Ependymin Peptide Mimetics That Assuage Ischemic Damage Increase Gene Expression of the Anti-Oxidative Enzyme SOD

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ABSTRACT

Ependymin (EPN) is a goldfish brain neurotrophic factor (NTF) previously shown to function in a variety of cellular events related to long-term memory formation and neuronal regeneration. Because of these functions, EPN and other NTFs have potential applications for treating neuro-degenerative conditions, including stroke. In previous experiments, our lab in collaboration with Victor Shashoua of Ceremedix Inc (Boston, MA), designed short synthetic peptide CMX-8933 (a proteolytic cleavage product of EPN) and CMX-9236 (an EPN-Calmodulin combination peptide) that mimic the action of full-length EPN. In a rat stroke model, administration of these peptides *i.v.* significantly lowered brain ischemic volume (Shashoua *et al*., 2003). Because oxidative stress is one of the primary mediators of cell damage following a stroke, we hypothesized that NTFs, and in particular our therapeutic peptides, may act in part by reducing neuronal oxidative stress. Thus, the purpose of this thesis was to determine whether CMX-8933 and CMX-9236 increase the cellular titers of anti-oxidative enzymes.

A hybridization array was used as a "hypothesis generator" to obtain candidates for further analysis. This approach applied to rat primary brain cortical cells treated with CMX-8933 identified superoxide dismutase (SOD) as strongly upregulated. SOD immunoblots on whole cell lysates, and RT-PCR on total cellular RNA, were used to confirm this observation. In time-course and dose-response experiments, treatment of rat primary cortical cultures with either peptide showed an optimal 8.5 fold ($N = 5$, $p < 0.001$) increase in SOD protein, while administration of CMX-8933 to murine neuroblastoma cells caused a 6.5 fold ($N = 3$, $p = 0.001$) increase in SOD mRNA levels.

Previous work in other laboratories indicated that systemic (*i.v.*) administration of full-length NTFs allows only an inefficient delivery across the blood brain barrier (BBB).

We hypothesized that our short synthetic peptides may cross the BBB more efficiently. Immunoblot analysis of brains and hearts excised from mice treated *i.v.* with various doses of CMX-8933 confirmed the elevated SOD titers (10 fold in brain, and 8 fold in heart, at a 6 mg/kg dose for 5 hr; $N = 5$, $p < 0.001$). Furthermore, we hypothesized that conjugation of CMX-8933 to BBB carrier DHA, a natural neuronal membrane fatty acid shown previously to enhance the delivery of dopamine to the brain (Shashoua and Hesse, 1996), might further enhance the NTF therapy. Delivery of DHA-8933 increased SOD expression by 3 fold ($N = 4$, $p < 0.001$) relative to non-conjugated CMX-8933.

Recently, the use of special incubators that allow the culture of cells under low oxygen conditions (anoxia) has been used as an *in vitro* model for stroke. When we tested our peptides in this new *in vitro* model, surprisingly SOD was upregulated 3 fold ($N = 3$, $p = 0.003$) in rat primary cortical cells cultured for 24 hr under oxygen deprivation, compared to normoxic conditions. This implies that these rat cultures may have an endogenous cellular system for responding to oxygen stress, a finding worthy of further investigation. Treatment of anoxic cells with CMX-8933 increased SOD levels another 2.8 fold ($N = 3$, $p < 0.001$) compared to the levels for anoxia alone (for a total of 8.5 fold relative to normoxic cells). Altogether, the data from this thesis illustrate that small NTF EPN peptide mimetics increase the cellular titers of the mRNA and protein for the anti-oxidative enzyme SOD, which may be an important step in their known therapeutic benefits.

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BACKGROUND

Neurotrophic Factors

Neurotrophic factors (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 system (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.

Role of NTFs in Stroke Therapy

NTFs help assuage the destructive neuronal oxidative stress associated with stroke. Apoptotic-like cells predominate for several days after an ischemic insult in the internal region of the borders of the infarct, the so-called penumbra zone (Linnik *et al*., 1995). Several NTFs like nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), Neurotrophin-3 etc. have been found to localize in the penumbra following a stroke (Ferrer *et al*., 1998) presumably to aid neuronal recovery. Because of this, some NTFs have been tested as stroke therapeutics. Intracerebrally delivered BDNF is effective in reducing infarction volume, hippocampal cell damage and death, and cortical neuron death (Ferrer *et al*., 2001). BDNF therapy also prevents superoxide anion-induced death in cells expressing the Trk B Tyrosine kinase receptor, a receptor that mediates neurotrophin signaling (Yamagata *et al*., 1999). Studies show that GDNF alleviates oxidative stress in neurons by blocking nitric oxide, and/or scavenging free radicals that are produced from ischemia (Ikeda *et al*., 2000).

Ependymin

Ependymin (EPN) is a goldfish brain glycoprotein found predominantly in the brain extracellular fluid (ECF) and cerebrospinal fluid (CSF) in glycosylated and non glycosylated forms. Discovered in 1976 by Victor Shashoua (formerly of Harvard Medical School, McLean's Hospital, currently of CereMedix Inc.), EPN was identified as a protein whose turnover rate was enhanced in goldfish brains after various training events. Isolation and localization studies indicate that EPNs are synthesized in the *zona ependyma* of the goldfish brain (Shashoua and Benowitz, 1977) and in the meninges of goldfish and zebrafish brains (Hoffmann *et al*., 1990).

Ependymin Functions as a Neurotrophic Factor

Although the precise molecular mechanism by which the brain uses EPN remains unknown, there are certain functional aspects of EPN that closely resemble the properties of NTFs. Surprisingly addition of goldfish EPN to murine 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). So although EPN was originally discovered in goldfish, it may have mammalian counterparts.

Three behavioral experiments (swimming with a float, avoidance conditioning, and classical conditioning) in the goldfish indicate that EPN has a role in the synaptic changes associated with the consolidation process of long-term memory formation and the activity-dependant phase of the sharpening of goldfish retinotectal connections during neuronal regeneration (Shashoua, 1991). When given anti-EPN antisera prior to an optic nerve crush, retinotectal projection was blocked in goldfish (Schmidt and Shashoua,

1988). Similarly, an antiserum to EPN when injected into the brains of trained goldfish caused amnesia of a newly acquired behavior (Shashoua and Moore, 1978). A multi-functional protein, EPN was recently found to increase goldfish acclimation to cold temperatures (Tang *et al*., 1999). EPN also has a characteristic calcium-binding affinity comparable to the ubiquitous calcium-chelator Calmodulin (Schmidt and Makiola, 1991), which perhaps allows EPN to be used for stroke therapy helping to chelate calcium before it floods into a damaged cell. In addition, EPN is expected to show the signal transduction-induced changes in gene expression associated with all NTFs.

Our Lab's Contribution Towards EPN

The Adams lab, in collaboration with CereMedix, Inc. (Boston), has contributed significantly towards EPN research. The earliest work involved the discovery, cloning and characterization of genes encoding EPN in goldfish and carp (Adams and Shashoua, 1994), and in giant danio and golden shiner (Adams *et al*., 1996). Research using NTF-EPN peptide mimetics 8933 and 9236 (discussed below) began in 1998, when a Master's student (Hasson, 1998) showed that these therapy peptides stimulate the transcription factor activator protein (AP-1), a signaling molecule that turns on neuronal growth genes. This AP-1 activated by 8933 and 9236 was later proved to be functional in neuroblastoma NB2a cells using transfection experiments (El-Khishin, 1999). In the same year mouse C-127 cells were engineered to secrete peptides CMX-8933 or 9236 into the culture medium in an attempt to develop a cost effective cell culture-based *in vitro* production system for these peptides (Sleeper, 1999). In 2001, therapy peptide CMX-8933, a proteolytic cleavage product of EPN, was shown to activate AP-1 in mouse

neuroblastoma and rat cortical cultures (Adams *et al*., 2001), thus extending and combining the earlier student projects. Further investigations showed that 8933 activates AP-1 using the MAPK pathway (Adams *et al*., 2003). The activation of AP-1 by EPN mimetic 8933 appears to be significant since AP-1 was recently shown to be a master switch that functions upstream of all other transcription factors to control long term memory formation and synaptic plasticity (Sanyal *et al*., 2002) which are two processes that also describe EPN's function. The most recent work in our lab involves an analysis of the neuroprotective effects of the therapy peptide CMX-9236 in *in vitro* and *in vivo* models of cerebral ischemia (Shashoua *et al*., 2003), and this work is discussed below.

EPN Peptide Therapy

Because full-length NTFs cross the BBB inefficiently, even when coupled to a BBB-carrier, CereMedix, Inc. designed two short NTF-EPN peptide mimetics CMX-8933 (MW 1049 Da) and CMX-9236 (MW 1477 Da) as potential therapies. These short specific peptide sequences were selected in preference to the complete EPN molecule to avoid any possible contamination with NGF, a normal component of the ECF of goldfish from which EPN is isolated (Adams *et al*., 2001).

CMX-8933 is an 8 amino acid synthetic peptide whose sequence is directly derived from a charged region of EPN with a conserved sequence of "KKETLQFR" (fig. 1, red letters). The two N-terminal lysine residues (KK) were included to enhance the peptide's water solubility (Adams *et al*., 2003). This sequence is completely conserved among all the protein sequences derived from true-EPN genes sequenced to date from the order Cypriniformes, the only order in which functional EPN studies have been performed (Adams and Shashoua, 1994; Adams *et al*., 1996; Adams *et al*., 2003). CMX-

8933 activates AP-1 in a manner similar to the full-sized NTFs, NGF and BDNF, suggesting a parallel mechanism in neuronal protection (Adams *et al*., 2001). ECF proteins from goldfish brains contain proteases and the peptide equivalent of synthetic 8933 (Shashoua and Holmquist, 1986). The 8933 sequence is also flanked by known protease cleavage sites (Adams *et al*., 1996). Thus peptide CMX-8933 is believed to represent a proteolytic cleavage product of EPN in the ECF (Adams *et al*., 2003) and hence is likely to be biologically relevant.

Goldfish EPN sequence

```
  +   +                          +   +     +           +            +-  -   -    -  +    + +  - -
MHTVKLLCVVFS-CLCAVAWAS--SH---R-QPCHAPPLTSGTMKVVST------GGHDLESGEFSYDSKANKFRFVED
   +   +   +   -    + - -      - -   + -   + + -     + + +   -    -    + -  -           -     +  +   +
TAHANKTSHM-DVLIHFEEGVLYEIDSKNESCKKETLQFRKHLMEIPPDATHESEIYMGSPSITEQGLRVRVGKFP
 -  + +                        + - + + -   +         -   -  - -              -    - -   - -  +       -    + -
ELHAHYSMSTTSCGCLPVSGSYHGEKKD-LHFSFFGVETEVDD-LQVFVPPAYCEGFEEAPDDHS—FFDLFH-D      215aa
```

Fig 1. Rationale for the Design of Therapy Peptide CMX-8933. The completely conserved 8 amino acid sequence of CMX-8933, represented in red, is a proteolytic cleavage product of a 215 aa long Goldfish EPN, present in ECF. Charged amino acids are indicated by '+' or '-' above the sequence (Adams *et al*., 1995; Adams *et al*., 2003).

CMX-9236 is a 14 amino acid synthetic peptide derived in part from the sequence of EPN and the sequence of one of the calcium binding domains of calmodulin with a consensus sequence of "KKDGDGDFAIDAPE" (fig 2; Shashoua *et al*., 2003). The peptide was designed as a single molecule with properties that combined an affinity for calcium along with a capacity to activate AP-1 and thus promote synaptic plasticity. Its N-terminal end is usually coupled to a fatty acid, *cis*-docosahexanoic acid (DHA) to allow penetration across the blood-brain barrier (discussed in detail below).

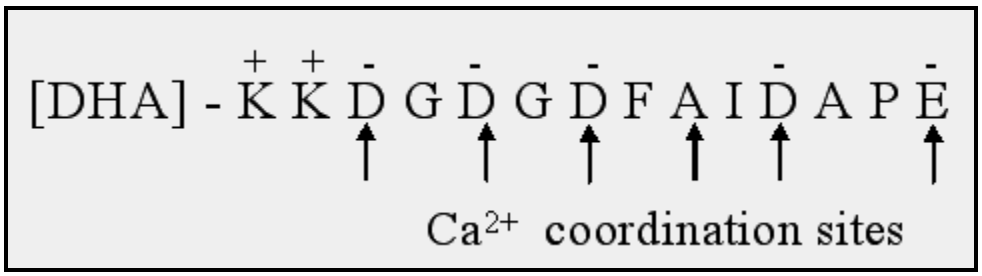


Fig 2. Structure of Peptide CMX-9236. It contains 6 calcium-binding sites, indicated by the arrows that are considered to be involved in calcium chelation (Shashoua et al., 2003).

In neurotrophic activity studies, both EPN peptides CMX-9236 and CMX-8933 were found to prevent the death of rat primary hippocampal neurons treated with excitotoxic levels (2 mM) of glutamate (figs 3 and 4; Shashoua, unpublished).

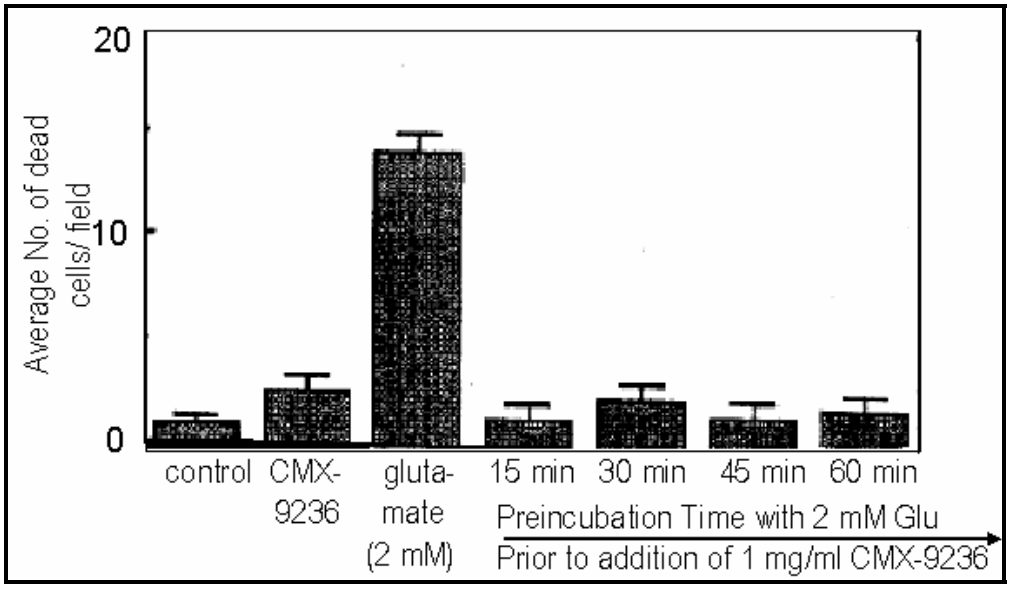


Fig 3. CMX-9236 Blocks Cell Death from Glutamate Excitotoxicity. Rat cortical cultures preincubated with excitotoxic levels of glutamate for 60 min showed a 5 fold increase in the number of dead cells/field relative to the same cultures post-treated with CMX-9236 for 15, 30, 45, or 60 min. Stain used was propidium iodide, which is an indicator of dead cells (Adapted from Shashoua, unpublished).

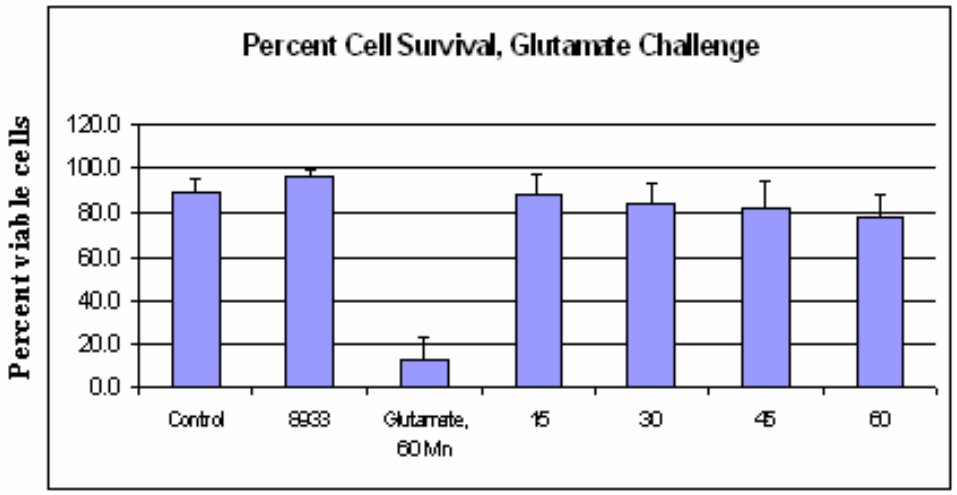


Fig 4. Effects of CMX-8933 on Neuronal Cell Survival *in vitro.* Legend is as described for fig. 3, except Y-axis shows percent viable cells (Shashoua, unpublished).

Increased accumulation of intracellular calcium following an ischemic insult initiates a cascade of biochemical changes that ultimately leads to cell necrosis and apoptosis. A fluoroprobe assay demonstrated the efficacy of CMX-9236 in restoring the increased intracellular calcium resulting from kainate (an excitotoxic glutamate analog) excitotoxicity back to normal (fig. 5, Shashoua *et al*., 2003).

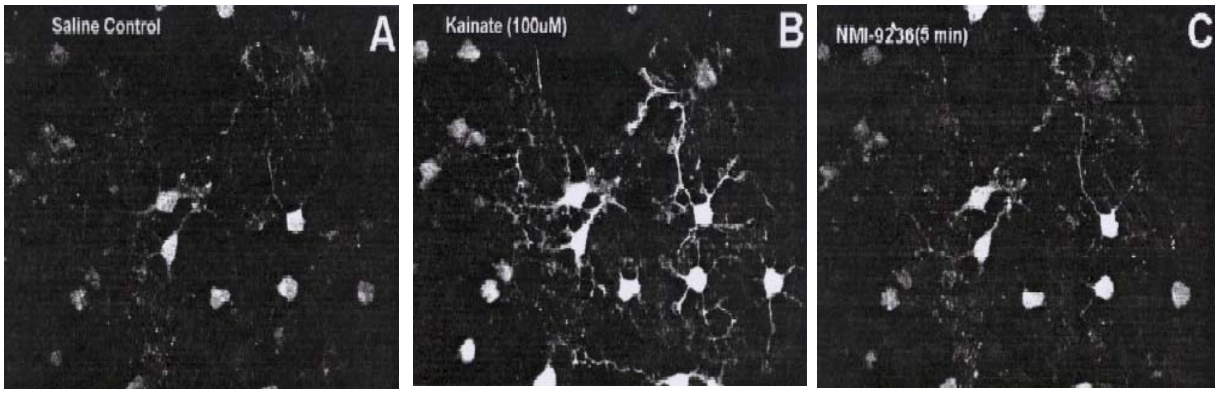


Fig 5. Calcium Chelating Capacity of CMX-9236 after Treatment with Excitotoxic Levels of Kainic Acid. Rat primary cortical cultures were preloaded with Fluo-3, a dye that fluoresces on binding to calcium. Panel A is the saline control. Panel B shows the treatment with 100 µM excitotoxic kainate indicating intense fluorescence due to increased intracellular calcium. Panel C represents the continuous infusion of 50 ng/ml of CMX-9236 to kainate treated cultures resulting in decreased fluorescence to about pre-stimulation stage within 5 minutes (Shashoua *et al.,* 2003).

In perhaps the most significant *in vivo* studies performed to date by the Adams and Shashoua labs, CMX-9236 and CMX-8933, in a rat stroke model, induced a dramatic reduction of infarct size following a 24 hour major occlusion of the middle cerebral artery (figs. 6 and 7; Shashoua *et al*., 2003; Shashoua, unpublished).

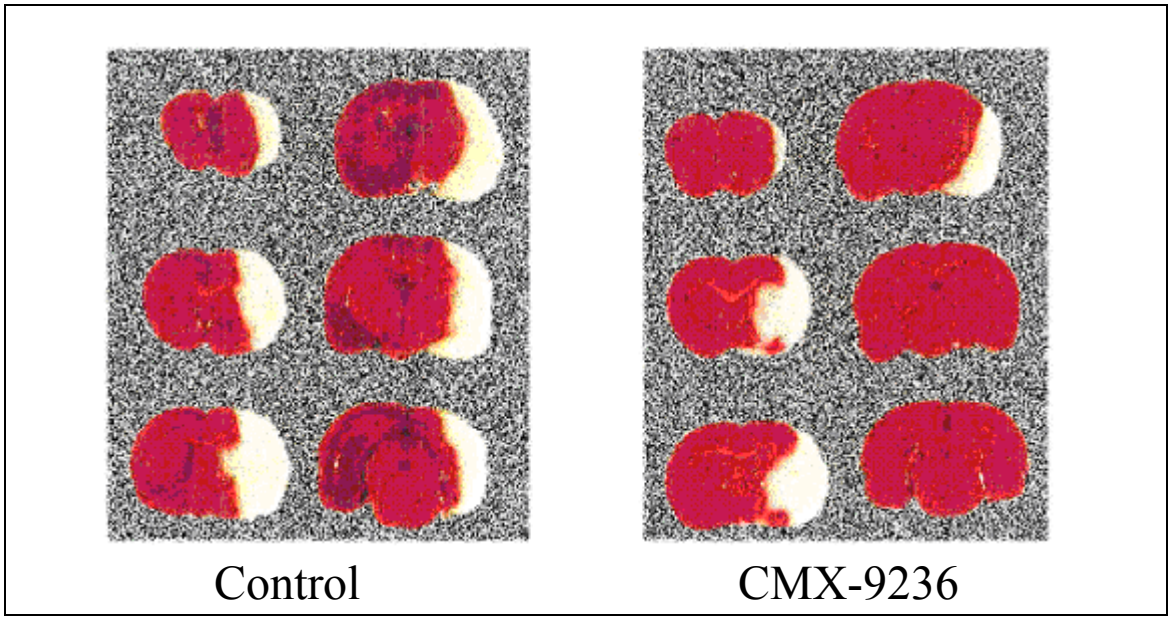


Fig 6. CMX-9236 Decreases the Size of Brain Infarct in a Rat Model for Stroke. The figure shows serial coronal brain sections (rostral to caudal) dissected from permanent MCA occlusion rat stroke models. Surviving tissue stains red with TPT (2, 3, 5-Triphenyltetrazolium) dye, while the dead cells remain white. The CMX-9236 treated brain (right) shows a substantial reduction in infarct size compared to the control brain (left) (Shashoua *et al*., 2003).

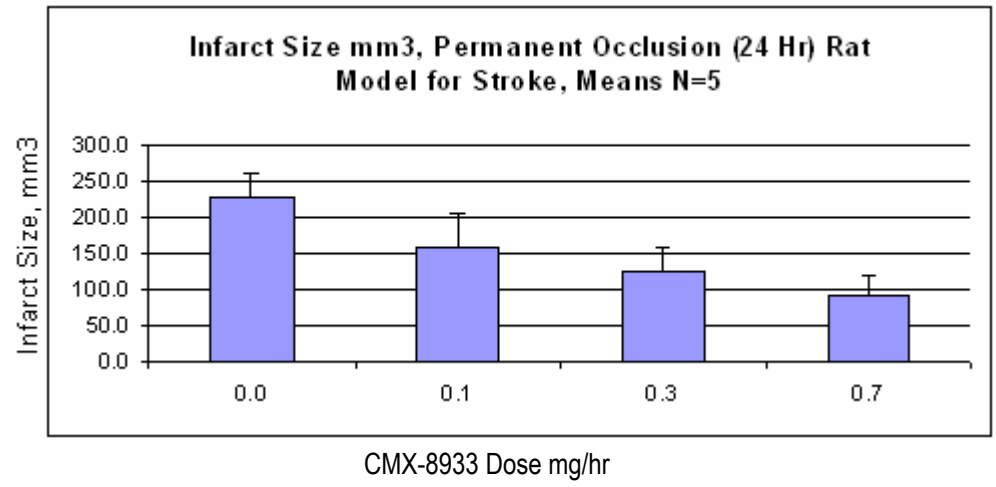


Fig 7. Effect of CMX-8933 on Infarct Size in a Rat Model for Stroke. CMX-8933 significantly reduces the size of brain infarct in comparison to the untreated brain (left) in the permanent (24 hr) occlusion stroke model (Shashoua, unpublished).

DHA-BBB Carrier

Neuroprotection following the systemic administration of full-length NTFs is generally not possible because these proteins are unable to cross the tight endothelial junctions lining the blood-brain barrier. Even the short synthetic peptides 8933 and 9236 cross the BBB inefficiently. One approach to the BBB problem is to couple the amino-terminal ends of these synthesized EPN peptides with *cis*-docosahexanoic acid (DHA), a lipid-soluble carrier molecule. DHA is a major fatty acid component of synaptic endings (Sun and Yau, 1976). Its lipophilic properties assist the diffusion of linked molecules across cell membranes, allowing the delivery of therapeutic molecules (like dopamine) into the brain (Shashoua and Hesse, 1996). Moreover DHA is essential for post-natal brain development, synaptogenesis, and photoreceptor membrane formation and function (Shashoua and Hesse, 1996) making it an attractive natural carrier.

Protarga, Inc. (Conshohocken, Pennsylvania) is a pharmaceutical company, founded by Dr. Shashoua, that makes use of this DHA-BBB carrier system to develop Targaceutical® compounds. Its targaceutical technology uses fatty acids as vectors to deliver more drug to targeted cells and achieve a sustained release of the therapeutic agent (Potera, 2001). The company's three lead drugs: Taxoprexin®, Doprexin® and Clozaprexin® are currently undergoing clinical trials. Taxoprexin®, a DHA-paclitaxel compound, which is an innovative new treatment for cancer, has recently proceeded to Phase III clinical trials (Protarga Homepage, 2003). Doprexin®, a DHA-Dopamine compound has applications for Parkinson's disease. Coupling DHA to dopamine increased brain uptake of dopamine by over 10-fold (Shashoua and Hesse, 1996). Clozaprexin®, made by coupling the potent anti-psychotic drug clozapine to DHA, is

used to treat psychotic disorders like schizophrenia. Studies show that clozaprexin is 10 times more potent and longer lasting than clozapine alone (Baldessarini *et al*., 2001).

Mechanism of Action of EPN-NTF Peptides

The precise mechanisms by which EPN peptides exert their neuroprotective actions *in vivo* are presently unknown. Work in progress in our lab is directed towards this goal. However, accumulating evidence suggests a biological mechanism, wherein the full length EPN, or the proteolytic cleavage products of EPN present in the ECF, may bind to a high affinity putative EPN receptor, initiating a signal transduction cascade involving series of biochemical steps (fig. 8, right side). The enzyme c-Jun-N-terminal kinase (JNK), a member of the MAP kinase family activated by 8933 (Adams *et al*., 2003), phosphorylates AP-1 leading to its activation and translocation to the nucleus. Activation of AP-1 could be a prerequisite for repair of brain injury, such as synaptic uncoupling that frequently occurs following an ischemic event. Previous studies in a number of laboratories have demonstrated an active role for AP-1 activating genes that promote cell proliferation, differentiation and survival mechanisms (summarized in Adams *et al*., 2001).

Alternatively, EPN peptides may also have an intracellular function (fig. 8, left side). Peptides coupled to the lipophilic DHA carrier may pass directly through the neuronal membrane into the cytoplasm. The DHA carrier is then cleaved by the normal cellular enzymes (Shashoua and Hesse, 1996) releasing the original peptide into the cytoplasm. The peptide then chelates increased intracellular calcium bringing the calcium levels to normal. DHA-8933 and uncoupled 8933 each activate AP-1, so the coupling of 8933 to the carrier does not appear to disrupt its receptor-binding properties.

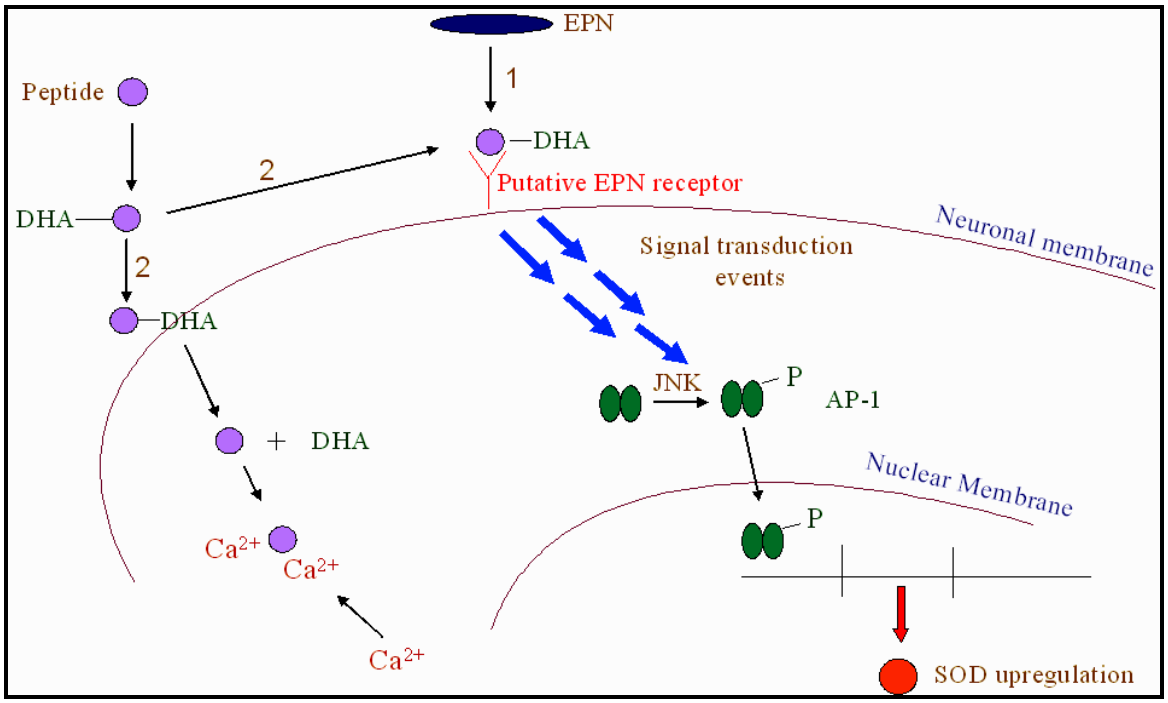


Fig 8. Working Hypothesis for NTF-EPN Peptide Mimetics. 2 pathways have been implicated in the peptide's neuroprotective effects. The biological pathway represented by '1' (right side) involves a signal transduction cascade leading to AP-1 upregulation via a putative ependymin receptor. The therapeutic unnatural (DHA) pathway represented by '2' (left side and right side) involves both the direct delivery of the DHA peptide across the neuronal cell membrane (left side) where it chelates intracellular calcium, as well as AP-1 activation triggered by ligand-receptor binding.

Anti-Oxidative Enzyme SOD

It is well known that toxic reactive species are overproduced in biological organisms following CNS injury, causing oxidation of cellular components. An increased production of superoxide radicals leading to oxidative stress is seen in neurons shortly after ischemic injury (Kim *et al*., 2002). Under normal physiological conditions, damage by superoxide radicals is kept in check by an efficient antioxidant system, which involves the production of free-radical scavengers like Superoxide dismutase (SOD), Catalase and Glutathione Peroxidase (GPX).

SOD is an endogenous anti-oxidative enzyme that catalyzes the dismutation of 2 superoxide anions to hydrogen peroxide, which is then broken down by Catalase or GPX

to water and molecular oxygen (Sun and Chen, 1998). Several investigators have reported that transgenic mice over-expressing SOD are resistant to injury that occurs during reperfusion following transient focal cerebral ischemia, as measured by smaller infarct size (Kinouchi *et al*., 1991; Chan *et al*., 1993; Yang *et al*., 1994). A variety of studies have revealed that transgenics over-expressing SOD appear to build resistance to cerebral ischemia (Chan *et al*., 1998; Sheng *et al*., 1999; Li *et al*., 2001). Moreover neuronal over-expression of SOD also plays a protective role in Parkinson's and Alzheimer's disease. Conversely, targeted disruption of the CuZn-SOD coding sequence causes increased edema and enlarged infarcts after murine middle cerebral artery occlusion (Sheng *et al*., 1999). An interesting finding by Araki *et al* (1992) suggested that the histoprotective action of SOD might also be due to its ability to attenuate the increase in intracellular calcium following stroke.

It is commonly known that the level of cellular oxidative stress increases with age and is a major causal factor in senescence. SOD has been implicated to have a potential role in deterring the aging process. Studies done in *Drosophila melanogaster* indicate a dramatic extension of Drosophila lifespan when the fly motor neurons were engineered to over-express human SOD-1 (Parkes *et al*., 1998). Similar studies done by Kitani *et al* (1996) suggested that neuronal over-expression of SOD and Catalase plays a major role in lengthening the mammalian life span.

Anoxia

Anoxia, a decreased oxygen condition commonly termed ischemic preconditioning, has emerged as a new topic in stroke therapy. Brief 'preconditioning' ischemia induces ischemic tolerance and protects the animal brain from subsequent

otherwise lethal ischemia. A cascade of signaling events initiated by sublethal stress proceeds during the latent period, resulting in a new, stress-resistant, biochemical makeup of brain cells (Liu *et al*., 2000).

Anoxia is produced *in vitro* in a cell culture environment consisting mostly of nitrogen, hydrogen and carbon dioxide for a short period of time, followed by reoxygenation (Jourdain, 2002). Studies show that after exposure to an anoxic environment, SOD and catalase levels in turtles dramatically increased, suggesting an anti-oxidant system was signaled (Willmore and Storey, 1997). Anoxia also resulted in an increase in the activities of brain GPX in goldfish by 79%, and after 14 hours of reoxygenation, brain GPX activities remained higher than control (Lushchak *et al*., 2000). An overall 30% reduction in infarct volume was seen when ischemic injury was introduced to anoxic preconditioned mice (Bernaudin *et al*., 2002). Tolerance was seen against focal transient and permanent cerebral ischemia in adult mice (Bernaudin *et al*., 2002).

The mechanism of ischemic preconditioning is unknown. Using anoxia-tolerant turtle as a model, the activity of the mitogen activated kinase family was analyzed in organs exposed to anoxia *in vivo*. The anoxic brain showed a 2 fold increase in the level of transcription factor c-Fos and increased JNK activity (Greenway and Storey, 2000) suggesting a possible role in the MAPK pathway mediating the adaptive response to oxygen deprivation. Alternately, Bernaudin *et al* (2002) suggested that expression of Hypoxia-inducible factor (HIF-1) along with its target genes erythropoietin and vascular endothelial growth factor (VEGF) could be important mediators of hypoxia-induced tolerance to cerebral ischemia.

THESIS PURPOSE

The purpose of this project was to test a three-part hypothesis. As mentioned earlier, CereMedix Inc. synthesizes small size Ependymin (EPN) peptide mimetics CMX-8933 and CMX-9236 that alleviate ischemic damage. It is well established that increases in oxidative stress result from ischemia, so we have hypothesized that our therapeutic peptide may alleviate oxidative stress. The primary goals of this project were to 1) determine whether CMX-8933 and CMX-9236 increase the cellular titers of the mRNA and protein for the anti-oxidative enzyme SOD. SOD was identified as a likely candidate for analysis based on early experiments in this thesis with hybridization arrays. 2) Since our previous studies indicated that DHA facilitates movement of dopamine across the blood brain barrier (Shashoua and Hesse, 1996), I wanted to test if conjugation of peptide CMX- 8933 to a DHA-BBB carrier further increases the expression of SOD. 3) Anoxia is a newly established *in vitro* system for studying stroke which uses a low oxygen cell culture environment to mimic *in vivo* ischemia. The third goal was to examine whether anti-oxidative events, specifically the upregulation of SOD, are a part of the cell's natural response to anoxia, and to determine whether anoxic neuroblastoma cells, when stimulated with peptide 8933 increase SOD levels beyond anoxic levels.

MATERIALS AND METHODS

Murine Neuroblastoma Cell Culture

The murine Nb2a neuroblastoma cell line (Neuro-2A) was purchased frozen from American Type Culture Collection (ATCC), Catalog # CCL-131 and thawed by rapid (~40-60 sec) agitation in a 37°C water bath. Other cell culture products, 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) were purchased from Life Technologies, Inc.

Complete Medium Preparation

500 ml of DMEM culture medium was supplemented with 5 ml of Non-essential amino acids (0.1 mM), 50 ml of 10% FBS and 250 µl of gentamicin (5 ug/ml) to make complete culture medium. The contents were mixed briefly and then filtered aseptically through a pre-sterilized filter using a vacuum pump.

Subculturing

Thawed NB2a cells were transferred to a vented T-25 culture flask and diluted with 10 ml of the complete medium. Flasks were incubated at 37°C in a humidified atmosphere containing 5% CO_2. When the cells attained confluency, the medium was removed, the monolayer was washed with old DMEM medium and the wash solution was discarded. The cells were then incubated with 2 ml of Trypsin-EDTA solution at 37°C

for 2 minutes with periodic, gentle agitation until the monolayer detached from the flask surface. The cell suspension was then flooded with an equal volume of complete DMEM to stop trypsinization, transferred to a 15 ml conical tube, and centrifuged (25°C, 5 min, 500 x g). Trypsin-containing medium was removed from the cell pellet, and cells were resuspended in fresh pre-warmed DMEM. The cells were then split and appropriate aliquots were dispensed into new flasks at 1:3 or 1:6 dilutions. Medium renewal was performed twice per week.

Drug Treatment

For RNA isolation experiments, a total of six confluent T-150 flasks were treated with peptide CMX-8933. The peptide CMX-8933 (in 5% xylitol HBSS) was added to the media at a final concentration of 10 ng/ml, and the 37°C incubations continued for 5 hours prior to isolation of total cellular RNA. Six untreated T-150 culture flasks were designated control flasks.

Anoxia Experiments

Anoxic cultures for Western blots were provided by CereMedix, Inc. Anoxic conditions (low oxygen) were induced for 24 hr in a special sealed cell culture incubator using a flush of N_2, H_2, and CO_2 gases in the presence of a palladium converter to remove trace amounts of O_2. Following the 24 hr induction of anoxia, peptide stimulations were performed as described in Culture Conditions.

Isolation of Total Cellular RNA

Total cellular RNA was isolated from murine NB2a neuroblastoma cells using the Clontech Atlas Pure Total RNA Labeling System (# K1038-1).

Cell Collection

A plastic cell scraper was used to dislodge the cells from the bottom of the T-150 culture flasks. The cell suspension was poured into a 50ml plastic tube and centrifuged at 2,000 rpm for 5 minutes to pellet the cells. The supernatant was discarded leaving a small volume for cell transfer into one 2 ml eppendorf tube per sample. The tubes were microfuged for 30 seconds to pellet the cells and to ensure complete removal of all the supernatant.

Cell Lysis

A volume of 500 µl of denaturing solution was added to the cell pellet. Cells were resuspended by pipetting up and down, and vortexing. The lysate solution was incubated on ice for 5-10 minutes. The solution was separated into two 2 ml eppendorf tubes (0.25 ml per tube). After vortexing the solution again, the lysate was microcentrifuged at 12,000 rpm (Sanyo Micro Centaur) for 5 minutes at 4°C to remove cellular debris. The entire supernatant was then transferred to new 2 ml centrifuge tubes (0.25 ml per tube).

Phenol Extraction

A volume of 0.5 ml of TE-saturated phenol was added to each tube. The tubes were capped securely, vortexed vigorously, and incubated on ice for 5 minutes. Next,

0.15 ml of chloroform was added per tube, and the sample was shaken and vortexed vigorously for 1-2 minutes. It was then incubated on ice for another 5 minutes. The organic/aqueous mixture was microcentrifuged at 12,000 rpm for 10 minutes at 4°C, and the upper aqueous phase containing the RNA was transferred to new 1.5 ml eppendorf tubes, making sure not to pipet any material from the white interphase or lower organic phase.

Isopropanol Precipitation

To the aqueous supernatant, a volume of 1 µl of 20 mg/ml glycogen was added per tube followed by the slow addition of 0.5 ml of isopropanol. The sample was mixed, incubated on ice for 10 minutes, and then microcentrifuged at 12,000 rpm for 15 minutes at 4°C to pellet the RNA. Supernatants were immediately removed without disturbing the RNA pellets.

Pellet Wash

A volume of 0.25 ml of 80% ethanol was then added per tube; the tubes were inverted several times, then microcentrifuged at 12,000 rpm for 5 minutes at 4°C to again pellet the RNA. Immediately the supernatant was carefully discarded, and the RNA pellet was dried in a speed vac-apparatus for 5 minutes.

Pellet Dissolution

The pellets from both tubes were resuspended in a total of 30 µl of RNase-free dH_2O. The tubes were heated at 50°C for 5 minutes, occasionally flipping the tubes to dissolve the RNA. In order to assess RNA yield, 1 µl of sample was added to 1 ml of

dH_2O, and the absorbance was taken at 260 nm. The remaining sample was aliquotted and stored at -80°C.

Assessing the Yield and Integrity of Total RNA

In order to assess the yield and integrity of the total RNA samples, a denaturing agarose gel was prepared and run. First, a mini-gel box and combs were thoroughly washed with deionized water. A 250 ml beaker containing a magnetic stir-bar was obtained, and 1 g of agarose was weighed and mixed with 82.5 ml of water. The solution was heated in a microwave oven for 2 minutes and placed on a magnetic stir-plate to stir slowly for 2 minutes to cool. While the solution was stirring, 10 ml of 10X MOPS buffer and 7.5 ml of formaldehyde were added. The stirring was continued for 1 minute, and then the solution was poured into the gel tray. The gel was left to solidify at room temperature for 1 hour, then the gel comb was removed and the gel was submerged in the gel box with 1X MOPS buffer.

The RNA loading solution was prepared immediately before running the gel by mixing the following (for 6-10 samples): 45 µl of formaldehyde, 45 µl deionized formamide, 10 µl 10X MOPS buffer, 3.5 µl EtBr (10 mg/ml), 1.5 µl 0.1M EDTA (pH 7.5), and 8 µl 10X bromophenol blue dye (in 50% glycerol). A volume of 15 µl of RNA loading solution was added to 2 µg (usually 2 µl) of total RNA and mixed well. The solution was heated at 70°C for 15 minutes and cooled on ice for 1 minute. The sample solution was then loaded onto the gel, and electrophoresed at 50-60 V until the blue dye reached the midpoint of the gel tray. RNA was visualized on a UV transilluminator.

cDNA Probe Synthesis for Rat Nylon Atlas Array 1.2 (catalog # 7854-1)

cDNA probe synthesis from total cellular RNA was performed in accordance with Clontech's Atlas cDNA Expression Array User Manual (# PT3140-1).

Preparation of Master Mix

First, a Master Mix was prepared at room temperature for all labeling reactions. The following reagents were combined in a 0.5 ml microcentrifuge tube at room temperature for each sample of RNA: 2 µl of 5X Reaction Buffer, 1 µl of 10X dNTP Mix, 3.5 µl of [α-^{32}P] dATP (3,000 Ci/mmol), and 0.5 µl of DTT (100 mM). Then, a PCR thermocycler was preheated to 70°C.

RNA Denaturation and Primer Annealing

For each reaction, 3 µl total cellular RNA (2-5 µg) and 4 µl CDS Primer Mix (a special combination of primers designed specifically for the Atlas filter) were combined in a labeled 0.5 ml PCR tube. Next, the reactions were mixed well by pipetting, and spun briefly in a microcentrifuge. Then, these tubes were incubated in the PCR thermocycler at 70°C for 2 minutes. After 2 minutes, the temperature of the thermocycler was reduced to 50°C, and the tubes were incubated for 2 more minutes. During this incubation, 1 µl MMLV Reverse Transcriptase was added per reaction to the Master Mix, it was mixed by pipetting, and incubated at room temperature for 2 minutes.

Reverse Transcription

After the incubation of two minutes was completed, 13.5 µl of Master Mix was added to each 7 µl annealed RNA sample, ensuring that the RNA samples in the 50°C

thermocycler were not removed for longer than was necessary to add the Master Mix. The contents of the tubes were mixed by pipetting and then immediately returned to the thermocycler. The tubes were then incubated in the PCR thermocycler at 50°C for 25 minutes. After incubation, 2 µl of 10X Termination Mix was added to each tube. The tubes were stored on ice until column chromatography.

Column Chromatography

To purify the labeled cDNA from unincorporated ^{32}P-labeled nucleotides and small cDNA fragments, the following procedure was performed. Before using the Buffer NT3, 15 ml of 95% ethanol was added to the bottle. First, the probe synthesis reactions were diluted by adding 177.5 µl of Buffer NT2. This makes a total of 200 µl. Second, a NucleoSpin Extraction Spin Column was placed into a 2 ml Collection Tube, and the sample was pipetted into the column. The tube was then microcentrifuged at 14,000 rpm for 1 minute. The Collection Tube and the flowthrough containing the waste were then discarded into the appropriate container for radioactive waste. Then, the NucleoSpin column was inserted into a fresh 2 ml Collection Tube, and 400 µl of Buffer NT3 wash was added to the column. The sample was microcentrifuged at 14,000 rpm for 1 minute, and the tube and flowthrough were discarded again. The addition of Buffer NT3 wash and the centrifugation steps were repeated twice more. The NucleoSpin column was then transferred into a clean 1.5 ml microcentrifuge tube, 100 µl of Elution Buffer NE was added, and the column was allowed to soak for 2 minutes. The tube was microcentrifuged at 14,000 rpm for 1 minute to elute the purified cDNA probe. The approximate radioactivity of the liquid eluted into the microcentrifuge tube versus that remaining in the column were assessed using a Ludlum hand-held Geiger counter to

monitor efficient elution from the column. The counts should be higher in the eluate tube than in the column, otherwise the elution was repeated.

Probe Hybridization

Hybridization of cDNA probe to the Rat Nylon Atlas Array filter was performed using Clontech's Atlas cDNA Expression Array Protocol (# PT3140-1). First, 5 ml of ExpressHyb was pre-warmed at 68°C. Next, 50 µl (0.5 mg) of sheared salmon testes DNA was heated at 97°C for 5 minutes to denature the DNA, then quickly chilled on ice to prevent snap-back. Then, the denatured salmon testes DNA and the 5 ml pre-warmed ExpressHyb aliquot were mixed, and kept at 68°C. A Tupperware container was filled with deionized water and the Rat Nylon Array 1.2 (catalog #7854-1, stored at -20°C) was placed in the Tupperware to wet it. All the water was then poured off and 5 ml of the 68°C testes DNA/ExpressHyb solution was added to the membrane. This step was performed quickly to ensure that the membrane would not dry. Then, the membrane was prehybridized for 30 minutes with continuous agitation at 68°C in an air incubator. Next, the probe was prepared for hybridization by adding 5 µl C_0t-1 DNA (1 mg/ml) to the entire 100 µl pool of labeled probe. The probe was then incubated in a boiling water bath for 2 minutes to denature it, and incubated on ice for 2 minutes. This mixture was added to the prehybridization solution and membrane in the Tupperware, ensuring that the probe was not directly added to the membrane, and that the two solutions were thoroughly mixed together. The membrane was then hybridized overnight with continuous agitation at 68°C. If necessary, 2-3 ml of extra pre-warmed ExpressHyb was added to the solution to ensure that the entire membrane was in contact with hybridization solution at all times.

The next day, Wash Solution 1 (2X SSC, 1% SDS) and Wash Solution 2 (0.1X SSC, 0.5% SDS) were pre-warmed at 68°C. The hybridization solution was discarded in an appropriate radioactive waste container, and was replaced with 200 ml of pre-warmed Wash Solution 1. The membrane was washed for 30 minutes with continuous agitation at 68°C. This process was repeated three more times. Then, one 30-minute wash was performed using 200 ml of pre-warmed Wash Solution 2 with continuous agitation at 68°C. One final 5-minute wash was performed in 200 ml of 25°C 2X SSC with agitation at room temperature. Using forceps, the membrane was removed from the container, and the excess Wash Solution was shaken off, never allowing the membrane to dry. The membrane was immediately wrapped in a plastic bag and sealed with a bag sealer. Finally, the membrane was exposed to BioMax x-ray film at -80°C with an intensifying screen for 5 days. When the exposure was complete, the membrane was kept wrapped in plastic, and stored at -20°C until stripped for re-use.

Probe Removal

To strip the cDNA probes from the membrane, 500 ml of 0.5% SDS solution was heated to boiling in a 2-L beaker. Then the membrane was removed from the plastic bag, and placed in the boiling solution for 5-10 minutes. The SDS solution was then removed from the heat and allowed to cool for 10 minutes. The Atlas Array filter was then removed from the solution and immediately re-wrapped in a new plastic bag and sealed.

To ensure the stripping procedure was effective, the membrane was checked with the hand-held Geiger counter, and by exposure to x-ray film for 3 days. Once the efficacy of the stripping was verified, the membrane was stored at -20°C until needed.

Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using Ambion's RETROscript, First Strand Synthesis Kit for RT-PCR Protocol (catalog # 1710) to verify the upregulation of specific candidates identified from the arrays.

Heat Denaturation of the RNA and Reverse Transcription

Each reaction was performed in a 0.5 µl eppendorf tube. First, 2 µg of total cellular RNA was added to 2 µl of the antisense primer and brought up to 12 µl with nuclease-free dH_2O. The tubes were then mixed, spun briefly in a microcentrifuge, and heated for 3 minutes at 70-85°C in the thermal cycler. The tubes were then removed to ice, spun briefly, and the following components were added (for each 12 µl sample to make a 20 µl reaction volume): 2 µl of 10X RT Buffer, 4 µl of dNTP mix, 1 µl of RNase Inhibitor, and 1 µl of MMLV Reverse Transcriptase. The reaction was then mixed gently, spun briefly, and incubated at 42-44°C for one hour. Upon completion of the reverse transcription, the reactions were then incubated at 92°C for 10 minutes to inactivate the reverse transcriptase. After this point, the samples could be stored at -20°C, or analyzed by agarose electrophoresis.

PCR

The following components were mixed in a separate 0.5ml tube for each PCR reaction, 5 µl of the RT reaction described above, 5 µl of 10X PCR buffers, 2.5 µl of dNTP mix, and nuclease-free dH_2O to make 50 µl. Then the following were added: 2.5 µl of 50 µM sense primer and 0.4 µl (2 U) of thermostable Taq DNA Polymerase. The samples were mixed, spun briefly and then placed in the thermal cycler for the following

program: 94°C for 2 minutes to initially denature the templates, then 30 cycles of the following three steps: 94°C denaturation for 30 seconds, 60°C annealing for 30 seconds, and 72°C elongation for 40 seconds. After these 30 cycles, a final polishing extension for the samples was performed at 72°C for 5 minutes. The tubes were then stored at 4°C until the next day or analyzed by agarose gel analysis of RT-PCR products.

Analysis of RT-PCR Products

To analyze the RT-PCR products, 10 µl aliquots of the 50 µl reaction mixes were loaded onto a non-denaturing 2.5% agarose gel containing 0.5 µg/ml of ethidium bromide and 1X TBE. A total of 2 µl of the high-resolution gel loading solution was mixed with 10 µl of the RT-PCR mix and 10 µl of this mixture was loaded into the wells of the gel. 1X TBE was used as the electrode buffer. The marker used was λ/Pst I (10 µl of 0.2 µg/µl stock). The remaining RT-PCR products and the PCR reaction mixes were stored at -20°C.

Western Blot Analysis

Western blots were used to analyze SOD protein levels in total cell lysates prepared from *in vitro* cultured rat primary cortical cells or from mouse brain and heart treated *in vivo.*

Gel Polymerization

The gel was polymerized between two non-siliconized plates, one small (6-inch by 8-inch) back plate and one large (8-inch by 8-inch) front plate. Two side spacers and a bottom spacer, all 0.8 mm thick, separated the plates.

The lower resolving gel was prepared first by adding 7.6 ml distilled H_2O, 6.7 ml 30% Acrylamide (2.7 % cross-linking), 5.1 ml 1.5 M Resolving Gel Buffer (1.5 M Trizma base in distilled H_2O), 200 µl 10% SDS, and 400 µl 5% Ammonium Persulfate to a 50 ml conical tube. Immediately before the gel was poured, 10 µl of 100% TEMED was added to initiate polymerization. The tube was gently mixed by slow inversion and the solution was then poured between the plates to a height approximately 1.5 cm from the top of the small plate. Distilled water was used as an overlay to allow the gel to polymerize more evenly. The lower gel was allowed to polymerize for at least 20 minutes, then the water overlay was poured off.

The upper stacking gel was prepared by adding 5.52 ml distilled H_2O, 1.67 ml 30% Acrylamide (2.7% cross-linking), 2.5 ml 0.5 M Stacking Gel Buffer (0.5 M Trizma base in distilled H_2O), 100 µl 10% SDS and 200 µl 5% Ammonium Persulfate to a 15 ml conical tube. Immediately before pouring, 10 µl 100% TEMED was added to initiate polymerization. The contents were mixed by gentle inversion and then poured above the lower gel. A 20-stall comb was carefully inserted, avoiding any bubbles, to a depth of approximately 0.5 cm into the upper gel to create sample wells. The stacking gel was allowed to polymerize for at least 20 minutes. At this point, if the gel was to be left overnight, the open end was tightly wrapped in saran wrap to prevent dehydration.

Gel Electrophoresis

Just prior to electrophoresis, the clamps holding the plates together were removed along with the bottom spacer, and the back plate was cleaned and dried. The gel was placed in a V-16 vertical electrophoresis unit, using clamps to press the gaskets tightly against the upper reservoir of the unit. The upper and lower reservoirs were filled with

Protein Electrode Buffer (25 mM Trizma base, 0.192 M Glycine, 0.1 % SDS) and a syringe was used to remove any bubbles trapped at the base of the gel between the plates. The comb was then removed and the wells cleaned with reservoir buffer. Pre-electrophoresis was performed for one hour at approximately 150 Volts. Prior to loading, the protein samples were heated in a boiling water bath for 2 minutes, then microfuged briefly. A V16 pipette tip was used to load the samples (usually 5 µl) into the wells of the gel. Elecrophoresis was performed for approximately 3 hours or until the bromophenol blue dye had run about $^4/_5$ of the way down the gel.

Gel Transfer

After electrophoresis, the plates were removed from the V-16 unit and the side spacers were removed. The plates were very gently pried apart using a pizza cutter. With the gel still laying on one of the plates, the original lower right corner was marked by cutting away the corner of the gel.

A piece of nitrocellulose membrane (0.45 µm pore size) was cut to match the size of the gel. The lower right corner of the membrane was also cut to match the gel. The membrane was dipped in the pre-chilled transfer buffer (48 mM Trizma Base, 39 mM Glycine, 0.037% SDS, 20% methanol) and placed carefully over the gel. Air bubbles were removed by smoothing a gloved finger over the membrane. Two pieces of 3MM paper were then cut to match the size of the gel. The first was dipped in transfer buffer and placed over the membrane. Bubbles were again removed in the same manner as with the membrane. The gel and lower plate were flipped over and the plate was removed. A second piece of pre-soaked 3mm paper was placed over the gel. Again, bubbles were smoothed out. Sponges were placed on both sides of the gel/membrane/paper sandwich

and the plastic holders were locked into place around the sponges. The plastic holder was placed into the transfer unit, with the membrane side of the sandwich facing the positive anode so the proteins will transfer from the gel towards the positive anode onto the membrane. The transblot unit was placed in the refrigerator with the electromagnetic stirrer set on a low speed. The current was set to 50 V for 2 hours at 4ºC, usually on an electronic timer.

Membrane Blocking/Antibody Incubations

After gel transfer, the membrane was immediately moved to a tupperware container, the protein side was marked with a felt pen , and it was covered completely in freshly made casein blocker solution (1X PBS, 1% Casein powder, 0.2% Tween-20). The membrane was incubated at 25ºC on the red rocker shaker at low speed for one hour. The membrane was then transferred to a fresh tupperware container and submerged in 50 ml of fresh casein blocker solution for the primary antibody incubation. 50 µl of primary antibody (rabbit anti-SOD, Rockland # 100-4191) was added to the blocker solution to make a 1:1000 dilution and the membrane was incubated at 25ºC on the red rocker shaker at low speed for at least two hours. The membrane was then transferred to a fresh tupperware container and washed twice in PBS-Tween (1X PBS, 0.05% Tween-20) for five minutes each at vigorous speed on a gyratory shaker to lower the background during film development by removing loosely associated primary antibody.

The membrane was again moved to a fresh tupperware container and covered in approximately 50 ml blocker solution for secondary antibody incubation. 50 µl of secondary antibody (0.4 mg/ml Glycerol Stock of Goat Anti-Rabbit-HRP, Pierce, catalog # 31460) was added to the blocker solution, and the membrane was incubated at 25ºC on

the red rocker shaker at low speed for at least two hours. Following secondary antibody incubation, the membrane was transferred to a fresh tupperware container and washed three times in PBS-Tween for five minutes each at vigorous speed on a gyratory shaker, and then briefly washed once in 1X PBS.

Chemiluminescent Detection

During the final wash in 1X PBS, 5 ml Luminol/Enhancer Solution was mixed with 5 ml Stable Peroxide Solution (both solutions represent Pierce # 34080 Supersignal Chemiluminescent substrate for Westerns) in a 15 ml plastic tube. The washed membrane was placed on a piece of foil, protein side up, and the detection mix was poured over the membrane. A glass pipette was used to roll the solution evenly over the membrane, and it was incubated for five minutes at room temperature, rolling at one minute intervals to move aside oxygen bubbles caused by the HRP reaction.

After incubating, the corner of the membrane was dabbed on a kim-wipe to remove excess solution and then placed between two sheets of a clear plastic Photogene Development Folder (Gibco BRL # 18195-016). The holder was placed inside a light-tight cassette without a screen for transportation to the darkroom.

X-Ray Film Development

Kodak X-Omat AR film was used for x-ray detection, and exposed to the membrane for 1-5 seconds, or until the negative control band was just visible after film was developed. Signals easily observed on film after 1-5 seconds exposure were visible to the naked eye before developing the film. If band hollowing occurred due to substrate depletion, extra substrate solution was added to the membrane. Immediately after

exposure to the membrane, films were placed in GBX developer solution for 5 minutes. The film was dipped in a water bath briefly, and then placed in Kodak Rapid Fixer plus Hardener for 3 minutes with periodic agitation. Finally the film was washed for 5 minutes in tap water then allowed to air dry. Bands were quantified using Scion Image Software (NIH).

RESULTS

The therapeutic benefits of over-expressing the anti-oxidative enzyme SOD in attenuating free radical-induced oxidative damage prior to ischemic injury in transgenic animals is well established in the literature as mentioned in the background. The goal of this project was to investigate whether EPN peptide mimetics 9236 and 8933 that are both known to reduce ischemic volume in rat models for stroke, increase the cellular levels of SOD and its mRNA. We also wanted to investigate the pattern of SOD upregulation in rat cortical cultures treated with either unconjugated peptide 8933 or the 8933-DHA conjugate. Our final goal was to test whether anoxia or ischemic preconditioning includes the upregulation of SOD. Experiments were performed using a mouse neuroblastoma cell line and primary rat cortical cell cultures as examples of transformed and primary cultures, respectively.

Total Cellular RNA Isolation from Murine Neuroblastoma Cells

Cultured mouse Nb2a neuronal cells were selected because preliminary experiments indicated that addition of goldfish EPN to these mouse cells induces neurite sprouting (Shashoua, 1991), one of the hallmarks of neurotrophic factors. Cultures at 90% confluency were treated with 10 μg/ml of the peptide 8933 for a period of 5 hours prior to total cellular RNA isolation. Table 1 summarizes the various neuronal cell samples tested in this thesis and their corresponding RNA yields after the isolation procedure. Because the initial experiments with single T-75 flasks yielded only about 45 μg RNA, T-150 flasks were used for subsequent experiments (3 per sample).

Table 1. Neuronal Cell Sample Size and Corresponding RNA Yields

Date	Sample	Size of culture flask	No. of culture flasks	RNA yield µg	Conc. µg/µl
05-04-02	Control	T-75	1	45.6	0.45
05-04-02	8933-treated	T-75	1	43	0.43
09-20-02	Control (Set A)	T-150	3	421.2	14.04
09-20-02	8933-treated (Set A)	T-150	3	440.4	14.68
09-20-02	Control (Set B)	T-150	3	417.6	13.92
09-20-02	8933-treated (Set B)	T-150	3	428.4	14.28

In order to verify that the RNA samples were intact following the RNA isolation protocol, denaturing formaldehyde agarose gel electrophoresis was performed. Discrete 28S and 18S ribosomal RNA bands were used as evidence of intact RNA. Figure 9 displays an example of an RNA integrity gel for Set A and Set B. The 28S and 18S ribosomal RNA bands at 4.5 kbp and 1.9 kbp respectively can clearly be seen in each sample indicating that the RNA isolated was intact.

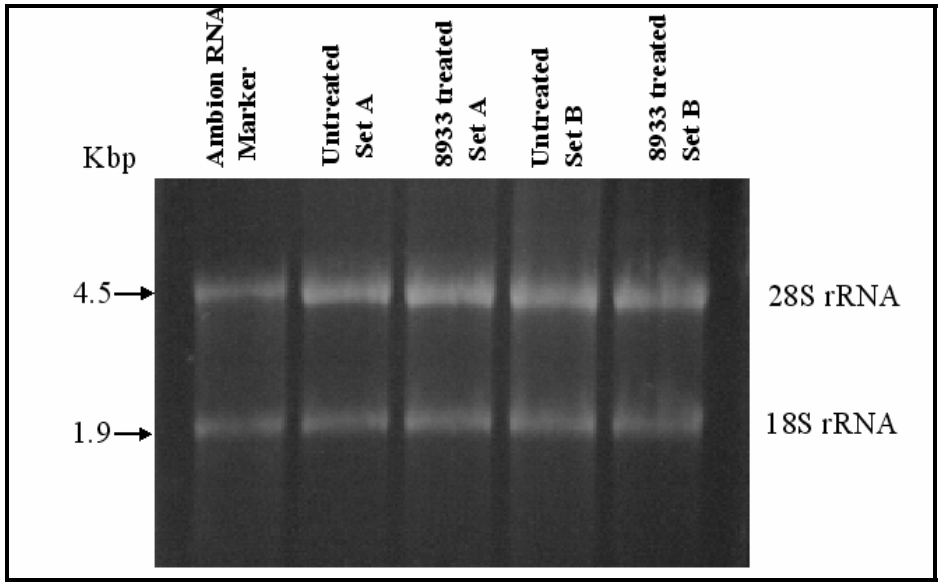


Fig 9. RNA Integrity Gel. Analysis of total cellular RNA isolated from Nb2a cells.

Multiple Gene Profiling Using Rat Nylon Arrays

Clontech's Atlas Nylon cDNA expression arrays are densely packed with sample dots of known cDNA sequences spotted on positively charged nylon membrane. Plasmid and bacteriophage DNAs are included as negative controls to confirm hybridization specificity, along with several housekeeping cDNAs as positive controls for normalizing mRNA abundance (Clontech User Manual). We selected Rat Nylon Atlas Array 1.2 (catalog # 7854-1) because it provided a total of 1,176 rat genes, which play a key role in a variety of different biological processes. Radiolabeled cDNA was synthesized from RNA isolated from primary rat cortical cultures treated for 3 hours with 5 ng/ml of the peptide 8933 and from untreated cultures. The radiolabeled samples were hybridized to duplicate array filters. Figure 10 shows the side-by-side comparison of the expression profiles of RNA isolated from untreated (upper panel) and 8933-treated (lower panel) samples. Equal signals were observed for all the true housekeepers (β-actin, polyubiquitin, GAPDH and S29 Ribosomal protein). Because of the high cost associated with these rat nylon arrays ($1,400), I was unable to perform additional independent observations, so used this single array experiment specifically as a "Hypothesis Generator" for my thesis to identify candidates worth further experimentation.

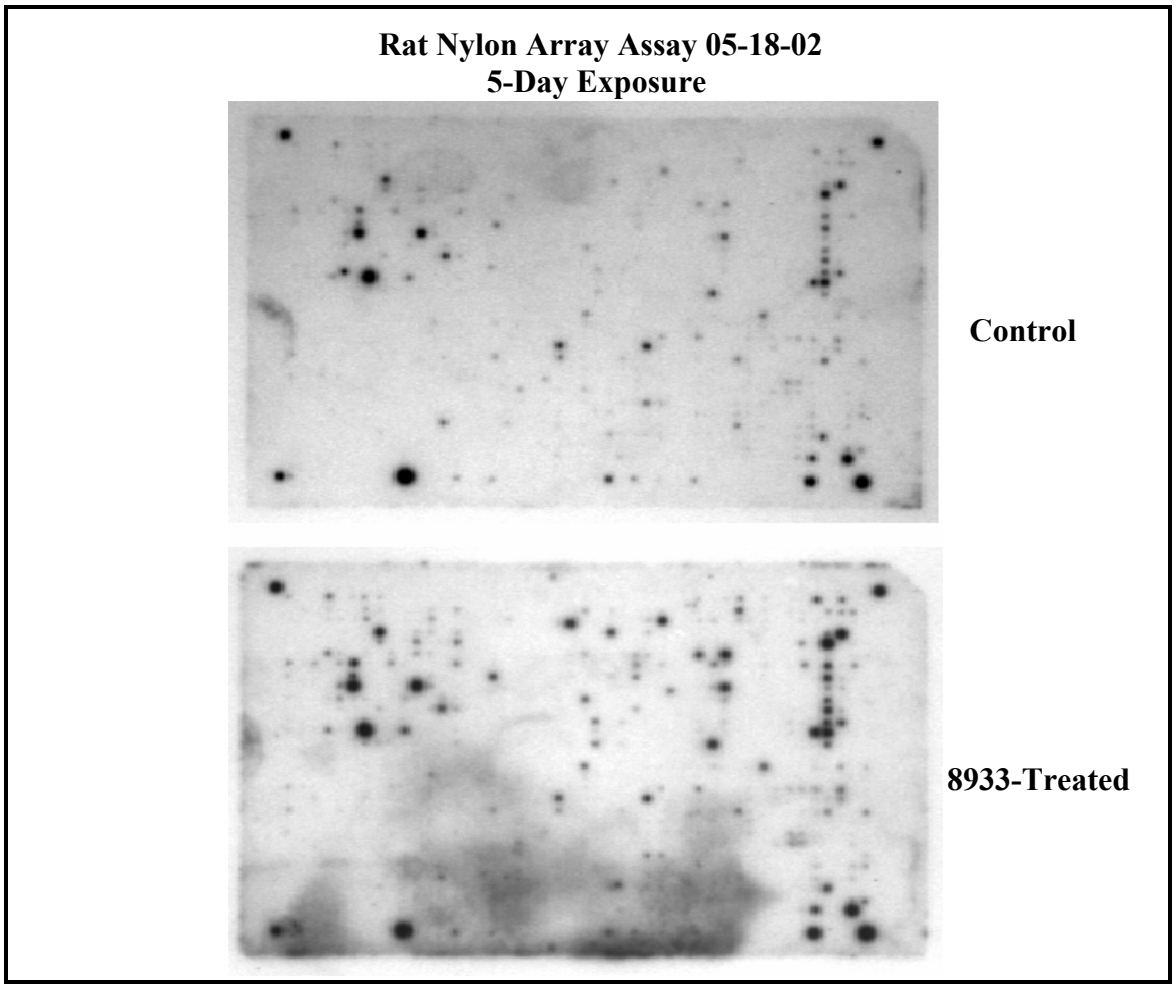


Fig 10. Rat Nylon cDNA Expression Array Used as a 'Hypothesis Generator'. The arrays were exposed for 5 days at –80°C. A grid provided with the kit was used to identify specific mRNAs upregulated. The 5 dots on the lower right corner in both the membranes represent the housekeeper with equalized signal intensity. The 8933-treated nylon membrane (lower panel) shows upregulation of numerous candidates indicated by strong darker dots compared to the untreated (upper panel).

Array Summary

The signal intensities for some specific interesting candidates have been summarized according to their biological function in Table 2. The symbol +++ (or more) signifies a strong signal (dark dots on the membrane), ++ signifies a moderate signal (medium dots), + signifies a low signal (light dots) and 0 signifies no observable signal. For both the membranes the positive controls (second group in the table) showed a positive signal, the negative controls (first group in the table) showed no signal and the housekeepers (third group in the table) had equal signals.

The most exciting candidate of direct interest to this thesis was the anti-oxidative enzyme SOD (fourth group in the table). It showed at least three times higher signal in the drug treated sample compared to the control. Three calcium-binding proteins (fifth group in the table), which could theoretically mediate ischemic damage by counteracting calcium accumulation, were also upregulated following drug treatment. In addition, other groups of gene involved in stress response, protease inhibition, energy metabolism and translation were also upregulated in the 8933-treated sample. Many scientists worry that since we are stimulating neuronal cell division with NTF therapy, we may induce cancer in these cells. However this array data showed no upregulation of any of the seven oncogenes tested (one above the last group in table). In fact, one metastasis inhibitor factor was upregulated.

Table 2. Summary of Hybridization Array Data

Array Coordinate	Genbank Accession	Gene/Protein	Classification	Array Signal Ctrl	Array Signal 115-2	RT-PCR Primers	Expected Amplicon
Negative Controls							
A5k	L26267	NF-kB (previously assayed)	Transcription Factor	0	0		
G4	x	M-13 DNA	Neg Ctrl	0	0		
G20	x	Lambda Phage DNA	Neg Ctrl	0	0		
G36	x	pUC-18 Plasmid DNA	Neg Ctrl	0	0		
Positive Controls							
G2	x	Rat Genomic DNA	Pos Ctrl	+	+		
G18	x	Rat Genomic DNA	Pos Ctrl	+	+		
G34	x	Rat Genomic DNA	Pos Ctrl	+	+		
Housekeepers							
G11	D16554	Polyubiquitin	Housekeeper	+++	+++		
G27	M17701	GAPDH	Housekeeper	+	+		
G43	V01217	b-Actin	Housekeeper	+++	+++		
G47	X59051	S29 Ribosomal Protein	Housekeeper	+++	+++		

Table 2, continued

Array Coordinate	Genbank Accession	Gene/Protein	Classification	Array Signal Ctrl	Array Signal 115-2	RT-PCR Primers	Expected Amplicon
Anti-oxidative Enzyme							
B1h	Y00404	SOD (previously assayed in primary cells)	Metabolism	+	+++		
Calcium-Binding Proteins							
A1g	M21730	Calphobindin-1, CBP-1	Ion Channel	+	++		
C10a	X13817	Calmodulin	Calcium-Binding Protein	+	+++		
C11b	M86870	Calcium-Binding Protein-2, CABP-2	Calcium-Binding Protein	0	+		
Stress-Response Proteins							
A13k	S45392	Heat Shock Protein HSP-90	Receptor-Assoc Protein	+	++		
A14e	J02592	Glutathione-S-transferase, Yb2 subunit	Stress Resonse Protein	+	++		
A14g	X02904	Glutathione-S-transferase, P subunit	Stress Resonse Protein	0	++		
F14e	D44495	DNA Damage Repair Protein APEX	DNA Repair	0	+		
Protease Inhibitors							
F10n	L31883	Tissue Inhibitor of metalloprotease-1, TIMP-1	Protease Inhibitor	+	++		
F11e	U40260	Tissue Carboxypeptidase Inhibitor TCI	Protease Inhibitor	0	+		
Receptors							
B7c	M80570	Dopamine transporter-1, DAT-1	Symporter/Antiporter	0	+++++	1, 2	224 bp
B7k	D50306	Proton-Coupled Dipeptide Co-Transporter	Symporter/Antiporter	0	+		
B8j	S59158	Excitatory aa Transporter-1, EAAT-1	Symporter/Antiporter	+	++		
D12b	M96377	Non-processed Neurexin-lib	Receptor	0	+		
F12c	L19181	Receptor-Linked Protein Tyrosine Phosphatase	Protein Phosphatase Receptor	+	++		
Energy Metabolism							
B8b	D12771	ATP Translocator-2	Symporter/Antiporter	0	+		
B9l	M28647	ATPase Alpha-1 Subunit	ATPase Transporter	+	++		
B9n	D10874	Vacuolar ATP Synthase	ATPase Transporter	+	++		
B10d	J04629	ATPase Transporter	ATPase Transporter	+	+++		
B12f	D13123	ATP Synthase Lipid binding protein P1	Energy metabolism	0	++		
B12g	D13124	ATP Synthase, Subunit-C	Energy metabolism	0	++	3, 4	230 bp
C4b	M19044	Mitochondrial ATP Synthase, Subunit b	Energy Metabolism	0	++	7, 8	197 bp
C2g	M12919	Aldolase	CHO Metabolism	0	+		
C3f	S79304	Cytochrome C Oxidase 1, COX-1	Energy Metabolism	+	++++		
C3i	X14209	Cytochrome Oxidase, subunit-4	Energy Metabolism	+	+++		
C9e	L38615	Glutathione Synthase	Amino Acid Metabolism	0	+		
C9g	U38419	Dopa/Tyrosine Sulfotransferase	Amino Acid Metabolism	0	++	5, 6	235 bp
C10m	D10854	NADP Alcohol Dehydrogenase	Xenobiotic Metabolism	++	+++	9, 10	236 bp
Transcription Factors							
A5i	X63594	IF-1 Transcription Factor	Transcription Activator	0	+		
A5j	X91810	STAT-3 Trancription Factor	Transcription Activator	0	+		
A10m	D17512	Cysteine-Rich Protein-2	Basic Transcription Factor	+	++		
Fatty Acid-Binding Proteins							
B14c	U13253	Fatty Acid-Binding Protein-5	Targeting Protein	+	++++		
B14i	U07870	Fatty acid binding protein-9	Targeting Protein	0	+		
C1f	U02096	Brain fatty acid binding protein FABP-7	Targeting Proteins	+	++++		

Table 2, continued

Array Coordinate	Genbank Accession	Gene/Protein	Classification	Array Signal Ctrl	Array Signal 115-2	RT-PCR Primers	Expected Amplicon
Translation							
C11e	M18547	Ribosomal Protein S12	Ribosomal Protein	++	+++		
C11g	X62146	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	++	+++		
Oncogenes (no upregulation)							
A10h	Y00396	c-Myc	Oncogene	0	0		
A11e	D13374	Metastasis **Inhibitor** Factor NM-23	Nucleotide Metabolism	+	++		
A12f	M15427	c-Raf	Oncogene	0	0		
E13f	D89863	m-Ras	Oncogene	0	0		
E14d	J02999	Ras-Related Protein, RAB-2	Oncogene	0	0		
E14e	X06889	Ras-Related Protein, RAB-3a	Oncogene	0	0		
E14f	X06890	Ras-Related Protein, RAB-4a	Oncogene	0	0		
E14g	Y14019	Ras-Related Protein, RAB-3b	Oncogene	0	0		
Misc							
A4m	M69139	Peripheral myelin protein-22, PMP-22	Other transducers, modulators	0	++		
A7a	D14014	G1/S-Specific Cyclin D1	Cyclins	0	+		
A7b	D16308	G1/S-Specific Cyclin D2	Cyclins	0	+		
A7c	D16309	G1/S-Specific Cyclin D3	Cyclins	0	+		
B12h	M11185	Myelin Proteolipid Protein PLP	Extracellular Matrix Protein	0	+		
D13k	X97375	Nociceptin	Neuropeptides	++	+		
E8a	M61177	ERK-1 Extracellular Signal Related Kinase	Kinase Network	+	++		
E11l	M23591	Ser/Thr Protein Phosphatase 2Ab subunit	Intracellular Prot Phosphatase	0	++		
E13i	M17528	Guanine Nucleotide-Binding Protein G1a	G-Protein	0	+		
F4k	M84416	Mitochondrial Import Stimulation Factor	Kinase Activator	0	+		

CMX-8933 Increases the Titer of SOD mRNA in Nb2a Cells

During array analysis, because of variations inherent in any hybridization reaction, a further confirmation using Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) is always recommended. I chose the potentially upregulated anti-oxidative enzyme SOD for further analysis by RT-PCR because of its known function in assuaging ischemic damage. Figure 11 shows the RT-PCR result for SOD performed in triplicate. As predicted, the levels of the housekeeper Polyubiqutin (amplicon size 231 bp) were equal between treated and untreated samples, but SOD mRNA (amplicon size 265 bp) was highly elevated in the CMX-8933 treated cells.

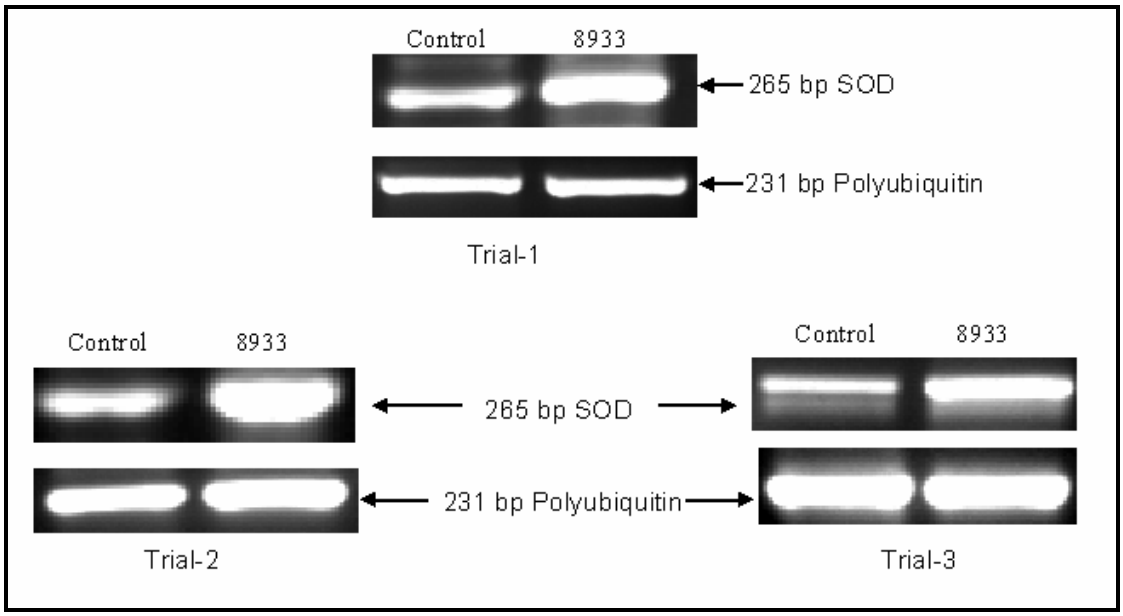


Fig 11. CMX-8933 Elevates SOD mRNA Levels in Nb2a Cells. Polyubiquitin (amplicon size 231 bp) was used as housekeeper. The results of 3 independent trials indicate that the SOD bands (amplicon size 265 bp) were stronger in samples treated with 10 ng/ml of CMX-8933 for 5 hours and hence this data correlates well with the results obtained from the hybridization array.

The means +/- standard deviation for the SOD RT-PCR experiment are shown in figure 12. The histobars illustrate the mean fold increase in a triplicate determination. The figure shows a 6.5 fold average increase in SOD mRNA levels for the 8933 treated samples compared to the control. Error bars denote sample variation within the triplicate set. A statistical analysis was performed using the Analysis of Variance (ANOVA) test. It indicated that the SOD upregulation was significant ($p = 0.001$).

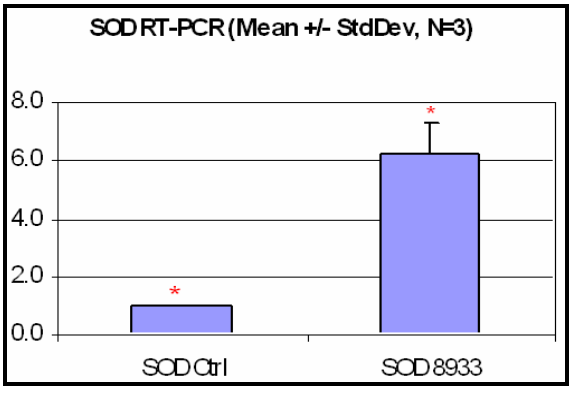


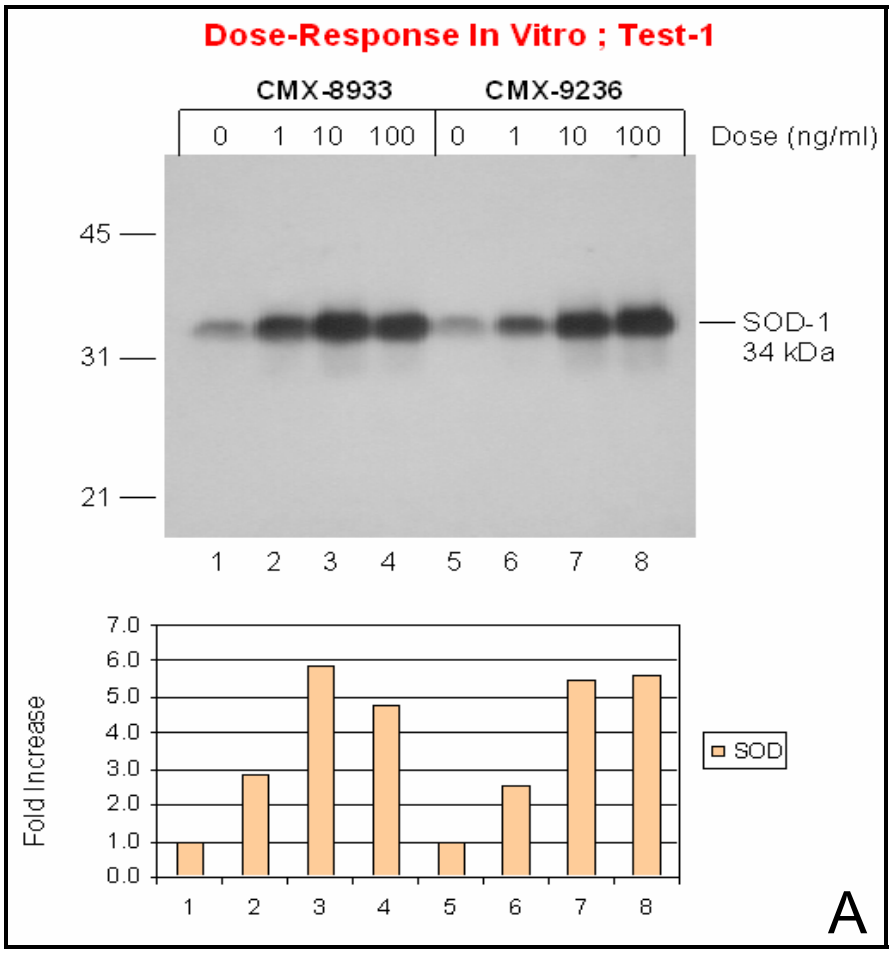
Fig 12. Quantitation Data for SOD RT-PCR. Histobars indicate means for 3 trials. Error bars denote standard deviations in a triplicate set. Asterisks denote the two histobars compared for the p value.

CMX-8933 and CMX-9236 Increase the Cellular Titers of SOD Protein

Western blots were used to determine whether SOD protein also increases like its mRNA when treated with EPN peptide mimetics. Five different SOD Western experiments were performed using whole cell lysates prepared from either in *vitro* cultured rat primary cortical cells or from mouse brain and heart treated with 8933 via *i.v.* administration *in vivo.* The SOD protein levels were compared to that of vehicle-treated control groups to determine effectiveness of treatments.

In Vitro Dose-Response Experiment

To analyze the effect of 8933 and 9236 on SOD levels at increasing doses of the peptides, rat primary cortical cultures were pre-incubated with 1, 10 and 100 ng/ml of either peptide for a period of 5 hours. Figure 13 shows the results for this *in vitro* dose-response experiment. A dose of 10 ng/ml showed the darkest SOD band at the expected size of SOD protein (34 kDa). Hence a dose of 10 ng/ml may be the optimal dose for these Nb2a cultures. A further increment of dose to 100 ng/ml did not show a further increase in SOD levels, indicating a possible post-saturation effect of the putative peptide receptors. The bands were quantified using Scionimage Software. Histobars shown below each panel indicate mean signal intensity. The results of four additional independent experiments (panel B) correlate nicely with test 1.



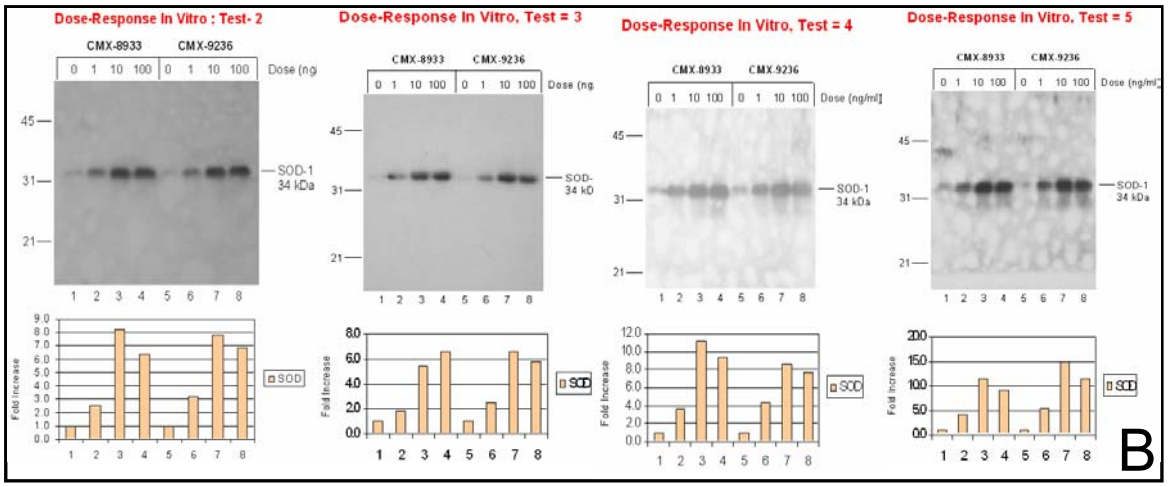


Fig 13. Peptides 8933 and 9236 Increase SOD Levels in a Rat Primary Cortical Cells in a Dose-Dependant Manner. Panel A represents the Test-1 western blot for CMX-8933 and CMX-9236. Peptide stimulations were carried out for 5 hours. Panel B shows the results of four additional sets of the same experiments. Arrows on the right denote the positions of 34 kDa SOD-1. Arrows on the left denote the positions of biotinylated markers. Histobars represent the signal strength quantitated using Scion image.

The means of trials 1 through 5 for the *in vitro* dose response experiment are shown in figure 14. The figure shows that both peptides 8933 and 9236, at an optimal dose of 10 ng/ml, cause an average increase of 8.5 fold in SOD levels over the vehicle-treated controls with a significant p value ($p < 0.001$).

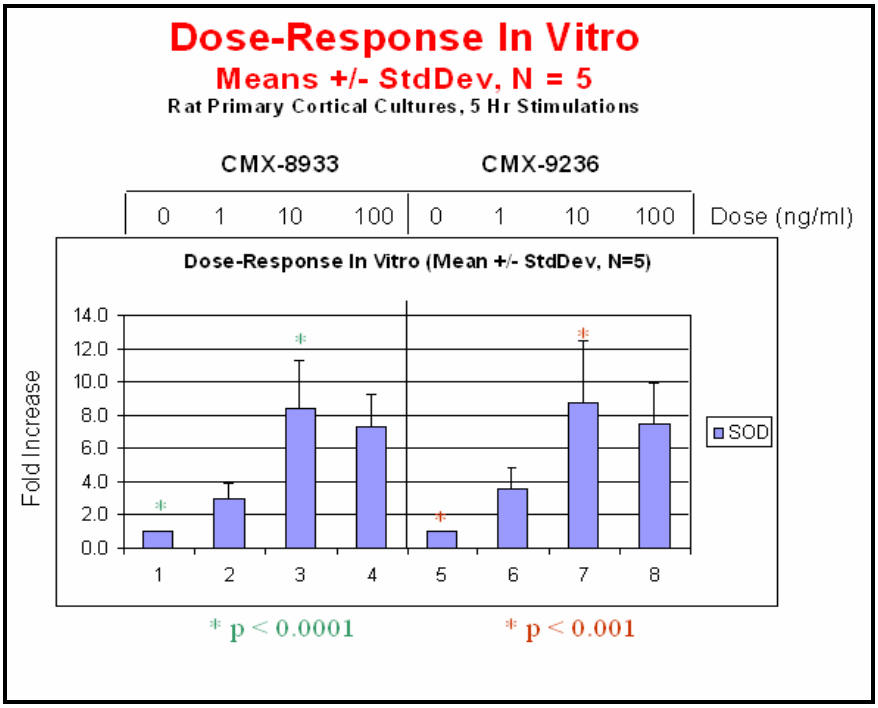


Fig 14. Statistical Analysis of Five *In Vitro* Dose-Response Experiments. Error bars denote standard deviations. Asterisks denote p values on selected sample pairs.

In Vitro Time-Course Experiment

To determine the optimal time period at which the SOD response occurs following treatment of rat cortical cultures with therapy peptides 8933 and 9236, a time-course experiment was performed. Figure 15 shows the result of an *in vitro* time-course experiment wherein rat primary cortical cultures were treated with 100 ng/ml of peptide 8933 or 9236 for a period of 1, 5 and 24 hours. A maximal increase in SOD levels was observed at 5 hour peptide treatment indicating the optimal time point. A prolonged

treatment for 24 hours did not further increase the SOD levels, again indicating a post-saturation effect. Figure 15 B shows the results of two additional independent trials with an identical pattern of SOD upregulation, thus confirming reproducibility of the data.

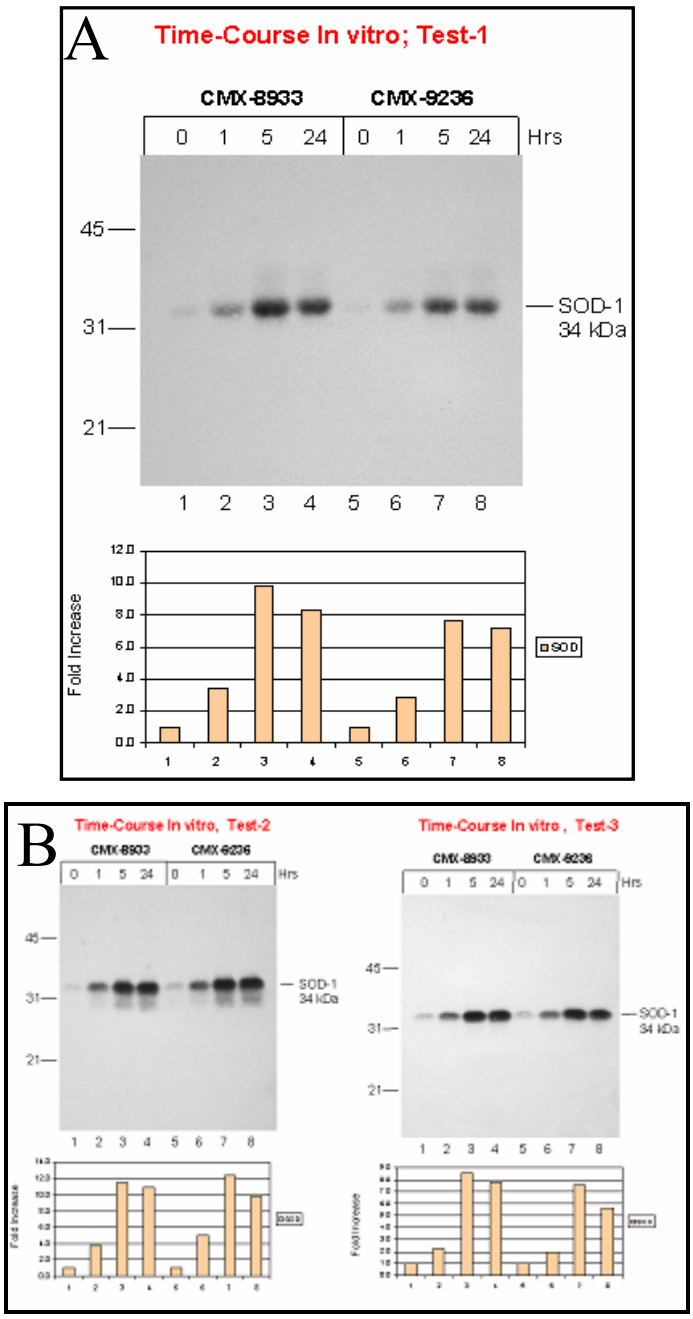


Fig 15. Time-Course of Rat Cortical Cultures Treated with Peptide 8933 or 9236 Show Maximum SOD Elevation at 5 Hours. Panel A represents Test-1 western blot for CMX-8933 and CMX-9236 at 100 ng/ ml. Panel B shows the results of two additional independent trials.

The means of test 1 through 3 for the *in vitro* time-course experiment are shown in figure 16. The figure shows that both peptides 8933 and 9236 increased SOD levels compared to the vehicle-treated control optimally at the 5 hour time point with average increases of 10 fold and 8.5 fold for CMX-8933 and CMX-9236, respectively ($p < 0.001$ in each case).

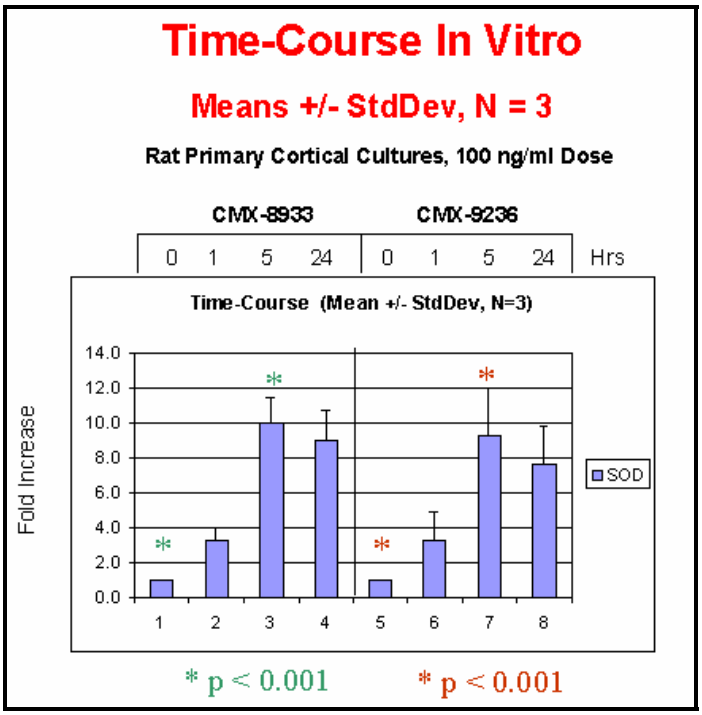
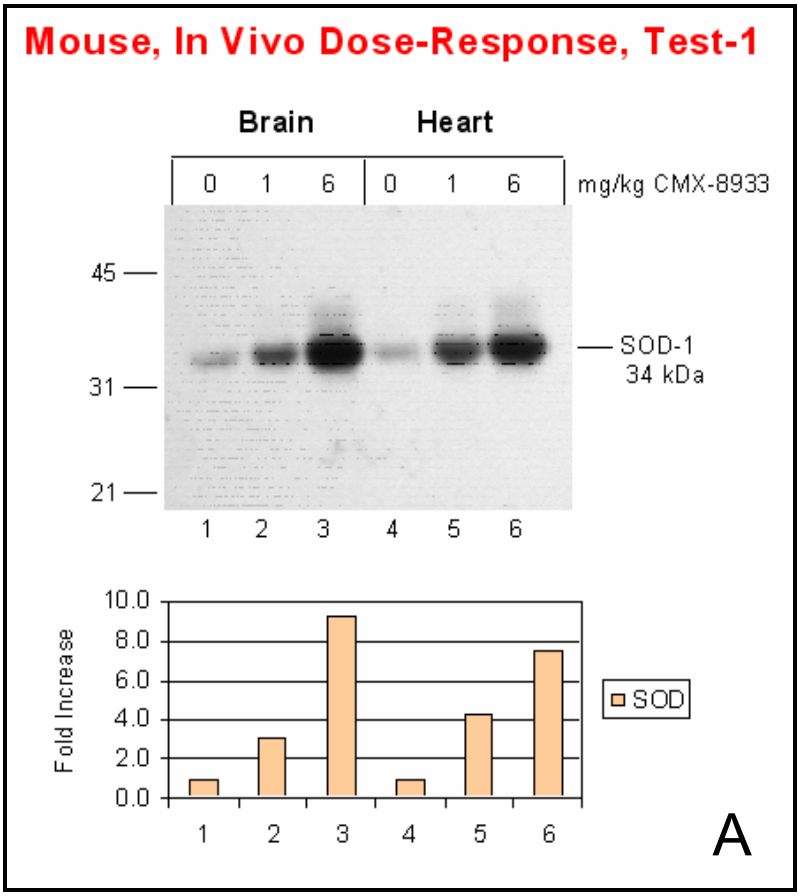


Fig 16. Statistical Analysis of Three *In Vitro* Time-Course Experiments.

Mouse, In Vivo Dose-Response Experiment

Our next goal was to perform a mouse, *in vivo* dose response experiment to investigate if there were any changes in SOD protein levels in the brain and heart of mice treated i.v. with the therapy peptide. Mice were treated for five hours with either 1 or 6 mg/kg of peptide 8933. Total cellular proteins were then isolated from brain and heart post-treatment and examined for SOD upregulation using western blots. Figure 17 shows a dose-dependent upregulation of SOD levels in both the brain and heart of mice. The

results were reproduced in four separate experiments (fig 17B), each of which is consistent with the data shown in fig 17A.



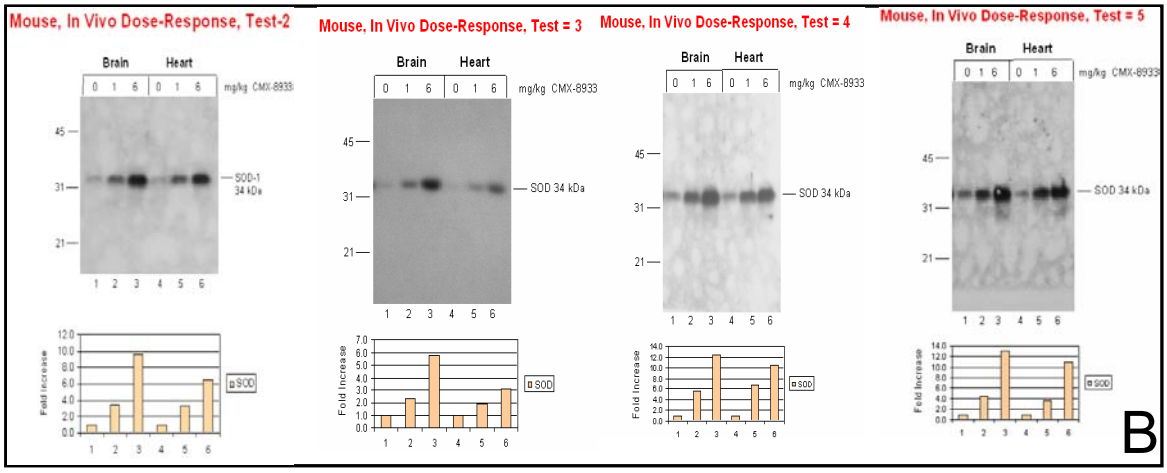


Fig 17. CMX-8933 Elevates SOD Levels in the Brain and Heart of a Mouse. Mice were injected *i.v.* with various doses of peptide, then sacrificed 5 hours later. Total cellular proteins isolated from mouse brain and heart post-peptide treatment were analyzed using immunoblots. Panel A shows SOD elevation in a dose-dependent manner in each organ. Panel B shows the results of four additional independent trials.

The means of trials 1 through 5 for the mouse *in vivo* dose response experiment are shown in figure 18. The figure shows a 4 fold elevation in SOD levels in both the brain and heart at a 1 mg/kg dose of peptide CMX-8933 compared to untreated controls. The highest dose given, 6 mg/kg, increased SOD levels an average of 10 fold in the brain and 8 fold in the heart ($p < 0.001$ in each case).

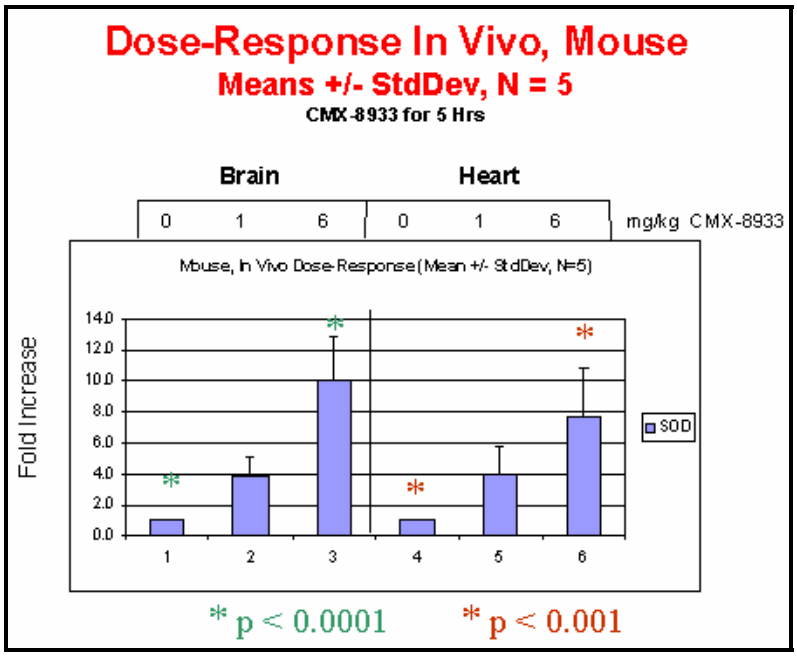
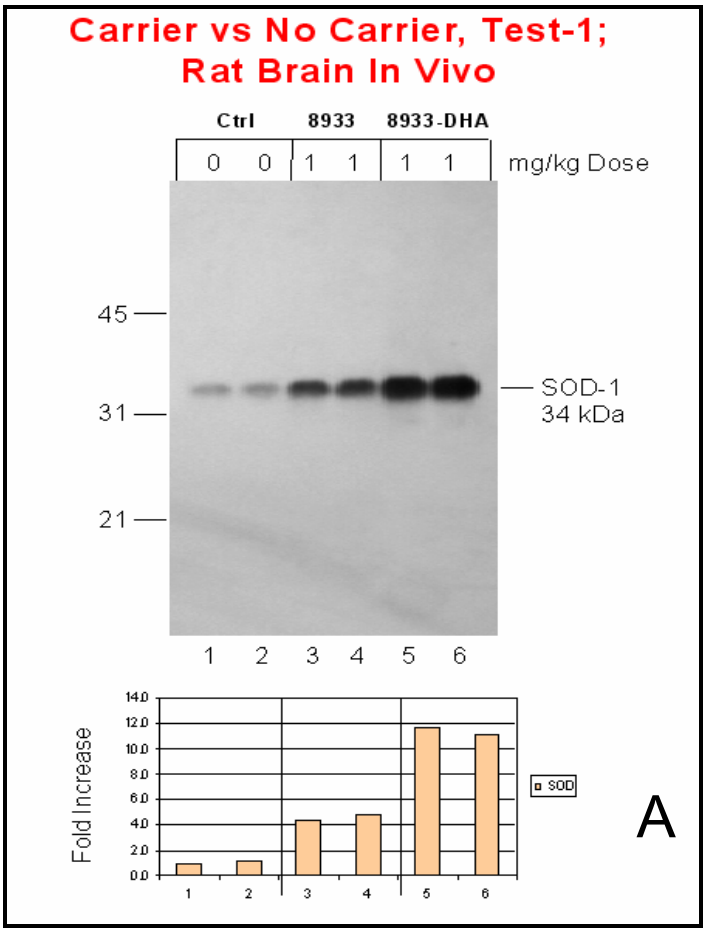


Fig 18. Statistical Analysis of Five Mouse, *In Vivo* Dose Response Experiments.

8933-DHA-BBB Conjugate Can Further Increase the Expression of SOD

Since previous data indicated that BBB carrier DHA could facilitate the delivery of dopamine to the brain (Shashoua and Hesse, 1996), we wanted to examine if the DHA carrier had any potential effect on the ability of 8933 to increase SOD levels. To analyze this, rats were injected *i.v.* with 1 mg/kg of DHA-8933 (peptide 8933 coupled to a DHA carrier) or unconjugated 8933 for a period of 5 hours. Figure 19 clearly indicates that, following systemic administration, the DHA-8933 conjugate is capable of further increasing the SOD titers in rat brain compared to the unconjugated peptide.



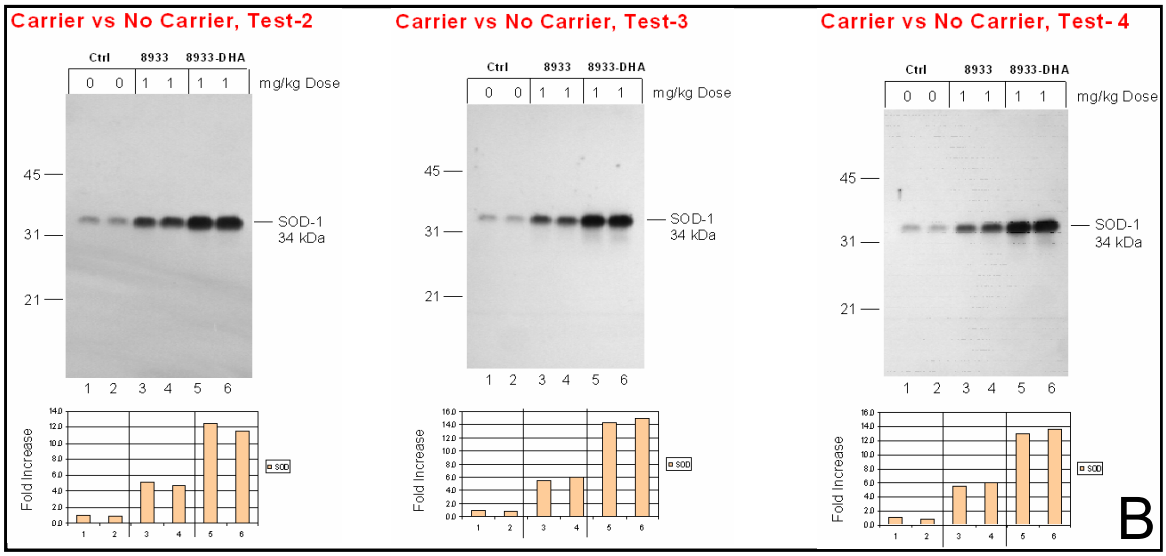


Fig 19. The 8933-DHA Conjugate Shows Further Increased SOD Expression Compared to Unconjugated 8933. Mice were injected *i.v.* with 1 mg/kg peptide (on vehicle control) and animals were sacrificed 5 hours later. Western blot analysis was performed using total cellular proteins isolated from peptide treated rat brains. The experiment was performed with duplication of doses. Panel B shows the results of three additional trials with similar observations.

The means of trials 1 through 4 for the *in vivo* carrier experiment are shown in figure 20. The uncoupled peptide 8933 at a dose of 1 mg/kg alone causes an average 5 fold increase in SOD levels compared to the vehicle-treated control (p=0.001). However coupling the peptide 8933 to DHA can bring about a further 2.4 fold increase (12 fold overall) in SOD levels at the same dose compared to the uncoupled 8933 (p<0.0001).

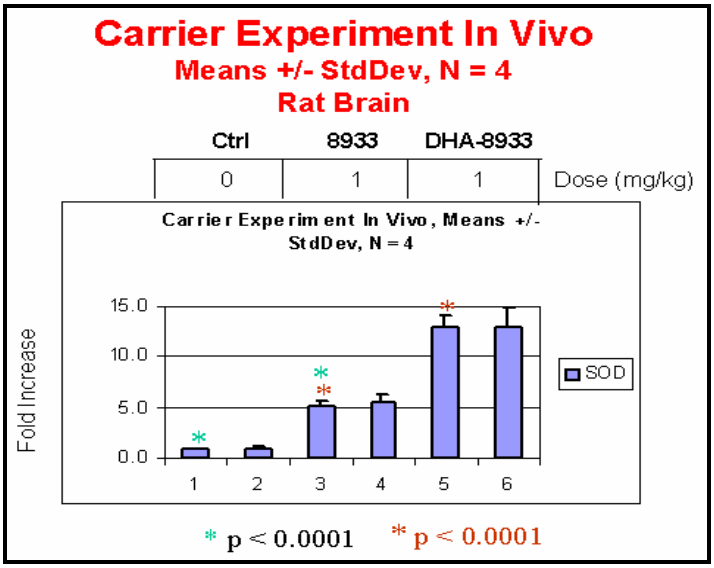


Fig 20. Statistical Analysis of Four Carrier vs Non-Carrier Experiments.

CMX-8933 Can Increase SOD Levels Beyond Normal Anoxic SOD Levels

Special cell culture incubators have recently been used as *in vitro* models for ischemia. We tested whether peptide therapy worked in this "*in vitro*" stroke system. Rat primary cortical cultures were conditioned in an anoxic environment by preincubating the cultures for a period of 24 hours in an environment of N_2, H_2, and CO_2 gases followed by reoxygenation. Peptide stimulation was then performed for 5 hours. The results of SOD Western blots performed on cells grown in anoxic conditions and treated with different doses of peptides 8933 and 2115 (8933 + two more N-terminal aa) are shown in figure 21. Lane 1 represents culture grown under normal oxygen conditions used as a negative control. SOD band in untreated anoxic cultures (lane 2) shows a 2.5

fold increase compared to the normoxic cultures (lane 1). This data directly indicates that anoxic cultures may have an inherent defense mechanism by which they upregulate SOD (and potentially other anti-oxidative enzymes) when placed in an anoxic environment. Both therapeutic peptides CMX-8933 and CMX-2115 also upregulate SOD levels in a dose dependent manner beyond the levels caused by anoxia alone (approx 8 fold totally).

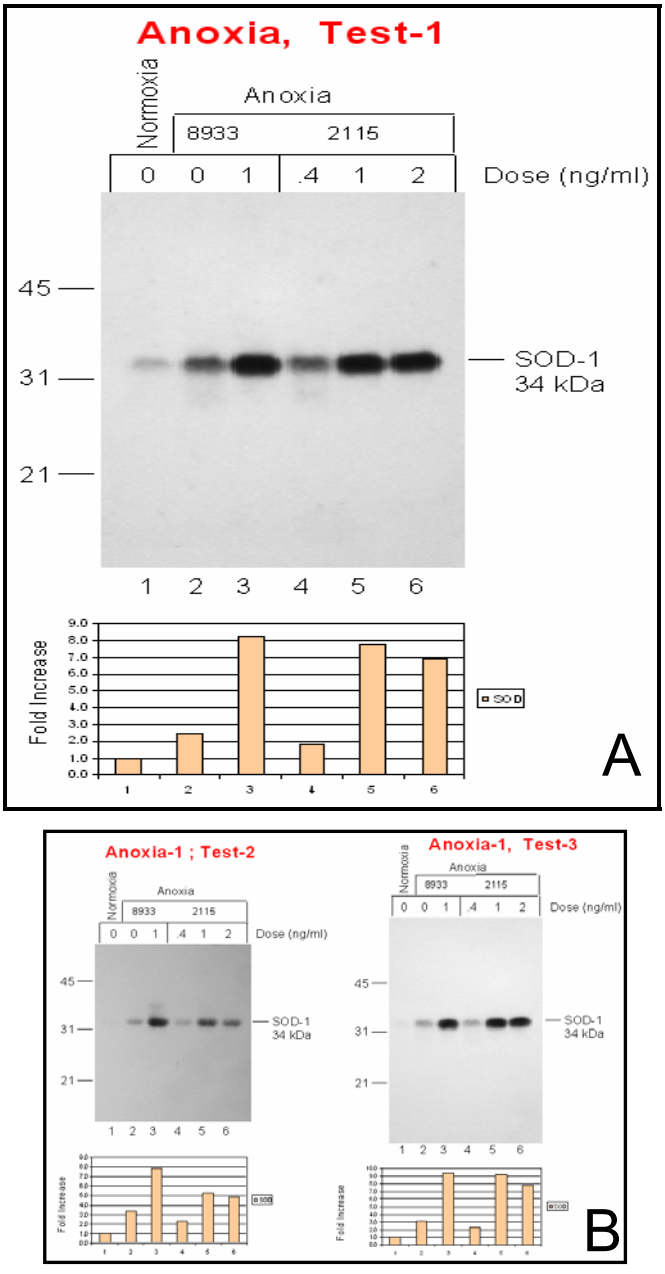


Fig 21. Western Blot Analysis Using Anoxic Neuroblastoma Cells Treated with Therapy Peptides 8933 and 2115. Anoxic untreated cultures (lane 2) shows SOD upregulation in contrast to normoxic untreated cultures (lane 1). SOD levels are elevated in a dose-dependent manner by 5 hours of peptide therapy. Panel B shows results of 2 additional trials.

The means of trials 1 through 3 for the anoxia experiment are shown in figure 22. The figure shows that anoxia alone (lane 2) causes an average 3 fold increase in SOD levels over the negative control grown under normal oxygen conditions (lane 1) (p= 0.003). A dose of 1 ng/ml of peptide 8933 caused a further 2.5 fold increase in SOD levels (8.5 fold totally) compared to untreated anoxic cultures (lane 2) ($p<0.001$). Peptide 2115 at the same 1 ng/ml dose (lane 5) caused an overall 7.5 fold increase in SOD levels compared to the negative control. A post-saturation effect was observed as the dose of peptide 2115 was doubled to 2 ng/ml (lane 6).

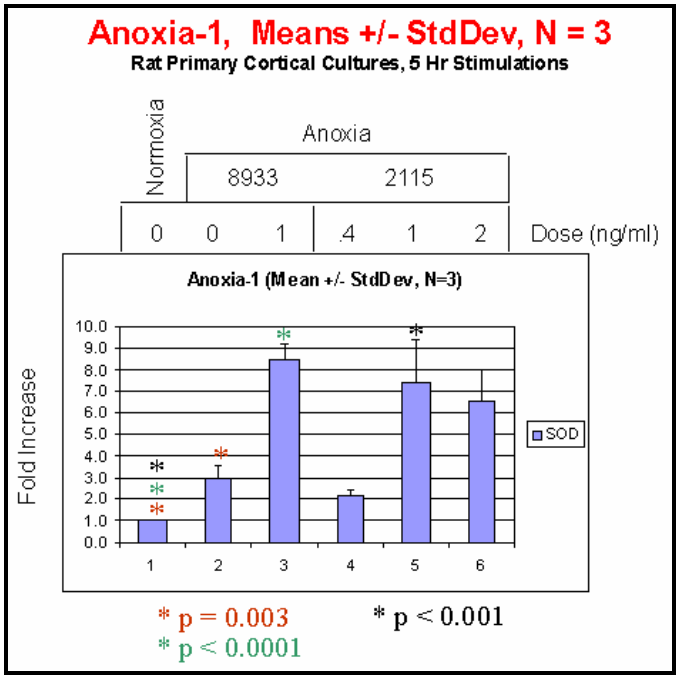


Fig 22. Statistical Analysis of Three Anoxia Experiments.

EPN Sequences, Both Left and Right of 8933 Fail to Upregulate SOD

CMX-8933, an 8 amino acid peptide, is a proteolytic cleavage product of ependymin and exists naturally in the cerebrospinal fluid. We wanted to examine if other

8 amino acid sequences present in goldfish ependymin also behave like 8933 to upregulate SOD. We chose negative peptides which were 8 amino acids left or right of the 8933 consensus sequence. Figure 23 shows the result of the negative peptide western experiment performed on rat primary cortical cultures to analyze SOD upregulation. As expected, a 5 hour stimulation with peptides 8 aa left of 8933, or 8 aa right of 8933, at any dose tested (including 10 fold higher than tested with 8933) failed to produce any detectable elevation in SOD levels. Hence it is likely that only specific portions of EPN (such as 8933) actually bind the putative receptor to induce signal transduction.

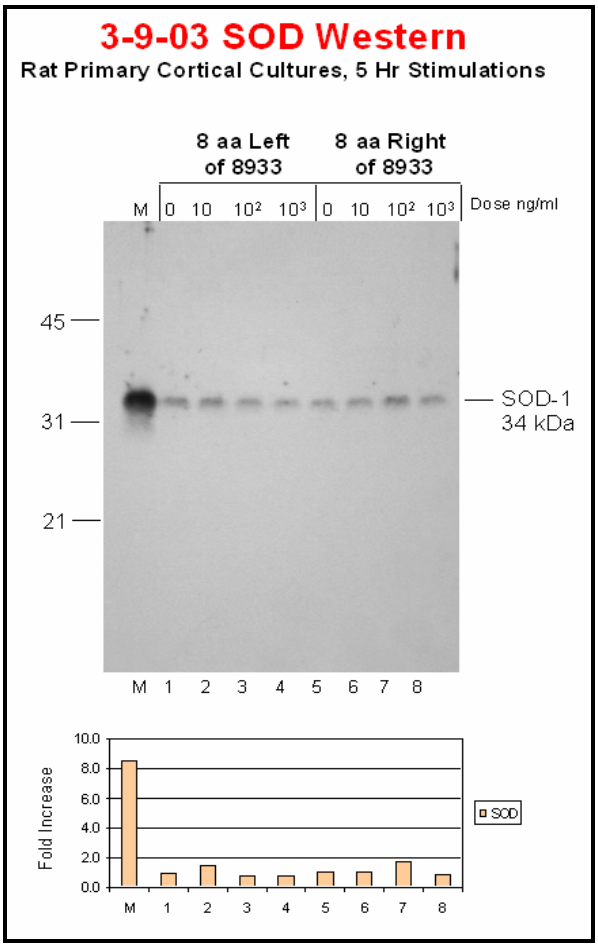


Fig 23. Western Blot Analysis Using Primary Rat Cortical Cultures Treated with Negative EPN Peptides. Lane M represents the SOD marker as a positive control (8933 10 ng/ml). The negative peptides failed to increase SOD signals.

In summary, the data shows that 1) treatment of primary rat cortical cultures with goldfish peptides 8933 and 9236, representing a portion of the EPN sequence increases the cellular titers of SOD mRNA and SOD protein; 2) Coupling 8933 to DHA BBB-carrier further increases SOD expression in the brain compared to 8933 alone; 3) anoxia induced *in vitro* by oxygen deprivation shows SOD upregulation as part of the cell's natural response to anoxia; 4) treatment of anoxic Nb2a cells with 8933 further increases SOD levels compared to the levels for anoxia alone.

DISCUSSION

The data from this study demonstrate that EPN peptide mimetics CMX-8933 and CMX-9236 that are therapeutic in a rat stroke model are also capable of upregulating the antioxidative enzyme SOD. This conclusion is based on three independent criteria; Treatment of primary rat cortical cultures with CMX-8933 or CMX-9236 increased SOD protein levels in 1) *in vitro* dose-dependent and time-course studies; 2) *in vivo* rat brain and heart; 3) an *in vitro* axoxic neuroblastoma model as a further stimulant to ischemic pre-conditioning. In addition the data from this thesis demonstrates that *i.v.* administration peptides conjugated to DHA, a blood-brain barrier carrier, further elevates the expression of brain SOD. Stimulation of anoxic cultures with CMX-8933 elevated SOD levels beyond natural anoxic levels. These latter experiments were not performed with CMX-9236.

Superoxide dismutase, Catalase and GPX are three important enzymes utilized by cells to keep oxidative stress at 'safe' levels. Because SOD converts superoxide (or reactive oxygen species, ROS) to hydrogen peroxide (also a toxic ROS precursor), it is important that either catalase or GPX which reduce hydrogen peroxide to water and oxygen, are also upregulated or remain highly active to accommodate the increased H_2O_2 production from SOD. Previous experiments in our lab have shown elevated catalase and GPX levels in neuroblastoma cells when stimulated with other EPN peptide mimetics (Armistead, 2001). However SOD is of utmost importance since it is directly involved with reducing oxidative stress and hence this project focused predominantly on SOD. The *in vitro* time and dose-response data show an average of 8.5 fold increase in SOD levels after 10 ng/ ml peptide treatment for 12 hours over the vehicle-treated controls with a significant p value. Higher doses of the peptide or longer

duration of peptide treatment did not further elevate the SOD levels indicating a possible post-saturation effect of the putative EPN receptors. The mouse *in vivo* dose response experiment also showed that peptide CMX-8933 is indeed effective in a whole animal. A dose-dependent increase in SOD was observed in both the brain and heart, with a dose of 6 mg/kg of CMX-8933 raising levels an average of 10 fold in the brain, and 8 fold in the heart. This analysis was not performed with CMX-9236. RT-PCR analysis to assess SOD mRNA elevation in addition to SOD protein level also gave fruitful results, with a 6.5 fold elevation in SOD mRNA level observed at a dose of 10 ng/ ml CMX-8933 for 5 hours. Altogether these data demonstrate SOD upregulation following EPN peptide treatment in *in vivo* and *in vitro* model systems.

Preliminary studies in a large number of laboratories have shown that SOD plays a major role in neuronal protection after a focal ischemic insult. Studies have shown that ischemic infarct is significantly increased in mice that lack the SOD gene (Mikawa *et al*., 1995). A variety of studies have revealed that transgenics over-expressing SOD appear to build resistance to cerebral ischemia (Chan *et al*., 1998; Sheng *et al*., 1999; Li *et al*., 2001). However reports of the effect of cerebral ischemia on SOD expression and activity *in vivo* are contradictory. One study conducted to measure SOD activity in the serum of patients with acute ischemic stroke observed a decreased SOD activity within 24 hours after the onset of symptoms (Spranger *et al*., 1997). Therefore, it seems likely that antioxidants are depleted as a consequence of an excessive production of oxygen free radicals very early after the ischemic insult, and hence supplementation with endogenous SOD may be beneficial. Because SOD is upregulated by our peptides, we predict that oxyradicals and oxidative stress are reduced. Although some cells may use oxyradicals

as signaling molecules (like heart cells), elevation of SOD levels does not appear to damage such cells in transgenic animals expressing SOD in all tissues.

I next addressed the issue of using DHA as a brain delivery vehicle for these EPN peptides. DHA is an abundant fatty acid in synaptic endings (Sun and Yau, 1976), and hence it is rightly termed a 'naturally occurring carrier'. DHA also facilitates rapid transport of dopamine into the brain (Shashoua and Hesse, 1996). The data from this thesis reveal that rats treated *in vivo* with 8933-DHA-conjugate doubled the SOD expression in the brain compared to the same dose of uncoupled 8933. This finding is in agreement with previous research wherein coupling DHA to dopamine increased the brain uptake of dopamine by over 10-fold (Shashoua and Hesse, 1996). Moreover Protarga, Inc. uses DHA with a variety of therapeutic drugs (currently in clinical trials). DHA by itself has no effects on AP-1 signaling (Shashoua *et al*., 2001), however the potential effects of DHA on SOD were not analyzed in this thesis. Some researchers believe that administration of neurotrophic factors intravenously would allow for widespread distribution of the drug throughout brain parenchyma even without using BBB carriers (Zhang and Padridge, 2000). However specific brain uptake of full length NTFs has not yet been demonstrated. One strategy for obtaining neuroprotective effects in brain following the noninvasive (intravenous) targeting of NTFs is the chimeric peptide technology (Padridge, 1997).

Recent studies have emerged with a new *in vitro* model for stroke. This model uses an induced ischemic environment *in vitro* in a special cell culture incubator. Anoxia experiments were designed to model *in vitro* the effects of a naturally occurring stroke. My western anoxia experiments strongly reveal that untreated anoxic cultures show a 3 fold SOD upregulation compared to the normoxic untreated cultures, thus indicating that

these rat primary cells have an inherent mechanism by which they increase SOD levels when placed in a low oxygen environment. This is particularly interesting because even without NTF treatment, rat neurons appear to naturally possess a mechanism for up-regulating a protective enzyme that reduces free radicals levels following a stroke. This finding extends and solidifies earlier findings in turtles exposed to an anoxic environment in which SOD and catalase levels dramatically increased (Willmore and Storey, 1997). However, no one to date has studied SOD upregulation in anoxic mammals and hence this present study done on rat primary cultures is the first of its kind. Cells grown under anoxic conditions then treated with either peptide CMX-8933 or CMX-2115 (CMX-8933 plus two extra amino acids) showed an additional 2.5 fold increase in SOD levels. Although many researchers have tried to elucidate the molecular pathways involved in mediating the adaptive response to oxygen deprivation, the precise mechanism of the resulting ischemic tolerance in stroke therapy remains a mystery.

A good future experiment for measuring the SOD's anti-oxidative activity would be the TBARS (thiobarbituric acid reactive substances) assay. This dye measures the overall oxidative stress on a cell lysate. Anoxic cultures are already subjected to oxidative stress and hence should contain a mixture of TBARS reactive lipid hydroperoxidase and aldehydes. Treatment of these cultures using EPN peptides shown here to elevate SOD levels should lower TBARS reactivity. This sensitive assay allows for screening and monitoring lipid peroxidation, and a direct measurement of the anti-oxidative activity of EPN peptides by fluorometry or spectophotometry.

Previous work has provided evidence that CMX-8933 and CMX-9236 promote activation of transcription factor AP-1 (Shashoua *et al*., 2001; Adams *et al*., 2003). These studies demonstrate that these small peptides can have properties similar to those

of large protein neurotrophic factors such as BDNF and NGF, which also stimulate neuronal growth via the activation of AP-1 (Gaiddon *et al*., 1996; Tong and Perez-Polo, 1996). Because goldfish EPN is difficult to isolate free from contaminating NGF, the study to prove that full-length EPN activates AP-1 is in progress. Inhibition experiments performed in our lab have shown that AP-1 is activated via the mitogen-activated protein kinase (MAPK) pathway (Hasson, 1998; Adams *et al*., 2003). Based on these known findings, and analogies to findings in other systems, we proposed a working hypothesis for these peptides. CMX-8933 representing a naturally cleaved product of EPN, exists extra-cellularly, and may interact with a cell surface receptor that initiates a cascade of biochemical steps leading to AP-1 activation and SOD upregulation. In contrast during therapeutic administration of DHA-8933 these peptides exert their biological effects not only by interacting with presumed EPN receptors, but also by direct delivery through the cell membrane via the DHA carrier. In this latter case, it could chelate excitotoxic calcium rather than interact with EPN receptor. However a detailed analysis of the precise pathways and genes activated by EPN will aid our understanding of its mechanism.

The study presented here provides solid evidence for SOD upregulation in neuronal cells by EPN peptides CMX-8933 and CMX-9236. However the negative peptide experiment performed in this study showed that peptides 8 aa left of 8933 and 8 aa right of 8933 do not show significant SOD upregulation at doses 10-fold higher than optimal 8933 doses, hence it is likely that only specific portions of EPN (such as 8933) actually bind the putative receptor to induce signal transduction. Thus future analysis for SOD upregulation using the full-length ependymin molecule needs to be tested.

Since SOD upregulation by itself is not enough to alleviate destructive neuronal cell death following stroke, more light should be thrown on various other proteins/genes also upregulated by these peptides. The rat nylon hybridization array data generated an interesting expression profile with a large category of genes, each playing different roles in many biological processes. The fact that calcium-binding proteins like calmodulin, calphobindin etc. were upregulated in the array data indicates a possible role of CMX-8933 in counteracting calcium accumulation following stroke. However analysis by RT-PCR to confirm this upregulation did not give positive results, and produced bands of equal intensity for the control and drug treated. One reason for this failure could be that we used rat nylon arrays hybridized to rat primary cortical cell cDNAs, but performed RT-PCR with RNA isolated from murine neuroblastoma cells using mouse primers. Hence the RT-PCR could be repeated in rats. The other reason for this disconnect with the array data for calcium-binding proteins could be the obvious variations encountered in array data. Additional repeated array experiments to confirm reproducibilty could have helped us build a strong case, but the high costs restricted us to just one trial. Immunoblots using calmodulin antibody (purchased from Zymed) to analyze calmodulin protein upregulation by EPN peptides in primary rat cortical cultures failed to produce any bands. We learned after consultation with some western experts that calmodulin, a low abundance protein is very difficult to visualize using any calmodulin antibody.

In conclusion, the data in this thesis are consistent with the proposed role of EPN peptides CMX-8933 and CMX-9236 in increasing the cellular titers of the antioxidative enzyme SOD, and thus hold potential as a future treatment in the battle to reduce brain damage induced by stroke. Research will continue to investigate the possibility of newer and more effective neurotrophic peptides derived from goldfish ependymin.

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