish species, each presumed EPN protein sequence contains five highly conserved cysteine domains (presumed to form disulfide bonds), one completely conserved tryptophan domain, and two highly conserved glycosylation sites. Results of this study indicated that molecules structurally related (highly conserved functional domains) to the very well characterized Cypriniform EPN's exist, even if their overall sequences are not highly conserved. These alignments of fish EPN protein sequences were later extended to include a few mammalian species (Apostolopoulos *et al*, 2001) providing evidence for a family of mammalian homologs of ependymin called "ependymin-related proteins" (ERPs) (for non-Cypriniform non-mammalian EPNs), and mammalian homologs as "mammalian

ependymin-related proteins” (MERPs) (Apostolopoulos *et al*, 2001). Thus, by extending the teleost alignment originally deduced by Orti and Meyer (1996), Apostolopoulos *et al* (2001) identified mammalian EPN-related proteins (MERP’s).

MERPs are found in the hippocampal and dentate granule cell neurons of brain. The entire coding region of human *MERP-1* has been cloned and mapped to chromosome 7p14.1. Mouse *MERP-1* and *MERP-2* genes have also been cloned (Apostolopoulos *et al*, 2001) and mouse *MERP-2* has been localized to chromosome 13. Furthermore, monkey *MERP-1* and frog ERP have also been identified (Apostolopoulos *et al*, 2001). Another novel EPN-related gene, UCC1, highly expressed in colorectal tumor cells (so it may have a role in cell growth), also showed significant sequence similarity to the highly divergent piscine ependymins (Nimmrich *et al*, 2001). Sequences for these and additional mammalian homologs from monkey and mouse, have recently been added to the GenBank database and so it is clear that MERP-1-like genes exist (Gregorio-King *et al*, 2002).

Despite relatively low amino acid sequence conservation between piscine ependymins, toad ERP, and MERPs, several amino acids (including four key cysteine residues) are found to be strictly conserved, and the hydropathy profiles and glycosylation sites are remarkably alike, suggesting the possibilities of similar protein conformation and function (Orti and Meyer, 1996; Apostolopoulos *et al*, 2001). As with fish ependymins, frog ERP and the MERPs contain a signal peptide typical of secreted proteins. The MERPs have been found to be expressed at high levels in several hematopoietic cell lines and in nonhematopoietic tissues such as brain, heart, and skeletal muscle, as well as several malignant tissues and malignant cell lines (Nimmrich *et al*, 2001). The complete human *MERP-1* sequence, composed of 344 amino acid residues, has been very recently

characterized and found to be expressed in hematopoietic progenitors and other human tissues (Gregorio-King *et al* 2002). These findings suggest that MERPs have several potential roles in a wide range of cells and tissues, so it may function in places other than the central nervous system.

### *Human EPN-1 and Human EPN-2 Peptides*

With the bioinformatic “discovery” of human MERP, our lab decided to investigate the possible biological activity of peptides derived from its sequence. The human EPN peptide mimetics that I used in this project are two different portions of *MERP-1*. Human EPN-1 (hEPN-1) comprises the 8 amino acid peptide "SKMTLTQP", corresponding to positions 234-241 in the mature *MERP-1* sequence (see Figure 2), which represents the exact same region as CMX-8933’s "KKETLQFR". Only 3 of the amino acids are conserved with CMX-8933, but our working hypothesis is that this region might represent the same receptor-binding region. The "K" in human EPN-1 is completely conserved in all known EPN's.

MPPRMLVAPLPLHFLLA AVSS ENRSGRRQWQQAVAAGSGPGRNSSRAIHWSLPRALVPATGTRASGSQKRQRP PKGQEALWSRPHSRHSAERAGGSHSDPGRLSAPLGLGLPSGRGRLTAMPGRAPLRTVPGALGAWLLGGLWAWT LCGLCSLGAVGAPRPCQAPQQWEGRQVMYQQSSGRNSRALLSYDGLNQVRVLDERKALIPCKRLEFYILLYK DGVMFQIDQATKQC <b>SKMTLTQP</b> WDPLDIPQNSTFEDQYSIGGPQEQITVQEWSDRKSARSYETWIGIYTVKDC YPVQETFTINYSVILSTRFFDIQLGIKDPSVFTPSSTCQMAQLEKMS EDCSW	344 aa
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**Figure 2 – Amino Acid Sequence of Human MERP-1 (Accession Number: AY027862) and Peptide Mimetics Tested in This Thesis.** The complete 344 aa of Human *MERP-1* (Apostolopoulos *et al*, 2001). The 8 amino acids shown in red represent mimetic hEPN-1 which precisely corresponds to the exact same region as CMX-8933, and includes a lysine residue (K) conserved in all known EPNs. The 14 amino acids in the box represent mimetic hEPN-2 which includes a cysteine residue at site 6 completely conserved in all known EPNs.

Human EPN-2 (hEPN-2) comprises the 14 amino acid peptide "QATKQCS-KMTLTQP", corresponding to positions 228-241 in the mature *MERP-1* sequence (see Figure 2), which simply extends hEPN-1 upstream to include a cysteine that is also completely conserved in all known EPN's.

### **Activator Protein (AP-1) Transcription Factor**

The activator protein (AP-1) transcription factor family is a ubiquitous, fundamental class of transcriptional regulators (Wagner, 2001). In fact, AP-1 was one of the first mammalian transcription factors to be identified, but its physiological functions are still being unraveled (Angel and Karin, 1991). It is a central component of many signal transduction pathways, and is activated in response to an incredible array of stimuli, including mitogenic growth factors, inflammatory cytokines, growth factors of the TGF-beta family, UV and ionizing irradiation, cellular stress, antigen binding, and neoplastic transformation (Wisdom, 1999). The pleiotropic nature of a variety of inducers raised an issue as to how could AP-1 possibly mediate the cellular response to such a wide array of stimuli? Peptide growth factors (which lead to cell cycle progression) and UV irradiation (which leads to cell cycle arrest) appear to be the two most potent inducers of AP-1 activity (Wisdom, 1999). AP-1 has been a paradigm for transcription factors, and acts like an environmental biosensor (Wagner, 2001).

#### *AP-1 Functions*

AP-1, depending on its exact protein composition, regulates the promoter activity of a large number of genes associated with developmental, proliferative, inflammatory, and



homeostatic processes in many types of cells. AP-1 is involved in such diverse processes as cellular proliferation, transformation, and death (Shaulian and Karin, 2002).

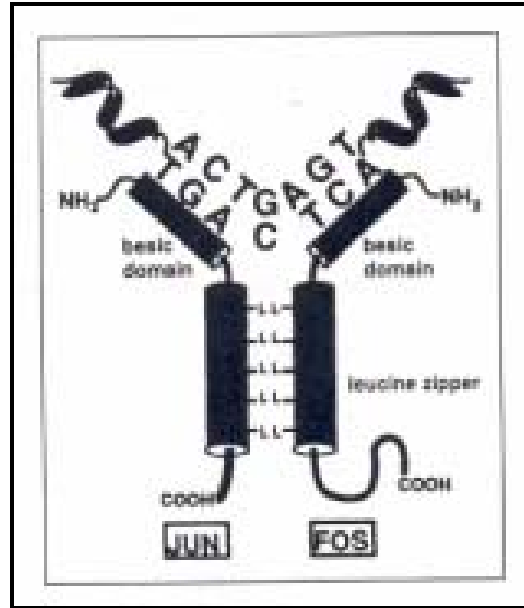
Biological functions of the dimeric transcription factor complex AP-1, in particular in its role in skeletal development, revealed that some components, such as c-Fos, are key regulators of bone cell differentiation, whereas others, like c-Jun, JunB and Fra-1 are essential in embryonic and/or postnatal development (Wagner, 2001). AP-1's activation by a variety of neurotrophic factors suggests its role in short- to long-term synaptic plasticity and memory, and in regulating long-term neural plasticity (Adams *et al*, 2003). AP-1 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).

AP-1 is a redox-sensitive transcription factor (Zhang *et al*, 2002). Cells evolve efficient strategies for dealing with stress responses by activating the transcription of genes that encode for defense and repair proteins. In yeast, members of the AP-1 family of proteins are required for the transcriptional response to oxidative stress. Many of the features of this response appear to be evolutionarily conserved (Zhang *et al*, 2002).

AP-1 activation has been shown as an important component of brain responses to oxidative stress (Tong *et al*, 2003). Increased expression of AP-1 transcription factors after neurotoxin insults is seen in rat hippocampus and cerebellum (Xiao *et al*, 1999). The important structural features of this factor, signaling pathways controlling its activity, and the nature of the target genes it control has made AP-1 a very important transcription factor (Toone *et al*, 2001).

### *Components of AP-1*

Biochemical purification showed that AP-1 is not a single transcription factor, but instead, a family of related dimeric complexes of Fos and Jun family proteins which are required for DNA-binding activity specific for the palindromic sequence 5' TGAGTCA 3' (Wisdom, 1999). This family appears in virtually all vertebrate cell types. Since the DNA recognition sequence is palindromic, DNA recognition occurs only when the Jun and Fos form dimeric complexes, which is mediated by the basic-leucine zipper (bZIP) motif to bind the AP-1 site in the promoter of target genes, which in turn regulate the expression of late response genes that produce long-term changes in cells (Yu *et al*, 1995). In fact, the DNA-binding activity of Jun is increased through its interaction with Fos (Allegretto *et al*, 1990). The properties of the Jun and Fos leucine zippers dictate the nature of the dimeric complexes that will form: the leucine zipper of Jun family proteins can form homodimeric complexes, as well as Jun–Fos heterodimers, while the leucine zipper of Fos proteins is capable only of heterodimer formation (Wisdom, 1999). The leucine zipper and DNA-binding regions are highly conserved among the c-fos and c-jun families of related inducible genes. Thus, multiple protein complexes can be formed that may interact with AP-1 binding sites in numerous genes to affect gene expression in response to environmental signals. (Abate and Curran, 1990).



**Figure 3 - Activator Protein-1.** This diagram depicts the leucine zipper motif (ladder “rungs”) in a JUN/FOS heterodimer complex. Also shown are the basic (positively charged) domains binding to the target DNA sequence (Gass *et al*, 1997).

c-Jun and c-Fos are some of the immediate early genes that are induced within minutes to hours in response to perturbations in the cellular environments (Zawia and Harry, 1993). Their induction has shown to cause sprouting or synaptic plasticity (Gass and Herdegen, 1995). Mostly, c-Jun acts as a positive regulator of cell proliferation unlike other members of the family. The growth-promoting activity of c-Jun is mediated by repression of tumor suppressors, as well as upregulation of positive cell cycle regulators. Yet it is interesting to note that its induced cell growth, for example during development, is not always accompanied by tumor growth.

c-Fos is involved in the response of the brain to the neurotoxin TMT in an age-dependent and region-specific manner (Zawia and Harry, 1993). The rapid and transient expression of c-Fos mRNA after exposure of cells to peptide growth factors indicates AP-1 is a nuclear target of growth factor signaling pathways (Wisdom, 1999). AP-1 influences a

variety of positive and negative cellular processes, which is determined in part by its exact protein composition and mechanism of activation (Adams *et al*, 2003).

Although we have come a long way from discovering AP-1's major components, we still lack a detailed molecular knowledge of how these factors interact with DNA to activate or repress genes in the nucleus. It is also not clear how the response of AP-1 to growth factor signaling from the cell surface to the nucleus is interpreted at the molecular level, and whether AP-1 is relevant for human disease (Wagner, 2001).

#### *Transcriptional Activation and Translocation of AP-1*

Like most transcription factors, Fos and Jun proteins are modular; the DNA binding and transcriptional activation domains are physically separable and are subject to control by different signaling pathways. Jun family proteins contain a single activation domain located amino-terminal to the bZIP motif. In the case of c-Jun, the activation domain is regulated to a large degree by the JNK family of MAP kinases (Karin, 1995). The docking site for JNK on c-Jun (the delta domain) is physically separable from the phosphorylation sites, and the substrate binding site on JNK is distinct from the active site (Hibi *et al*, 1993). One interesting feature of this mechanism is that JNK binding to c-Jun may or may not lead to c-Jun phosphorylation.

Fos family proteins contain activation domains both amino-terminal and carboxy-terminal to the bZIP motif, which is centrally located. The Fos C-terminal activation domains are regulated by phosphorylation, although there is not a lot of information about them. Although, the N-terminal activation domains of Fos proteins are required for

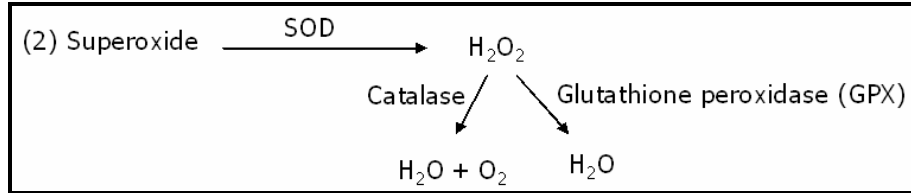
biological activity, there is no evidence that the function of the N-terminal domain is regulated in response to extracellular signaling.

AP-1 translocation to the nucleus, and binding to promoters containing one or more AP-1 binding sites, leads to the transcription of those genes that promote the survival of damaged neuronal cells. Stimulation of AP-1 has been shown previously to activate neurotrophin genes in addition to being activated by them.

### **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 Glutathione Peroxidase (GPX) into water and molecular oxygen, as shown in Figure 4. 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). 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 4 - Biological Function of Superoxide Dismutase (SOD).**

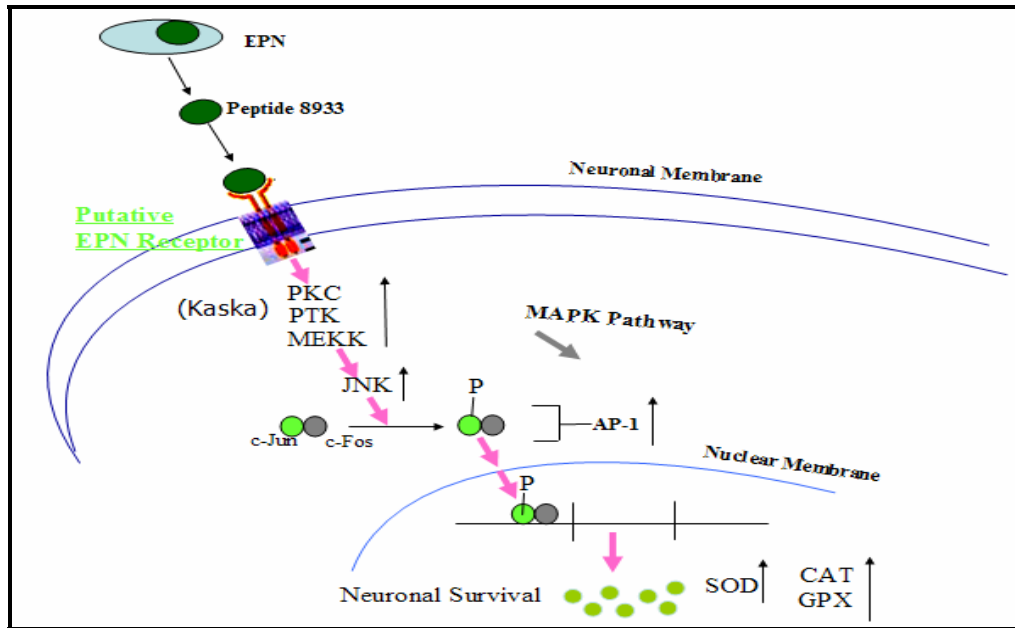
When designing therapeutics to over-express SOD, it is important to determine whether SOD's over-expression might have deleterious effects. It has been shown in the transgenic models that over-expression of SOD by as much as 30-fold in all tissues, not only resists ischemia but the animals remain healthy (Przedborski *et al*, 1992; Chan *et al*, 1993; Chan and Kawase, 1998; Sheng *et al*, 1999b; Li *et al*, 2001). In addition, SOD has been found to have a potential role in deterring the aging process, thus over-expression of SOD in transgenic models shows multiple positive effects (Parkes *et al*, 1998).

### **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. CMX-8933, a synthetic peptide created to mimic a peptide naturally cleaved from EPN, has been shown by our lab 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*, 2003). 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 5). Since all known growth factors and NTFs act via cell surface receptors, EPN may too (Adams *et al*, 2003). 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 5. Activation of protein kinase C (PKC), protein tyrosine kinases (PTKs), and MEK kinase (MEKK) (Hasson, 1998; Kaska, 2003; inhibition experiments), leads to the activation of JNK kinase (Adams *et al*, 2003; substrate assays). JNK phosphorylates the c-Jun component of AP-1 (Adams *et al*, 2003; c-Jun phospho-westerns), inducing AP-1's translocation into the nucleus (Hasson, 1998; Adams *et al*, 2003; nuclear isolations). A functional nuclear AP-1 (El-Khishin, 1999; Adams *et al*, 2003; luciferase reporter assays) 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). Three of the above MAPK components (PKC, PTK, and MEKK) were shown by inhibition experiments to be required for SOD upregulation (Kaska, 2003). A key purpose of this thesis was to use an AP-1 inhibitor to demonstrate that the AP-1 activation is also required for SOD upregulation.



**Figure 5 - Our Lab's Working Hypothesis for the Mechanism of Action of Ependymin.** 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 other therapeutic proteins. This is thought to ultimately lead to an increase of antioxidative enzymes within the cell, a lowering of oxidative stress, and increased neuronal survival.



## THESIS PURPOSE

Our lab previously showed that treatment of mouse neuronal cell lines or rat primary cortical cultures with peptide CMX-8933 activates several components of the MAPK pathway (PKC, PTK, MEKK), increases the phosphorylation of the c-Jun component of transcription factor AP-1, increases nuclear translocation of AP-1, increases the DNA-binding affinity of AP-1, and increases cellular levels of SOD mRNA and protein. Using SOD protein upregulation as an end-point, our lab has recently focused on determining which of the above events is *required* for SOD upregulation. Inhibitors of PKC, PTK, and MEKK showed that those three components are required (Kaska, 2003). The first part of this thesis will test a new chemical inhibitor of AP-1 transactivation, TFPC, to determine whether AP-1 activation is required for SOD upregulation, or is an unrelated event.

The second part of this thesis will test the biological activity of two new peptides homologous to a newly “discovered” human mammalian ependymin-related protein MERP-1, identified using bioinformatics (Apostolopoulos *et al*, 2001). The thesis will test whether treatment of a mouse neuronal cell line with these human peptides induces the translocation of c-Jun and c-Fos (two AP-1 components), and up-regulates mRNAs involved in translation and growth, as expected for any NTF that functions in neuronal growth.

## MATERIALS AND METHODS

### **Murine Neuroblastoma Cell Culture Conditions**

The neuronal cell line grown and maintained for this project was mouse neuroblastoma (Neuro-2a). It was originally established in 1969 by Klebe and Ruddle from a spontaneous brain tumor of a strain-A albino mouse. These cells were purchased frozen from American Type Culture Collection (ATCC) (catalog #CCL-131). This cell line is adherent (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 Invitrogen 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 Neuro-2a cells by rapid (<1 min) agitation in a 37°C water bath. They were then transferred to a vented T-25 culture

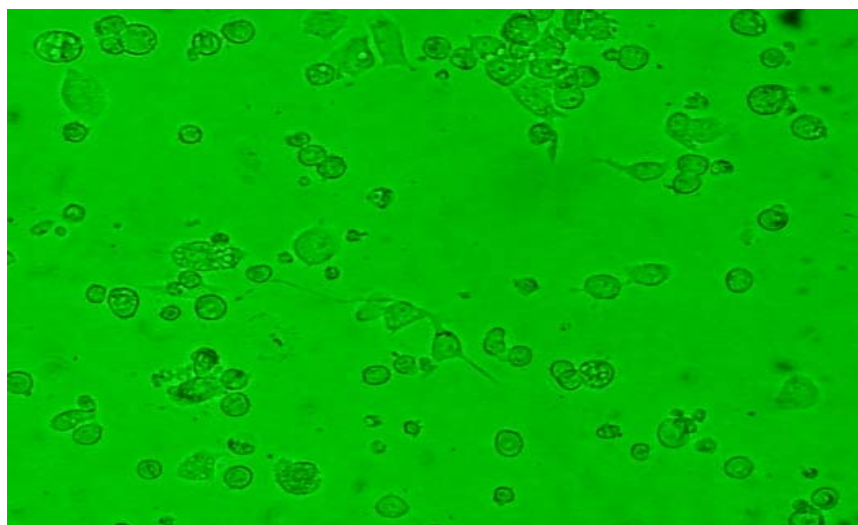
flask diluted with 6 ml of complete medium. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> overnight, and then the toxic freezing medium containing DMSO was replaced with normal complete medium. When the cells reached confluency, the medium was removed, and the 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 minutes, 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- Summary of Various Cell Culture Reagent Volumes**

<b>Flask Type</b>	<b>Amt. Used For Trypsinizing</b>	<b>Amt. Used For Washes</b>	<b>Normal Total ml in Flask During Culture</b>
T-25	2 ml	3 ml	6 ml
T-75	5 ml	10 ml	15 ml
T-150	7 ml	20 ml	25 ml

The normal split ratio specified by 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. At low density, the Neuro-2a cells had a spherical morphology (Figure 6).



**Figure 6 – Neuro-2a Cells.** The neuro 2a cells are about 25  $\mu\text{m}$  in size. Cells were plated at low density and visualized under a 40X objective.

For SOD inhibition experiments, rat primary mixed cortical cultures were provided by Ceremedix, Inc. For AP-1 translocation experiments and RT-PCRs, mouse Neuro-2a cells were used.

#### *CMX-8933 Preparation*

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 powder. The structure was confirmed by amino acid analysis and mass spectroscopy. Mass spectroscopy was also used to verify the absence of trifluoroacetic 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.

### *Human EPN Peptides*

Human peptides hEPN-1 and hEPN-2 were purchased from Abbott Laboratories. hEPN-1 was stabilized in a solution of 1% BSA, and hEPN-2 was stabilized in complete DMEM culture medium.

### *Drug and Inhibitor Culture Treatments*

For inhibition experiments with AP-1 inhibitor TFPC (Trifluoromethyl pyrimidine carboxylate) (Palanki *et al*, 2000), TFPC (Calbiochem) was added directly to the rat primary cortical culture media to make the following final concentrations 0, 0.1, 1, 10 and 100  $\mu$ M, as shown in Table 2 (samples 2 – 6), five minutes prior to the addition of CMX-8933 to 10 ng/ml, then the incubation was continued for 5 hours. The IC<sub>50</sub> of TFPC on neuronal AP-1 is 1.0  $\mu$ M (Palanki *et al*, 2000). Whole cell lysates were then prepared (see below) and SOD activity was analyzed by Western Blots.

**Table 2 - CMX-8933 / TFPC Inhibitor Amounts Used**

<b>Samples</b>	<b>CMX-8933</b>	<b>TFPC Final Conc (<math>\mu</math>M)</b>
<b>1</b>	-	<b>0</b>
<b>2</b>	+	<b>0</b>
<b>3</b>	+	<b>0.1</b>
<b>4</b>	+	<b>1.0*</b>
<b>5</b>	+	<b>10</b>
<b>6</b>	+	<b>100</b>

\* = IC<sub>50</sub> of TFPC on neuronal AP-1.

For AP-1 translocation experiments with human EPN peptides, nuclear extracts were prepared (see below) on seven confluent T-25 flasks. Quiescent (serum-free 12 hr) neuroblastoma cells were treated with hEPN-1 (in BSA) or hEPN-2 (in culture medium) to make final concentrations of 0.1  $\mu$ g/ml, 0.2  $\mu$ g/ml and 1.0  $\mu$ g/ml, for low, medium and high

doses, respectively. The 37°C incubations were continued for 3 hours prior to extraction of nuclei. AP-1 translocation was verified by analyzing the increase in cellular titers of c-Jun and c-Fos nuclear proteins by Western Blots.

For RT-PCR experiments with Human EPN peptides, RNA extractions were done on two confluent T-150 flasks. Quiescent (serum-free 12 hr) neuroblastoma cells were treated with Human EPN 1 peptide mimetic at a final concentration of 1.0 ug/ml and the 37°C incubations continued for 24 hours prior to isolation of total cellular RNA. Increases in mRNA levels were verified by RT-PCR.

### **Whole Cell Extracts**

Whole cell extracts were prepared from *in vitro* plated rat primary mixed cortical 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  $\mu$ l 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 eppendorf tubes on ice. The suspension was incubated on ice for at least 10 minutes to thoroughly lyse the cells.

### *Lysate Clarification*

The pellet was then microcentrifuged at 4°C for 5 minutes to pellet cell debris. The supernatant was put into 0.5 ml eppendorf tubes (about 40  $\mu$ l per tube) and 1  $\mu$ l of the supernatant 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  $\mu$ g/ml), and 0.5 ml of each was added to 1.5ml eppendorf tubes. A blank was prepared with 0.5 ml of  $dH_2O$ . Next, 1  $\mu$ l of the sample plus 0.5 ml of  $dH_2O$  were put into 1.5ml eppendorf tubes. All tubes were then put into a water bath for 10 minutes to ensure that all were at the same temperature. The tubes were then microcentrifuged, and 0.5 ml of Coomassie Reagent (Pierce) was added to all tubes. The tubes were then vortexed to mix. Absorbance was read on a spectrophotometer at OD 595 nm zeroed against the  $dH_2O$  blank.

## **Nuclear Extracts**

Nuclear extracts were prepared from *in vitro* cultured Neuro-2a cells in T-25 flasks, in order to obtain nuclear protein for AP-1 translocation immunoblots.

### *Cell Harvesting*

The flasks were collected, 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-EDTA 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-EDTA was then discarded, and to the cell pellet was added 1 ml 1X PBS and the resuspended solution was transferred to a 1.5 ml eppendorf tube on ice. The eppendorf tubes were then microfuged for about 15 seconds to pellet the cells. Using a pipette tip, discard the wash to get rid of as much of the PBS as possible and place the tubes on ice.

### *Cell Lysis*

Once all the PBS wash was removed, 1 ml of Complete Buffer-A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, and freshly added 0.5 mM DTT and 0.5 mM PMSF) was added to each pellet from one T-25 flask and the pellets were resuspended in this hypotonic buffer and incubated on ice for 15 minutes for thorough swelling of the cells. 50 ul of 10% NP-40 was added to each 1 ml sample (to make 0.5% final concentration) gently mixed by inverting the tubes several times to lyse the cell membranes and not vortexing.



### *Nuclear Extraction*

The tubes were then microfuged for 30 seconds and the supernatant was discarded with a pipette tip to remove as much buffer as possible and samples were placed on ice. 50  $\mu$ l of the Complete Extraction Buffer (20 mM HEPES pH 7.9, 0.45 M NaCl, 1 mM EDTA pH 8.0, and freshly added 0.5 mM DTT and 0.5 mM PMSF) was added to each nuclear pellet from one T-25 cultured flask, resuspended thoroughly with a pipette tip and placed on ice for 10 minutes with intermittent vortexing.

### *Lysate Clarification and Storage*

The nuclear suspension was then microfuged at 4°C for 5 minutes to pellet nuclear debris. The supernatant (containing nuclear proteins) was put into 0.5 ml eppendorf tubes (about 50  $\mu$ l per tube) on ice and equal volume (50  $\mu$ l) of Dilution Buffer (20 mM Hepes pH 7.9, 0.1 M KCl, 0.2 mM EDTA pH 8.0 and 20% Glycerol) was added to lower the salt concentration. The samples were vortexed to mix and 5  $\mu$ l of the aliquot was put into 0.5 ml of dH<sub>2</sub>O for protein determination (1:100) using Coomassie Reagent (Pierce) as described above. The nuclear extract tubes were stored at -80°C.

### **Western Blots (Immunoblots) Analysis**

Western blots were used to analyze the levels of SOD protein in Neuro-2a whole cell lysates in inhibition experiments, and the levels of c-Jun and c-Fos proteins in Neuro-2a nuclear extracts in AP-1 translocation experiments.

### *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<sub>2</sub>O, 6.7 ml of 30% acrylamide (2.7% cross linking), 5.1 ml of resolving gel buffer (1.5 M Tris-HCl, pH 8.0), 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). The solution was poured between the two plates to about 1.5 cm from the top. A thin layer of dH<sub>2</sub>O was overlaid on the top in order 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 H<sub>2</sub>O, 1.67 ml of 30% acrylamide (2.7% crosslinking), 2.5 ml of stacking gel buffer (0.5 M Tris-HCl pH 6.8), 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). 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, about 0.5 cm into the upper stacking gel, making sure no air bubbles were near the comb teeth. 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 to prevent desiccation.

### *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 (5  $\mu$ l loads were used per lane corresponding to 5  $\mu$ g of protein unless otherwise noted), and electrophoresed for about 3 hours at 150 volts so that the dye traversed down  $\frac{3}{4}$  of the gel.

### *Gel Transfer*

After the 3 hours of electrophoresis, the side spacers were removed, and one of the glass plates was carefully removed. 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 (0.45  $\mu$ m, BA-85, VWR Scientific) 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. For c-Jun and c-Fos protein detection, 50 µl of primary c-Jun (rabbit anti-c-Jun polyclonal, Santa Cruz #SC-44, 200 µg/ml) and 50 µl of primary c-Fos (rabbit anti-c-Fos polyclonal, Santa Cruz #SC-52, 200 µg/ml) was added to the 50 ml blocker to make a final concentration of 0.2 µg/ml. The membrane was then left again to mix 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, 2 minutes each time, at a vigorous rate on a gyratory shaker. Next, the membrane was incubated with 50 ml of fresh blocker supplemented with 50 µl of the secondary antibody (goat anti-rabbit-HRP, Pierce #31460,

final concentration: 0.4 µg/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, then once in 1X PBS) for 2 minutes each on the gyratory shaker at vigorous speed.

#### *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 ~30 seconds for SOD, or for ~ 20 minutes for c-Jun and c-Fos proteins. 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).

### **Isolation of Total Cellular RNA From Mouse Neuroblastoma Cells For RT-PCR**

Confluent neuroblastoma cultures were treated with 1.0 µg/ml of hEPN-1 peptide for 24 hours, followed by RNA isolation. Total cellular RNA was isolated from the neuroblastoma cultures using the Clontech Atlas Pure Total RNA Labeling System (# K1038-1). Table 3 summarizes the two neuronal cell samples tested in this thesis, and their corresponding RNA yields.

**Table 3 - Mouse Neuroblastoma Cell Sample Size and Corresponding RNA Yields**

<b>Date</b>	<b>Sample</b>	<b>Flasks</b>	<b>RNA Yield (µg)</b>	<b>Conc. µg/µl</b>
03/29/2004	Ctrl	One T-150	100.8	2.5
03/29/2004	hEPN-1	One T-150	75.6	1.9

#### *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 2ml sterile eppendorf tubes. Typically 2ml eppendorf tubes were used for experiments using cell counts ranging from 10<sup>6</sup> to 10<sup>7</sup> total cells. The tubes were then microcentrifuged for 30 seconds at 4°C 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 eppendorf 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 minutes, and then re-vortexed. The tubes were microcentrifuged at 12,000 rpm for 5 minutes at 4°C in order to pellet cellular debris. Following centrifugation, the supernatant (a volume of approximately 0.5 ml) was removed and transferred to new sterile 2ml 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 4°C 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.

### *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 minutes, and then centrifuged at 12,000 rpm for 15 minutes at 4°C to pellet the RNA. Once centrifuged, the supernatant was carefully removed and discarded, without disrupting the RNA pellet.

### *Pellet Wash*

Then 0.25 ml of 70-100% ethanol was added to the pellet, the tube was vortexed, then centrifuged at 12,000 rpm for 5 minutes at 4°C. 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<sub>2</sub>O water was added, producing an RNA concentration of 1-2 µg/µl. The tube was then heated at 50°C 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<sub>2</sub>O prior to UV spectrophotometry. The rest of the RNA was aliquoted into several eppendorf tubes (about 10 µl per tube) to avoid repeated freeze/thaw which degrades nucleic acids. The samples were stored at -80°C for later use.



## **Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)**

To test whether the mouse Neuro-2a RNA samples expressed genes of particular interest, RT-PCR was performed using a modified version of Amersham's Two-Step RT-PCR with Ready-To-Go RT-PCR beads (#27-9266-01) in 0.5ml eppendorf tubes. Each tube contained one bead containing lyophilized and stabilized buffer, dNTPs, Taq, and reverse transcriptase. All primers used were purchased from Fisher Sigma Genosys, and were designed for the mouse sequences using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

### *Reverse Transcription of the RNA*

The following items were added to each 0.5 ml ready-to-go eppendorf tube containing one bead: 44  $\mu$ l dH<sub>2</sub>O (flip the tube to dissolve the bead, do not vortex which can denature the enzymes), 2  $\mu$ l of 50  $\mu$ M antisense primer, and 2  $\mu$ l of 1  $\mu$ g/ $\mu$ l RNA template. This was gently mixed, spun briefly in a microcentrifuge at room temperature, and then incubated in the thermal cycler at 42°C for 30 minutes to elongate, followed by 95°C for 5 minutes 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 reaction tubes from the reverse transcription step (already containing the antisense primer) were spun briefly in a microcentrifuge at room temperature and 2  $\mu$ l of 50  $\mu$ M PCR sense primer were added to make a 50  $\mu$ l reaction. The samples were gently mixed, spun briefly, and then placed in the thermal cycler for the following program: 95°C

for 4 min initial denaturation, 32 cycles of 95°C for 30 seconds, annealing for 30 seconds (usually at 55°C unless otherwise stated), and elongation for 72°C for 40 seconds. Finally a polishing step was performed at 72°C for 5 minutes. The samples were then stored at 4°C overnight in the thermal cycler, or used immediately.

#### *Analysis of RT-PCR Reactions*

1 µl of 10x DNA loading dyes was mixed with 10 µl of the PCR reaction, and 10 µl of this 11 µl mixture was analyzed by electrophoresis on a 2.5% agarose gel in 1X TAE buffer, containing about 0.5 µg/ml of ethidium bromide. Since the TAE-based loading dye contained TAE as its buffer, the same TAE buffer was used to make the gel and electrode buffer. Gels were visualized under UV light.

## RESULTS

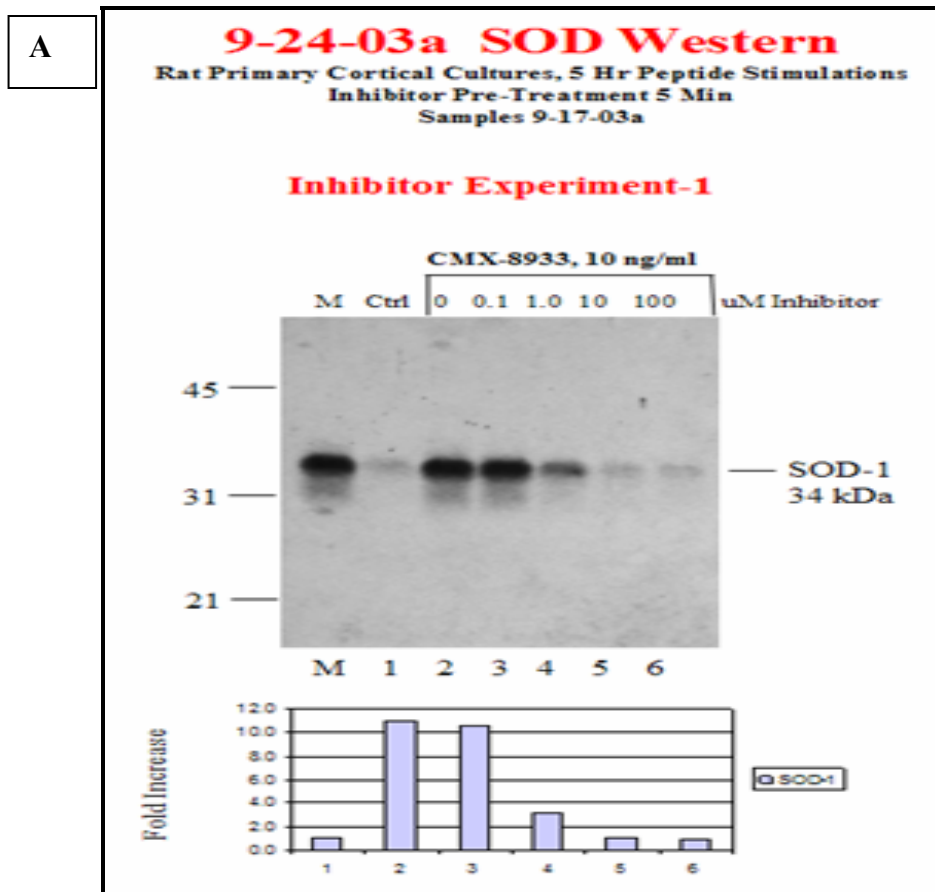
The goals of this thesis project were to extend our knowledge of the mechanism of action of goldfish EPN mimetic CMX-8933, to use bioinformatic approaches to identify candidates for human EPN, and to test human EPN mimetics for biological activity.

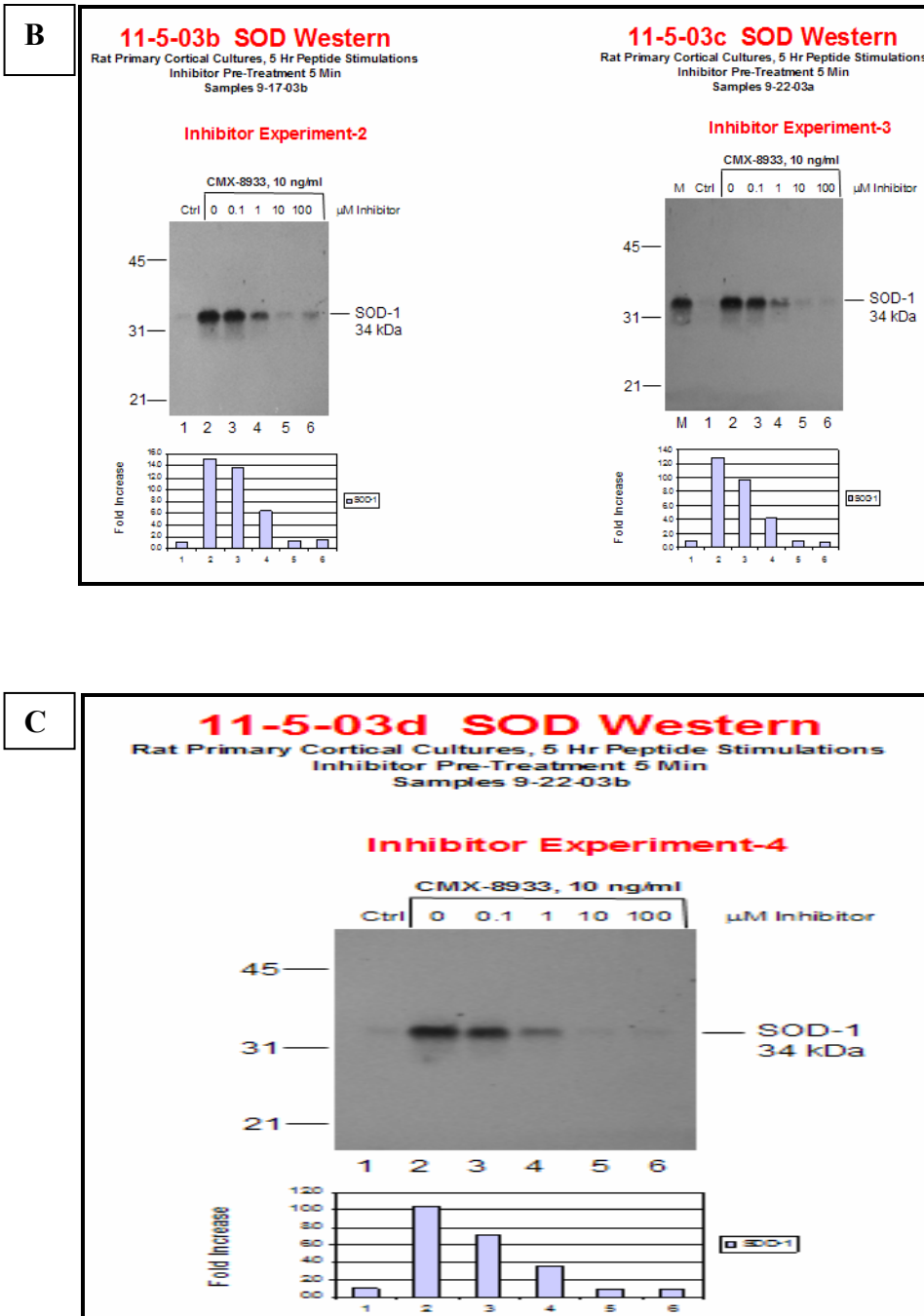
### **CMX-8933-Induced Upregulation of SOD Depends On AP-1 Activation in Rat Primary Cortical Cultured Cells**

The first part of the thesis investigated whether transcription factor AP-1 is required for CMX-8933's upregulation of SOD, or whether its activation is merely concurrent. Our lab previously showed that CMX-8933 activates JNK kinase, phosphorylates the c-Jun component of AP-1, increases AP-1 translocation to the nucleus, increases AP-1's DNA-binding affinity, and increases the transactivation of an AP-1-dependent promoter (Adams *et al*, 2001). We also showed that CMX-8933 upregulates SOD (Parikh, 2003) and that this upregulation depends on the activation of three kinases in the MAPK / AP-1 pathway (PKC, PTK, MEKK) (Kaska, 2003) in rat primary cortical cells.

Inhibition experiments were performed using trifluoromethyl pyrimidine carboxylate (TFPC), a specific AP-1 inhibitor with an  $IC_{50}$  of 1.0  $\mu$ M in neuronal cultures (Palanki *et al*, 2000). Four independent SOD Western experiments were performed (Figure 7) using whole cell lysates prepared from *in vitro* cultured rat primary cortical cells pre-treated for 5 minutes with TFPC inhibitor before a 5 hour treatment with 10 ng/ml CMX-8933. SOD protein levels were compared to a control with no treatment of peptide (lane "ctrl", low SOD levels) and to cultures treated with 8933 but no inhibitor (lane "0", high

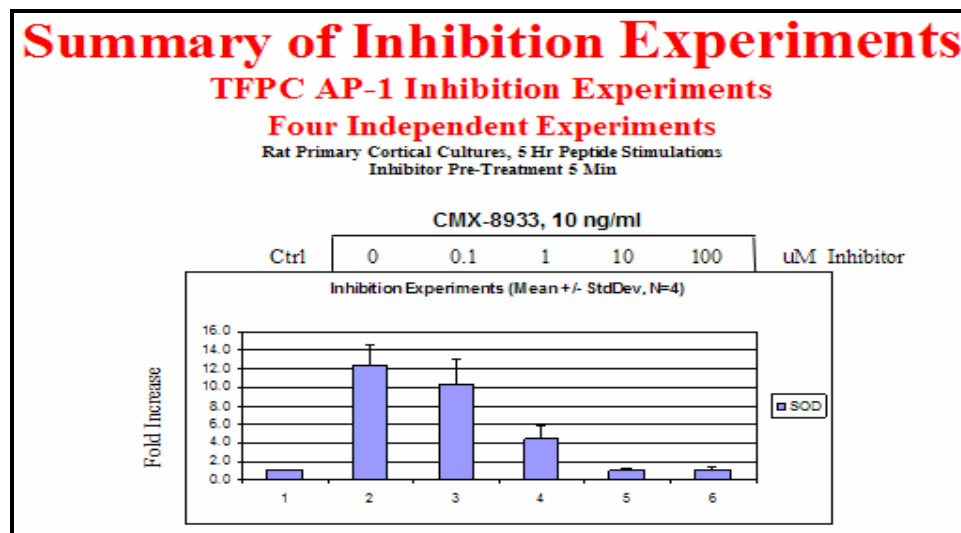
SOD levels). SOD bands were quantified using Scionimage software, shown as histobars below each panel (fold increase relative to the untreated sample). As expected (Parikh, 2003), the 10 ng/ml CMX-8933 treatment with no inhibitor (lane “0”) showed a dark SOD band at the expected size of SOD protein (34 kDa) that migrated just above the 31 kDa marker band. Increasing concentrations of TFPC inhibitor (0.1, 1.0, 10, 100  $\mu$ M) produced less SOD activation, in a concentration-dependent manner. The results of the three additional independent experiments (panels B (experiments 2 & 3) and C (experiment 4)), show similar trends as panel A (experiment 1).





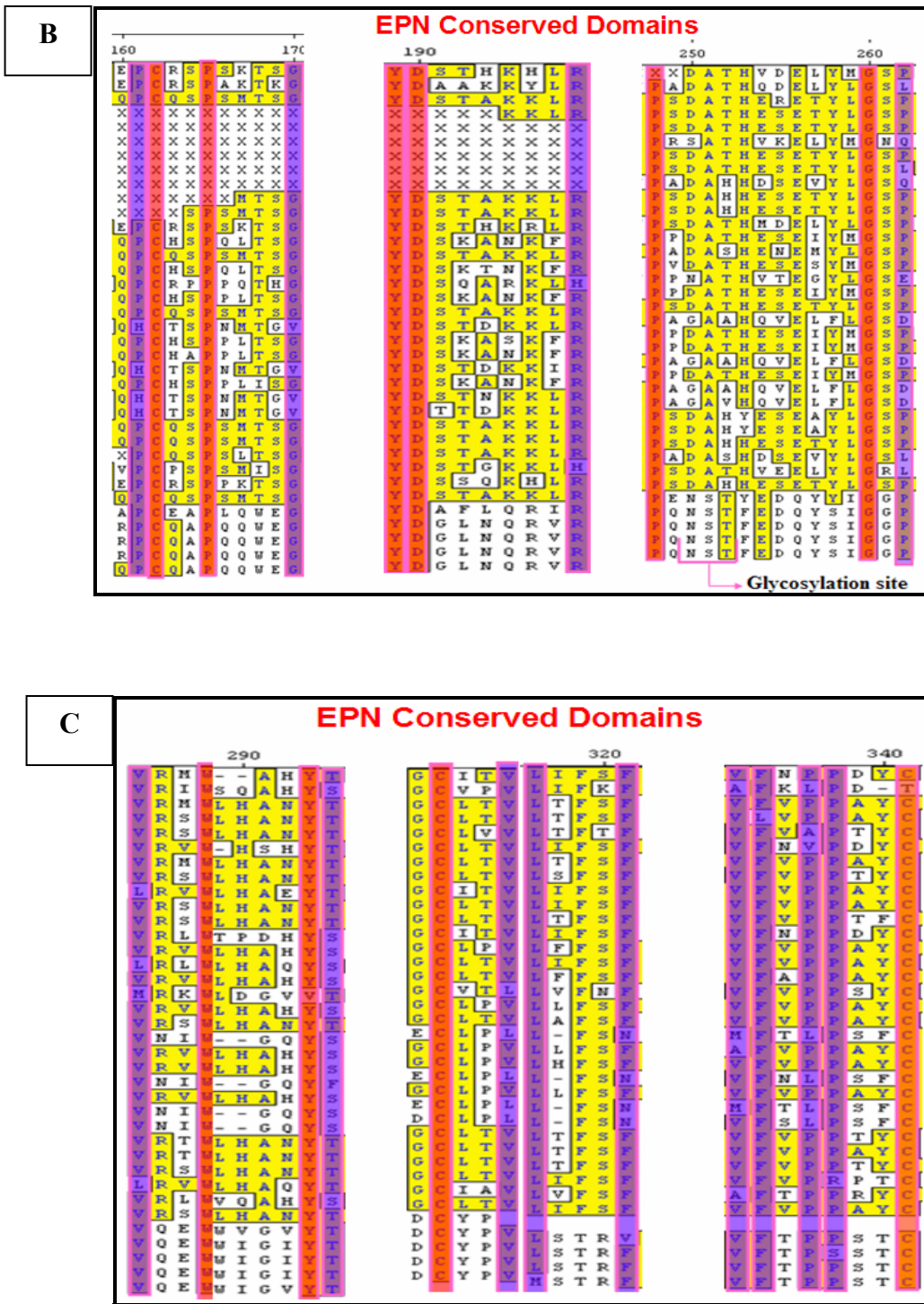
**Figure 7 - TFPC Inhibitor of AP-1 Inhibits CMX-8933-Induced Increases in SOD Levels in Rat Primary Cortical Cells.** Rat primary cortical cultures were pre-incubated with different concentrations of TFPC inhibitor (see Methods) for 5 minutes, then 10 ng/ml of CMX-8933 was added for a period of 5 hours. Panel A represents Experiment 1, while Panels B and C represent three additional experiments. 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 4 for the *in vitro* inhibition experiment are illustrated in Figure 8, with error bars representing standard deviation. P values were calculated by Microsoft excel using a two-tailed T-test. 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 ( $p = 0.06$ ) occurred between lanes “0” and “0.1  $\mu\text{M}$ ”, indicating a low concentration of inhibitor had no significant effect on SOD levels. Statistically significant differences occurred at TFPC concentrations of 1.0  $\mu\text{M}$  ( $p = 0.0004$ ), 10.0  $\mu\text{M}$  ( $p = 0.001$ ), and 100.0  $\mu\text{M}$  ( $p = 0.001$ ) relative to the uninhibited CMX-8933-stimulated lane “0”. The statistically significant 67% reduction in SOD signal at the 1.0  $\mu\text{M}$  TFPC concentration occurred at the published  $\text{IC}_{50}$  for cultured neuronal cells where the inhibitor retains full specificity for AP-1. No significant difference ( $p = 0.81$ ) occurred between lanes “10.0  $\mu\text{M}$ ” and “100.0  $\mu\text{M}$ ”, indicating the inhibitor’s effect was maximal at those concentrations. The data clearly demonstrate the requirement for AP-1 in CMX-8933’s activation of SOD.



**Figure 8 - Statistical Analysis of Four *in Vitro* SOD Inhibition Experiments.** Histograms denote the means of four independent experiments. Error bars denote standard deviation calculated in Excel. P values were calculated using a student’s two-tailed T-test in Excel. Histograms 2 and 3 did not show significant difference ( $p = 0.06$ ), whereas histograms 4, 5 and 6 showed significant differences in p values ( $p = 0.0004$ ,  $p = 0.001$  and  $p = 0.001$ , respectively) compared to the uninhibited CMX-8933-stimulated lane “0” (histogram 1).

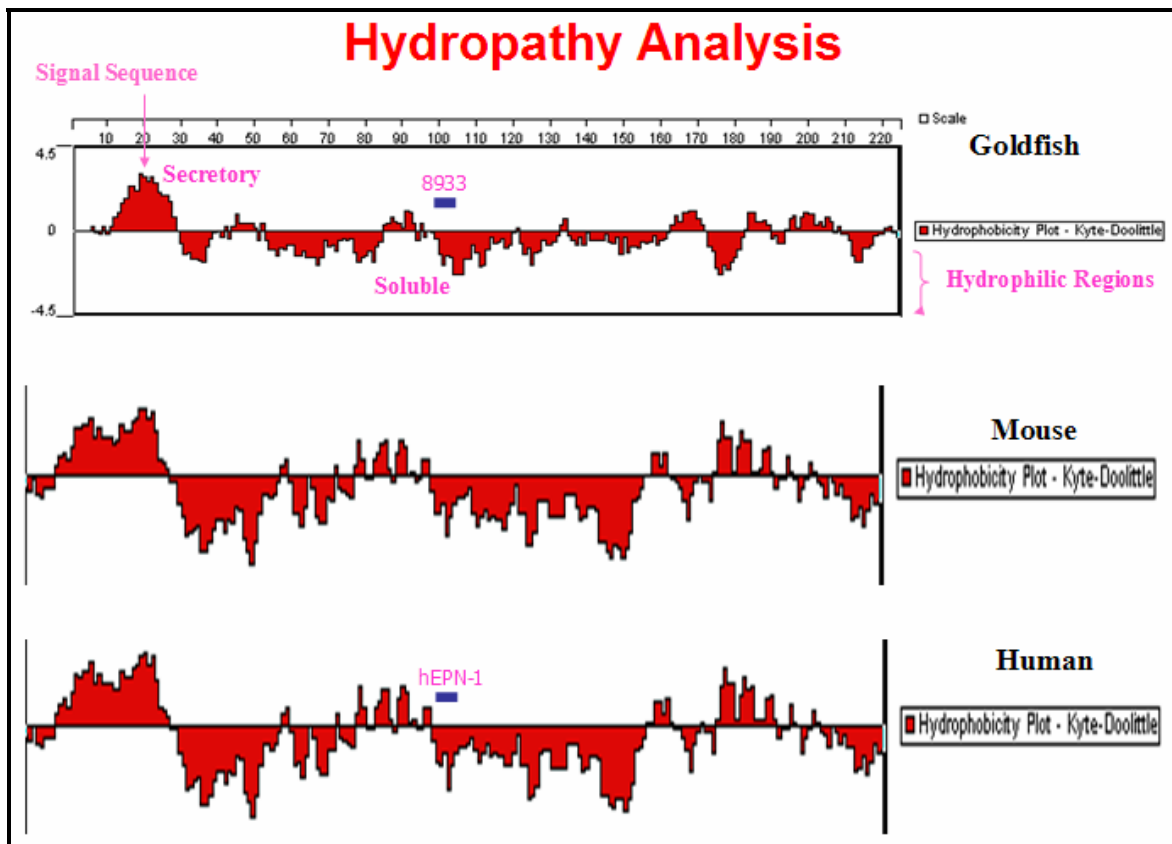




**Figure 9 – Verification of Previous Bioinformatics Alignment Studies.** Program MegAlign was used to align the EPN protein sequences of 31 species deposited in Genbank. Colors indicate areas of amino acid identity (yellow), completely conserved domains (red), and strongly conserved domains (purple). Portions of the full-length sequence alignments are shown in panels A, B and C. Panel A also shows 7 of 8 aa of goldfish mimetic CMX-8933 (red, underlined) and two human EPN mimetics, hEPN-1 (7 of 8 aa, red underlined) and hEPN-2 (13 of 14 aa, blue box). Panel B indicates one of the two potential mammalian glycosylation sites.



The analysis also included hydropathy plots which showed an amazing conservation in hydropathy profiles between human and goldfish EPN as shown in Figure 10.



**Figure 10 – Hydropathy Analysis of Goldfish, Mouse and Human EPN.** Kyte and Doolittle hydropathic profile comparison between goldfish and MERPs (mouse and human) ependymins. The vertical scale represents the hydropathic score for each amino acid, while the horizontal scale shows the amino acid position in goldfish. The heavy horizontal line in each profile represents an amino acid “score” of zero, with hydrophobic regions shown above the line, and hydrophilic regions shown below. The hydrophobic signal sequence and hydrophilic regions of goldfish EPN are denoted by pink arrows.

### **Human EPN Peptide Mimetics hEPN-1 and hEPN-2 Induce AP-1 Translocation in mouse neuroblastoma cells.**

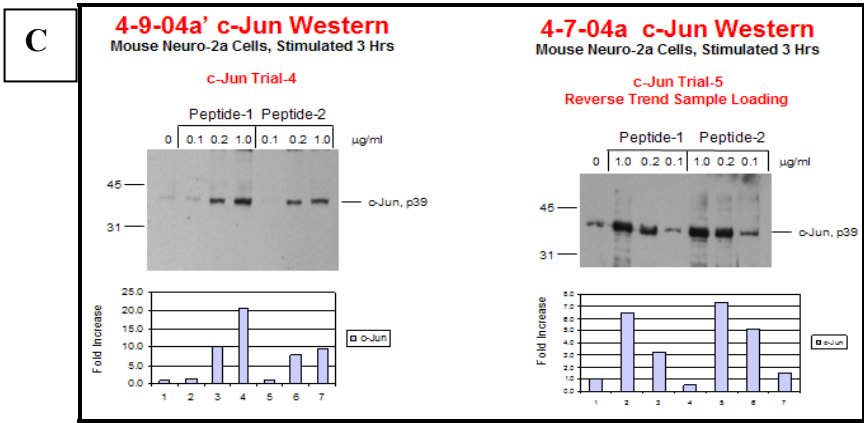
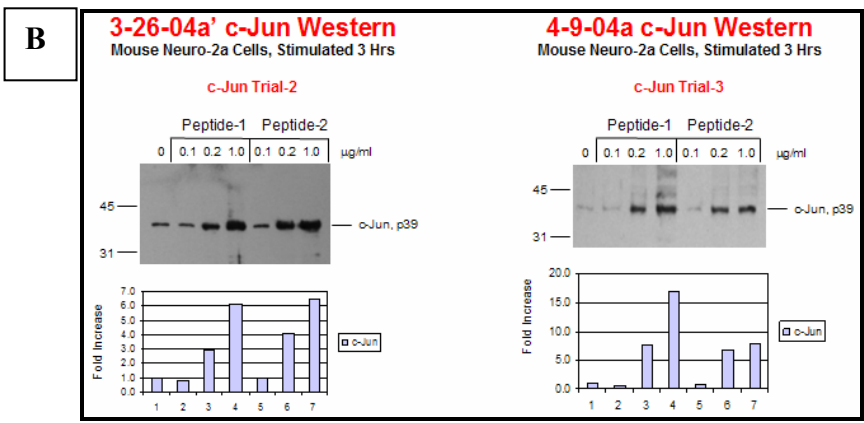
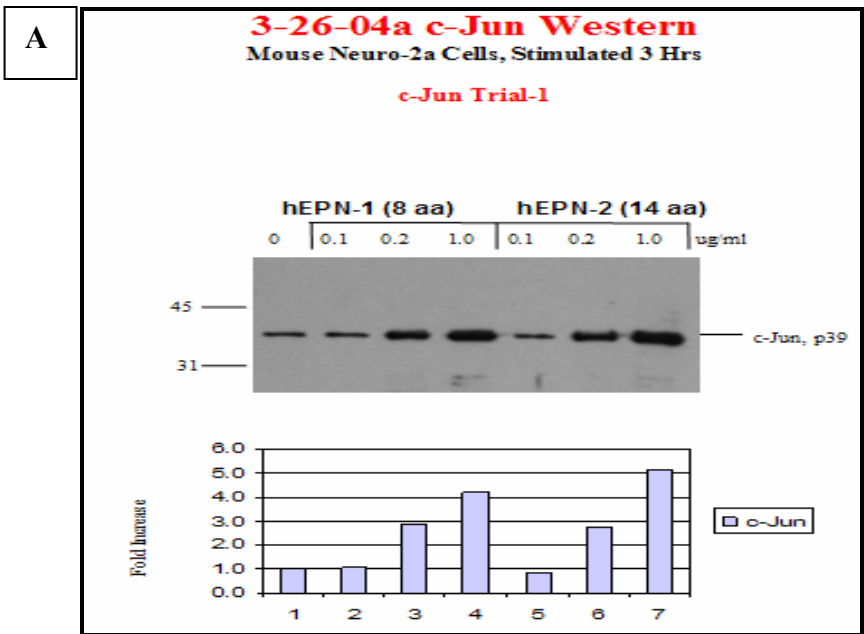
To test for potential biological activity of human EPN, we designed two human EPN peptide mimetics, hEPN-1 and h-EPN-2, based on our bioinformatic alignments. The former represents the same domain as goldfish CMX-8933 (strongly conserved in most fish

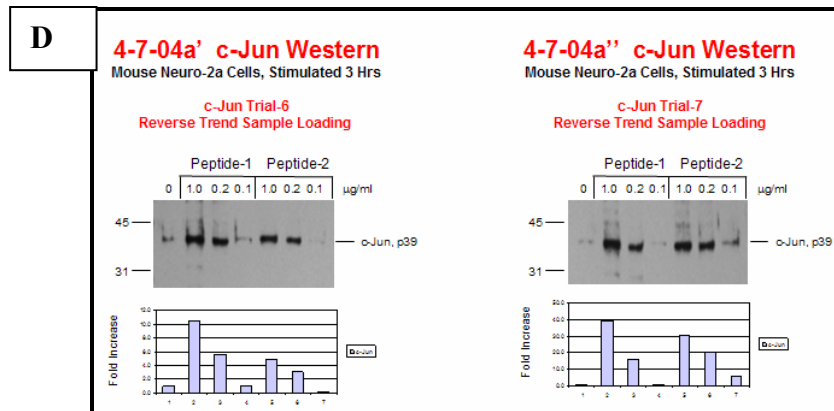
species, and with 3 residues perfectly conserved in all known EPN candidates), while hEPN-2 represents CMX-8933 plus 6 upstream aa (to include a perfectly conserved cysteine residue).

As an assay to test “biological activity” we chose AP-1 translocation, since AP-1 activation is clearly induced by goldfish CMX-8933, and translocation is a relatively easy to perform non-radioactive assay. Seven independent c-Jun, and five independent c-Fos, Western experiments were performed (Figures 11 and 13) using nuclear extracts prepared from *in vitro* cultured mouse neuroblastoma cells treated for 3 hours with various doses (0.1, 0.2, and 1.0 µg/ml) of hEPN-1 or hEPN-2.

#### **a) c-Jun Translocation**

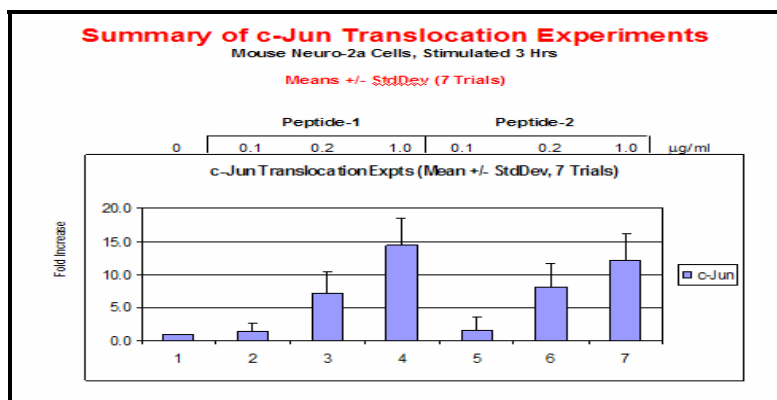
c-Jun nuclear protein levels were compared to a control with no peptide treatment (Figure 11, lane “ctrl”) to determine the effectiveness of the treatment. The bands were quantified using Scionimage software, and the histograms shown below each panel indicate the fold increase relative to untreated sample. Both peptides induce a strong dose-dependent increase in the nuclear levels of a c-Jun protein that migrates at the expected 39 kDa size. These results were reproducible, the results of six additional independent experiments (Panels B, C and D) correlate nicely with experiment 1 (Panel A).





**Figure 11 - Human EPN Mimetics hEPN-1 and hEPN-2 Increase c-Jun Translocation in Mouse Neuro-2a Neuroblastoma Cells in a Dose-Dependant Manner.** Mouse Neuro-2a cells were treated for 3 hours with various doses (listed) of hEPN-1 or hEPN-2, and nuclear extracts were analyzed by c-Jun immunoblot. Panel A represents trial-1. Panels B, C and D show the results of six additional experiments. Arrows on the right denote the positions of 39 kDa c-Jun protein. Arrows on the left denote the positions of biotinylated markers. Histograms represent the signal strength quantitated using Scion image software.

The means of c-Jun experiments 1 through 7 are shown in figure 12. The figure shows that both peptides at the highest concentration tested (1.0  $\mu\text{g/ml}$ ) cause a statistically significant ( $p = 0.0001$ ) average 12-15-fold increase in c-Jun levels over the untreated controls. There was little or no significant difference ( $p = 0.205$ ) between the untreated samples and the low dose samples for both peptides. The medium dose (0.2  $\mu\text{g/ml}$ ) produced a significant difference ( $p = 0.001$ ) compared to untreated samples. This data demonstrates that human EPN mimetics hEPN-1 and hEPN-2 increase nuclear levels of c-Jun.

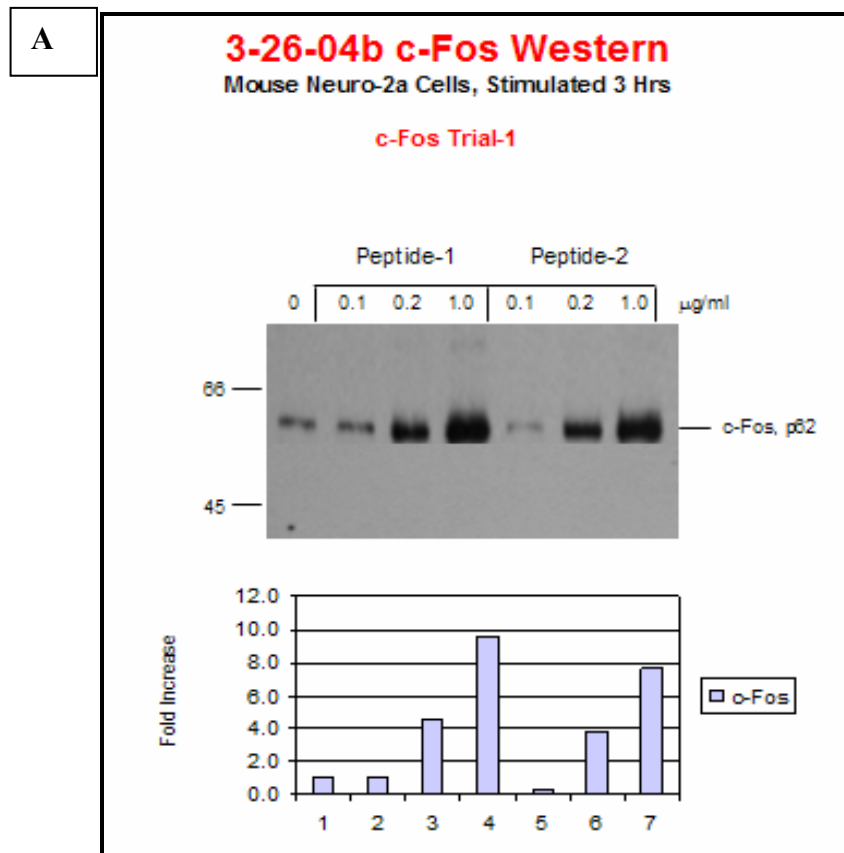


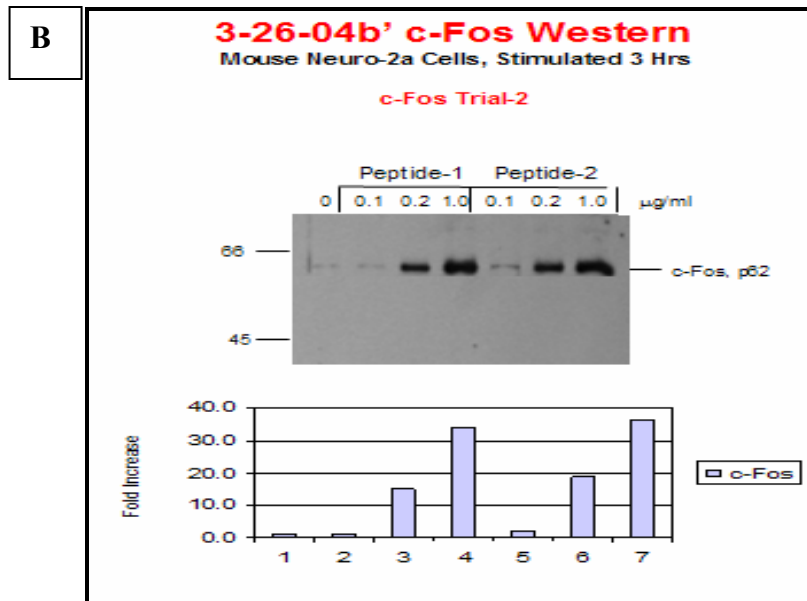
**Figure 12 - Statistical Analysis of Seven c-Jun Translocation Experiments.** This data summarizes the 7 experiments shown in figure 11. Histograms represent the means, and error bars denote standard deviations

calculated in Excel. P values were calculated using a student's two-tailed T-test in Excel. There was a significant difference between untreated versus medium dose samples (histobars 1 versus 3; and 1 versus 6) ( $p = 0.001$ ), and between the untreated versus high dose samples (histobars 1 versus 4; and 1 versus 7) ( $p = 0.0001$ ).

### b) c-Fos Translocation

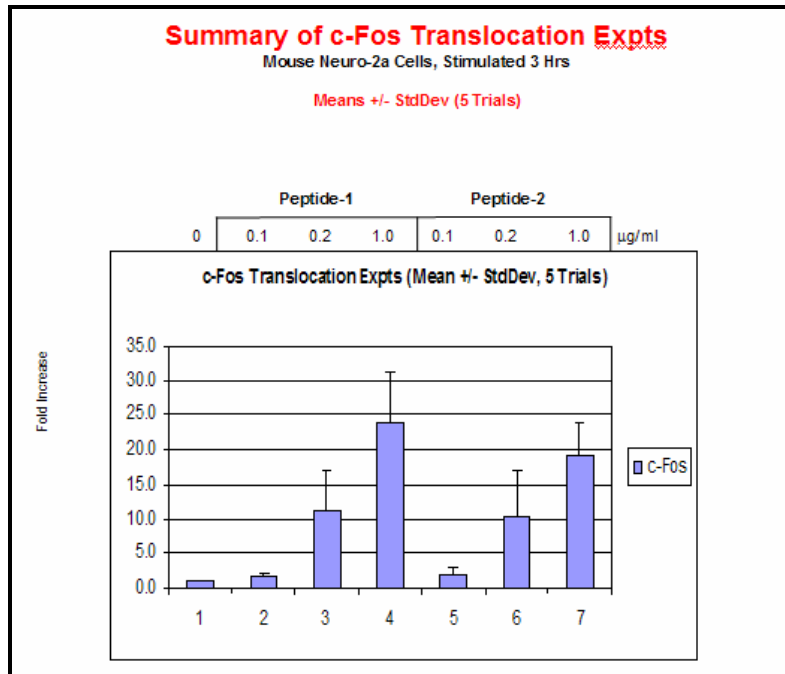
Since the AP-1 particle activated by CMX-8933 includes c-Fos in addition to c-Jun (Adams *et al*, 2003), the ability of hEPN-1 or hEPN-2 to translocate c-Fos was also tested (Figure 13). This data (experiment-1 in panel A, experiment-2 in panel-B) was similar to that previously described for c-Jun. Three other experiments were also done (not shown). The c-Fos protein migrated at the expected size (62 kDa).





**Figure 13 - Human EPN Mimetics hEPN-1 and hEPN-2 Increase c-Fos Translocation in Mouse Neuro-2a Neuroblastoma Cells in a Dose-Dependant Manner c-Fos Translocation Experiments.** Panel A represents experiment-1, while panel B shows experiment-2. Peptide stimulations were performed for 3 hours. Three additional sets of the same experiment were also performed (data not shown). Arrows on the right denote the positions of 62 kDa c-Fos protein. Arrows on the left denote the positions of biotinylated markers. Histograms represent the signal strength quantitated using Scion image.

The means of the five c-Fos experiments are shown in figure 14. Both peptides at the highest concentration tested (1.0 µg/ml) cause a statistically significant ( $p = 0.002$ ) average increase of 20-25-fold in c-Fos levels over the untreated controls. Little or no significant difference ( $p = 0.09$ ) was seen between the untreated samples and low dose (0.1 µg/ml) samples with either peptide. A substantial significant difference ( $p = 0.01$ ) in c-Fos levels was found between untreated versus medium dose samples.



**Figure 14 - Statistical Analysis of Five c-Fos Translocation Experiments.** Histograms denote the means of 5 independent experiments. Error bars denote standard deviations calculated in Excel. P values were calculated using a student's two-tailed T-test in Excel. There was a significant difference ( $p = 0.012$ ) between untreated and medium dose samples (histobars 1 versus 3; and 1 versus 6), and ( $p = 0.002$ ) between the untreated and high dose samples (histobars 1 versus 4; and 1 versus 7).

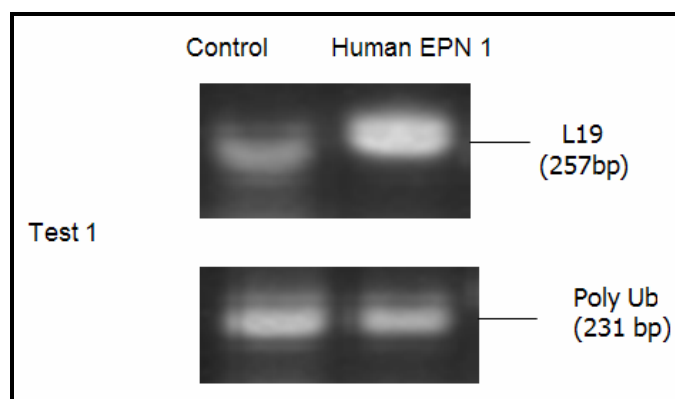
### hEPN-1 Upregulates mRNAs Related To Growth And Translation

The third part of this thesis was designed to determine whether treatment of mouse Neuro-2a neuroblastoma cultures with hEPN-1 increases the cellular titers of mRNAs related to growth and translation. We chose these areas to assay since previous data in our lab indicated treatment of rat primary cortical cultures with CMX-8933 upregulated mRNAs related to these functions (Kaska, 2003). In addition, a previous hybridization array performed in our lab (Parikh, 2003) indicated mRNAs for several ribosomal proteins increased in rat primary cortical cells treated with CMX-8933 (Table 4).

**Table 4. Hybridization Array Summary Table for Ribosomal Proteins and EF-2 (Parikh, 2003).** Bold text indicates candidate primers chosen for investigation in this thesis. +++ denotes a strong signal on the array, ++ is moderate, and + denotes a weak signal.

ARRAY COORDINATE	GENBANK ACCESSION	GENE/PROTEIN	CLASSIFICATION	ARRAY SIGNAL	
				CTRL	8933
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	+	++
<b>C11m</b>	<b>J02650</b>	<b>Ribosomal Protein L19</b>	<b>Ribosomal Protein</b>	++	+++
<b>C12d</b>	<b>K03502</b>	<b>Elongation Factor-2</b>	<b>Translation Factor</b>	++	+++

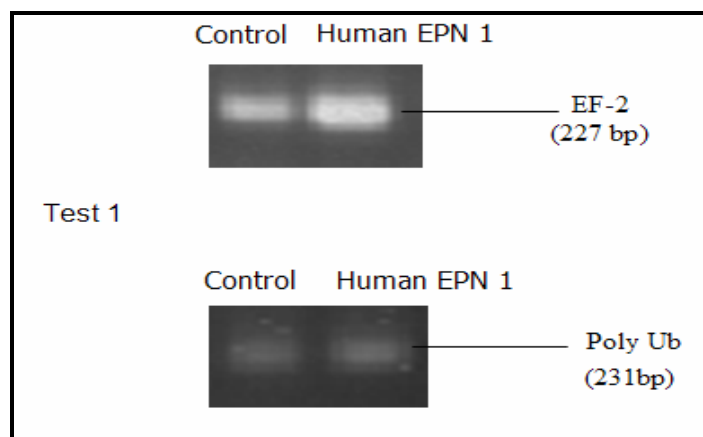
Reverse transcriptase-polymerase chain reaction (RT-PCR) was used to assay levels of ribosomal protein L19 (Figure 15), translational elongation factor-2 (EF-2) (Figure 16), and energy-related ATP Synthase-C (Figure 17). Each assay was performed only once, so no positive conclusions can be drawn from the data, however the findings from each RT-PCR experiment support our hypothesis that EPNs play a role in growth. In all experiments, the levels of housekeeper Polyubiquitin (amplicon size 231 bp) were equal between treated and untreated samples, while the levels of L19 mRNA (amplicon size 257 bp), EF-2 mRNA (amplicon size 227 bp), and ATP Synthase-C mRNA (amplicon size 227 bp) increased in hEPN-1-treated samples.



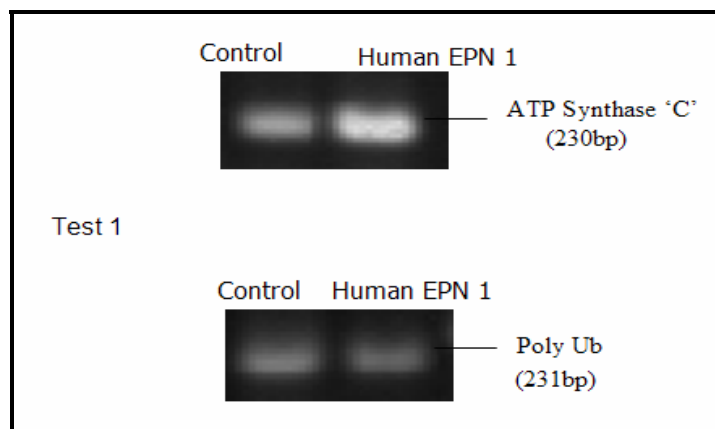
**Figure 15 - RT-PCR Analysis of L19 mRNA Levels in Mouse Neuroblastoma Cells Treated with hEPN-1.** Mouse Neuro-2a cells were treated for 24 hrs with 1  $\mu$ g/ml hEPN-1, then RT-PCR was performed on total cellular RNA. Polyubiquitin (amplicon size 231 bp, lower panel) was used as a housekeeper. The L19 signal



(amplicon size 257 bp, upper panel) was stronger in the hEPN-1-treated sample. This data correlates with the preliminary results obtained from the hybridization array.



**Figure 16 - RT-PCR Analysis of EF-2 mRNA Levels in Mouse Neuroblastoma Cells Treated with hEPN-1.** Mouse Neuro-2a cells were treated for 24 hrs with 1  $\mu\text{g/ml}$  hEPN-1, then RT-PCR was performed on total cellular RNA. Polyubiquitin (amplicon size 231 bp, lower panel) was used as a housekeeper. The EF-2 signal (amplicon size 227 bp, upper panel) was stronger in the hEPN-1-treated sample. This data correlates with the preliminary results obtained from the hybridization array.



**Figure 17 - RT-PCR Analysis of ATP Synthase-C mRNA Levels in Mouse Neuroblastoma Cells Treated with hEPN-1.** Mouse Neuro-2a cells were treated for 24 hrs with 1  $\mu\text{g/ml}$  hEPN-1, then RT-PCR was performed on total cellular RNA. Polyubiquitin (amplicon size 231 bp, lower panel) was used as a housekeeper. The ATP Synthase signal (amplicon size 230 bp, upper panel) was stronger in the hEPN-1-treated sample. This data correlates with the preliminary results obtained from the hybridization array.

## DISCUSSION

Transcription factor AP-1 was one of the first mammalian transcription factors identified (Angel and Karin, 1991), is activated in response to an incredible array of different stimuli (Wisdom, 1999), and is a central component of many signal transduction pathways. The term AP-1 actually refers to a family of related transcription factors whose members consist of dimers of the Jun and Fos family of proteins. Approximately 20 different AP-1 versions have been characterized so far that explain, in part, AP-1's diverse biological activities. The specific AP-1 upregulated by CMX-8933 consists of a c-Jun/c-Fos heterodimer (Adams *et al*, 2003). Full-length neurotrophic factors (NTFs), such as Nerve Growth Factor, Neurotrophin-3, and Brain-Derived Neurotrophic Factor promote the activation of AP-1 (Ip *et al*, 1993; Tong and Perez-Polo, 1996; Gaiddon *et al*, 1996), and in a positive feedback loop, AP-1 promotes their activation. Most importantly, AP-1 has been shown to function as a master-switch in short- to long-term synaptic plasticity, and long-term memory consolidation (Sanyal *et al*, 2002). That finding, plus ours, showing that EPN activates AP-1, may indicate that AP-1 is a key component of EPN's mechanism of action during its function in long-term neural plasticity (Shashoua and Moore, 1978; Schmidt, 1987; Piront and Schmidt, 1988; Schmidt *et al*, 1995).

Our laboratory studies EPN mimetic peptides whose sequences represent a small portion of full-length EPN. Treatment of rat primary cortical cultures with mimetic CMX-8933, an 8 aa peptide based on a middle portion of goldfish EPN, was previously shown to upregulate therapeutic enzyme superoxide dismutase (SOD) (Parikh, 2003). The first part of this thesis tested whether our previously observed CMX-8933-induced SOD

upregulation depends on AP-1 activation. Previous inhibition experiments in our lab (Kaska, 2003) showed that the SOD upregulation depends on three key components of the MAPK pathway (PKC, PTK, and MEKK), so we hypothesized that AP-1 activation known to be downstream from these components may also be required for SOD upregulation. In order to test this we chose TFPC (Trifluoromethyl Pyrimidine Carboxylate), a specific AP-1 inhibitor with a 50% inhibition concentration ( $IC_{50}$ ) of 1.0  $\mu$ M in neuronal cells (Palanki *et al*, 2000). Although the complete mechanism of action of TFPC remains unknown, cells treated with this poison do not transactivate AP-1-dependent genes. The inhibition experiments in Figure 7 illustrate that inhibition of AP-1 with 1  $\mu$ M ( $IC_{50}$  concentration) TFPC significantly decreased ( $p = 0.0004$ ) CMX-8933's induced SOD signal by 67%. Thus, CMX-8933-induced upregulation of SOD indeed depends on the activation of AP-1. In the future, these SOD activation pathway experiments could be extended utilizing specific kinase/substrate assays to determine which specific components of the PKC, PTK and MEKK families are involved in SOD upregulation.

Since our lab's 1994 publication of the first EPN sequence in goldfish (Adams and Shashoua, 1994), approximately 30 EPN-related genes (and their deduced proteins) have been identified by bioinformatic approaches. Initially, the bioinformatic approach was applied to 25 fish species (Orti and Meyer, 1996), but was eventually used to identify mammalian EPN-related proteins (MERPs) (Apostolopoulos *et al*, 2001). Recently, the human MERP-1 sequence was completed, and its expression in hematopoietic progenitors, brain, cardiomyocytes and muscle was reported (Gregorio-King *et al*, 2002). Expression of an EPN (usually associated with neuronal re-growth) in heart cells was initially quite surprising given cardiomyocyte's well known lack of growth in culture, but recent findings

demonstrate the expression of nerve growth factor (NGF), one of the best characterized NTFs, and its p75 receptor, in myocardium (Ieda *et al*, 2004; Zhou *et al*, 2004).

The second part of this thesis re-verified the bioinformatic alignments (Figures 9 and 10) discussed above to convince ourselves that human EPN might indeed exist, then based on those alignments, two human EPN mimetics were designed to test for biological activity. My alignments (Figure 9) did indeed find the same conserved cysteine domains (among others) as previous studies, indicating that primary sequence is not well conserved in this EPN family of proteins, but perhaps shape or key aa residues are. Hydrophathy comparisons of goldfish EPN versus human MERP-1 were strikingly similar (Figure 10). Two human EPN mimetics were designed, hEPN-1 (8 aa long, whose sequence domain is analogous to goldfish CMX-8933), and hEPN-2 (14 aa long, that extends 6 aa upstream of hEPN-1 to include a totally conserved cysteine residue). To test their potential biological activities, we chose an AP-1 translocation assay since it is relatively easy to perform, and is non-radioactive. The results (figures 11 through 14) indicate that treatment of mouse Neuro-2a cells with various doses (0.1, 0.2, and 1.0  $\mu\text{g/ml}$ ) of hEPN-1 or hEPN-2 induces a statistically significant dose-dependent translocation of c-Jun and c-Fos proteins to the nucleus. The c-Jun increases at the highest doses tested were 12-15-fold ( $p = 0.0001$  hEPN-1;  $p = 0.0004$  hEPN-2). The c-Fos increases at the highest doses tested were 20-25-fold ( $p = 0.002$  hEPN-1;  $p = 0.001$  hEPN-2). No significant differences occurred between untreated versus low dose samples (c-Jun:  $p = 0.205$  hEPN-1,  $0.467$  hEPN-2; c-Fos:  $p = 0.09$  hEPN-1,  $p = 0.161$  hEPN-2). Also no significant differences were observed between hEPN-1 versus hEPN-2 peptides at any dose tested (for example at a 1  $\mu\text{g/ml}$  dose comparison,  $p = 0.422$  c-Jun,  $p = 0.189$  c-Fos) indicating that both mimetics were equally

active, and that the extra 6 aa length of hEPN-2 does not result in greater activity. So the simplest hypothesis is that CMX-8933 and hEPN-1 contain a key receptor-binding element, and extra amino acids add nothing more. Thus, these two human EPN peptide mimetics stimulate AP-1 translocation in mouse neuroblastoma cells, a finding that represents the first reported biological activity for human EPN.

Future human EPN experiments could include the use of immunoprecipitation experiments to determine whether the translocated c-Jun and c-Fos proteins are linked together in one particle, as is the case for the AP-1 activated by CMX-8933 (Adams *et al*, 2003). Additionally, other human mimetics could be designed based on other regions of the proposed human EPN sequence, DNA hybridization arrays could be used to facilitate the identification of activated genes, or transcription factor arrays could be used to identify transcription factors activated in addition to AP-1.

Previously, it was shown that CMX-8933 upregulates mRNAs related to growth and translation in rat primary cortical cells (Kaska, 2003). The last portion of this thesis used RT-PCR to determine whether hEPN-1 activated similar mRNAs. Mouse Neuro-2a cells were treated for 24 hours with 1 µg/ml hEPN-1 (the highest dose tested in the AP-1 translocation experiments), then RT-PCR was performed on total cellular RNA template for the following candidates: ribosomal protein L19, translational EF-2, and ATP Synthase-C. Figures 15-17 show preliminary results (one trial each), indicating that each of these candidates is upregulated in response to hEPN-1 relative to housekeeper polyubiquitin. So hEPN-1 mimetic does indeed appear to upregulate mRNAs related to growth in mouse Neuro-2a cells.

Future RT-PCR experiments could include verifying these interesting preliminary findings, expanding the candidate list to include other growth-related mRNAs (such as L-11 and S-19, which gave moderately increased signals on a hybridization array), or using inhibition experiments to test whether the activation of these growth-related genes requires AP-1 or the MAPK pathway. Perhaps a profile of growth-related genes could be created for hEPN-1, and compared to the profile induced by another NTF like NGF, to test the uniqueness of EPN as a NTF.

The study presented here gives us a deeper understanding of the mechanism of action of EPN. The existence of mammalian MERPs opens up vast new research prospects. The expression of human MERP-1 in brain, heart, and muscle (Gregorio-King *et al*, 2002), and NGF in heart (Ieda *et al*, 2004; Zhou *et al*, 2004), indicates NTFs have broader roles outside the central nervous system, perhaps related to growth or regeneration of these tissues. Experiments will need to be performed to extend this understanding, including experiments to isolate and characterize the specific cell surface receptor(s), and investigating NTF signal transduction pathways in heart and muscle.

In conclusion, the data presented in this thesis demonstrates that 1) the CMX-8933-induced upregulation of SOD depends on AP-1 in rat cortical cultures, 2) a human EPN candidate exists (as identified by bioinformatics), 3) human EPN peptide mimetics hEPN-1 and hEPN-2 increase the nuclear titers of c-Jun and c-Fos proteins, and 4) the mimetics may increase cellular levels of mRNAs related to growth (L19, EF-2, and ATP Synthase-C). Human EPNs or their peptide mimetics may potentially be used for future treatments of neuronal (or even muscle or cardiac) degenerative diseases.

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