

**Mechanism of Reversal of Alzheimer's Disease A β -Induced Neuronal
Degeneration in Cultured Human SHSY Cells Using
A Neurotrophic Ependymin Mimetic**

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder that leads to dementia in adults. The mechanism of neurodegeneration is thought to involve the extracellular production of a highly toxic amyloid-beta ($A\beta$) peptide that engages cell surface receptors to induce cellular oxidative stress and apoptosis, but the signal transduction pathways that lead to $A\beta$ -induced cell death are unknown. We previously showed that a human ependymin neurotrophic peptide mimetic (hEPN-1) can promote cell survival in an *in vitro* AD model system. This initial observation was extended in this thesis by investigating the mechanism of $A\beta$ -induced apoptosis and hEPN-1-induced survival. Immunoblots were used to assay the total cellular levels of specific caspase proteins. The results show that $A\beta$ -induced apoptosis uses an extrinsic caspase pathway involving caspases-2 and -3, and that hEPN-1 treatment can reduce those caspase levels. A caspase activity assay showed that $A\beta$ increased caspase-3/7 activity, while hEPN-1 treatment lowered it. Moreover, *in vivo* studies with AD transgenic mice showed that hEPN-1 treatment increased antioxidative superoxide dismutase levels in brain. Thus, hEPN-1 holds potential as a therapeutic to treat the underlying neurodegenerative cause of AD, not merely its symptoms as with other currently approved AD drugs.

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PROJECT SUMMARY

Alzheimer's disease (AD) is a neurodegenerative disorder that leads to progressive cognitive deterioration. The main pathological lesion in AD is thought to be a 42 aa long highly neurotoxic amyloid- β (A β) peptide unnaturally cleaved by beta and gamma secretases from the normal neuronal cell surface protein amyloid precursor protein (APP). Released extracellular A β engages cell surface receptors to cause cell death through a series of events involving cellular oxidative stress and apoptosis. An *in vitro* model for AD uses cultured human SHSY-5Y (commonly abbreviated SHSY) neuroblastoma cells treated with Yankner peptide, an 11 amino acid peptide representing A β residues 25-35 that strongly binds receptor. Treatment of SHSY cells with 20 μ M Yankner peptide strongly induces cellular apoptosis. An *in vivo* model for AD uses a double transgenic Alzheimer's mouse with increased production of A β .

Ependymin (EPN) is a neurotrophic factor (NTF) initially shown to function in goldfish long-term memory formation and optic nerve elongation; however, mammalian versions of EPN have recently been shown to exist. Our lab, and our collaborators at Biotherapeutix Inc. (Waltham, MA), are currently investigating short peptide mimetics of human EPN (the short versions more efficiently cross the blood brain barrier) as potential therapeutics for neurodegenerative disorders. Previous experiments in our lab have shown that CMX-8933 (a short goldfish EPN mimetic) stimulates the MAPK signal transduction pathway to activate AP-1 transcription factor in mouse neuroblastoma cells (Adams *et al.*, 2003) which can function as a master switch in long-term memory consolidation. CMX-8933 has also been shown to increase expression of anti-oxidative enzymes such as superoxide dismutase-1 (SOD-1) in mouse neuroblastoma cells (Parikh, 2003). hEPN-1 (a short human EPN mimetic) was previously shown by our laboratory to upregulate neuronal growth genes (Arca, 2005), and has shown

partial restoration of cell population survival in an *in vitro* model for Alzheimer's disease (Stovall, 2006).

This thesis had two main hypotheses. First, since it is known that the Yankner peptide induces neuronal apoptosis *in vitro*, we hypothesized that the apoptosis is mediated by an increase in cellular caspase levels or activities, likely caspases-2 and -3 associated with an externally stimulated cell death pathway. Because hEPN-1 treatment can partially restore neuronal cell survival, we hypothesized that increases in caspase-2 and -3 levels or activities can be reversed by hEPN-1. Second, since A β -induced neuronal apoptosis is widely known to increase cellular oxidative stress, and ependymin mimetic CMX-8933 has previously been shown to increase antioxidative SOD-1 expression in mouse Neuro-2a cells, we hypothesized that hEPN-1 can upregulate SOD-1 in an Alzheimer's mouse model.

The initial set of experiments was designed to test the validity of the *in vitro* AD assay with respect to the ability of the Yankner peptide to induce neuronal apoptosis, and to determine useful hEPN-1 therapeutic doses. SHSY cell numbers were analyzed *in vitro* in microtiter wells treated at the time of plating with no peptides (control), 20 μ M Yankner peptide (based on previous data), or Yankner peptide + hEPN-1 therapeutic at various concentrations, then cultured for 3 days. After 3 days, the numbers of cells in each well were determined by microscopy. Statistical significance was determined using SPSS software and a One Way ANOVA test followed by LSD post-hoc tests. In six independent trials, the average cell numbers per microtitre well decreased 31.7% in the presence of 20 μ M Yankner compared to the control ($p < 0.001$). Upon simultaneous treatment with 20 μ M Yankner + 75 μ M hEPN-1, + 150 μ M hEPN-1, or + 225 μ M hEPN-1, the average cell numbers increased by 37.7%, 62.5%, and 51.6%, respectively compared to the Yankner treatment alone ($p < 0.001$, for all treatments). The control mean was statistically indistinguishable from the Yankner + 75 μ M hEPN-1 treatment, and

Yankner + 225 μ M treatment, demonstrating complete cellular recovery from the Yankner insult. In addition, the Yankner + 150 μ M hEPN-1 dose resulted in mean cell numbers statistically higher than the control (p values <0.05).

The 75 and 150 μ M hEPN-1 doses were also tested for caspase-3 upregulation (increase in cellular levels), a key end-stage effector caspase regulated by either the extrinsic or intrinsic cell death pathways. T-25 flasks were treated with peptides as described previously, then whole cell lysates were prepared for immunoblot analysis using an antibody that can detect both inactive and active forms of the enzyme. The immunoblots produced a strong band exactly the expected size of procaspase-3, whose intensity increased in cultures treated with staurosporine (a well known caspase-3 stimulator), and which disappeared in negative controls lacking the primary antibody. In five independent trials, 20 μ M Yankner treatment caused an average 5.96 fold upregulation in procaspase-3 levels compared to control (p < 0.001). Upon simultaneous treatment with 20 μ M Yankner + 75 μ M hEPN-1 or + 150 μ M hEPN-1, there was a 13.9% and 35.5% decrease, respectively, in cellular procaspase-3 levels. Although the 75 μ M treatment was on average lower than the Yankner treatment alone, it was not statistically distinguishable from that sample; however, the 150 μ M hEPN-1 treatment was statistically lower (p<0.05) than Yankner alone, demonstrating that hEPN-1 can indeed lower procaspase-3 protein levels induced by the Yankner peptide. All treatments were significantly different than the control mean (p< 0.001 Yankner alone; p< 0.001 Yankner + 75 μ M hEPN-1; p< 0.05 Yankner + 150 μ M hEPN-1). No band was observed on the immunoblots for active caspase-3, so perhaps that band represents only a small portion of the overall cellular levels, and is too low to be detected by this assay.

The cellular levels of caspase-2, a key initiator caspase in the externally stimulated cell death pathway, were also analyzed. These immunoblots produced a strong band exactly the size expected for procaspase-2, which disappeared in controls lacking the primary antibody. In five

independent trials, 20 μ M Yankner treatment caused a very strong average 67.21 fold upregulation in procaspase-2 levels compared to control ($p < 0.001$). Upon simultaneous treatment with 20 μ M Yankner + 75 μ M hEPN-1, or + 150 μ M hEPN-1, there was an average 21.6% and 45.2% decrease in cellular procaspase-2 levels, relative to Yankner alone. The larger decrease was statistically significant ($p < 0.05$). All treatments were significantly different ($p < 0.001$) than the control mean. Thus, the Yankner peptide appears to strongly elevate procaspase-2 protein levels, and treatment of SHSY cells with hEPN-1 can lower the procaspase-2 levels.

With the immunoblot analyses, no bands corresponding to active caspases-2 or -3 were observed, so no conclusion can be made using that assay about caspase activation. To address this activity question, the enzymatic *activity* of caspase-3 was analyzed using a commercially available caspase-glo assay. In three independent trials, 20 μ M Yankner treatment caused an average 16.01 fold increase in caspase-3/7 activity (the substrate can not distinguish between caspases-3 and -7, but both are end stage caspases) compared to control ($p < 0.001$). Upon simultaneous treatment with 20 μ M Yankner + 75 μ M hEPN-1, there was an average 57.7% decrease in caspase-3/7 activity compared to treatment with Yankner alone ($p < 0.05$). The control mean was statistically lower than the Yankner + 75 μ M treatment ($p < 0.05$). Thus, the caspase data overall support the first main hypothesis that A β uses an externally stimulated cell death pathway involving caspases-2 and -3, and that hEPN-1 can lower this caspase expression/activation.

Experiments were also performed *in vivo* with commercially available Alzheimer's disease transgenic mice that overexpress A β . Superoxide dismutase-1 (SOD-1) protein levels were analyzed in AD mouse brain lysates using immunoblots. In five independent trials, there was an average 1.4 fold and 2.04 fold increase in SOD protein levels when the mice were treated with 46

μM hEPN-1 or 60 μM hEPN-1, respectively. The 46 μM hEPN-1 treatment was not significantly different from untreated AD mice, but the 60 μM treatment was significantly higher ($p < 0.05$). Thus, this data supports the second main hypothesis, that hEPN-1 can raise the expression of antioxidative SOD levels in brain in an AD animal.

Several attempts were made to test the cellular levels of caspases in brain tissue *in vivo* using immunoblots, but most of the antibodies tested produced no bands. However, the antibody for caspase-7 produced a band larger than the expected size whose intensities behaved exactly as expected, so we termed the band caspase-7-related protein (C7RP). In six independent trials, there was an average 67% and 39% decrease in C7RP levels in brain lysates for AD mice treated with 46 μM and 60 μM hEPN-1, respectively. Both doses were significantly lower than untreated AD animals ($p < 0.001$).

Overall, the data in this thesis suggest that the Yankner peptide uses an externally stimulated cell death pathway involving caspases-2 and -3 to induce apoptosis in neuronal cells, that human ependymin mimetic peptide hEPN-1 can restore cell survival, and that the protection correlates with a lowering of caspase cellular levels and activity. Moreover, results from the *in vivo* data suggest that hEPN-1 is capable of upregulating anti-oxidative SOD-1 protein levels and lowering caspase-7-related protein levels in an Alzheimer's mouse model. As no current drug is known to block Yankner-induced neuronal toxicity, the hEPN-1 therapeutic shows potential in combating the underlying neuronal apoptosis of AD.

BACKGROUND

Alzheimer's disease (AD) is the most common neurodegenerative disease with more than 20 million cases worldwide (Goedert and Spillantini, 2006). One in ten persons over the age of 65, and nearly half of those 85 or older have the disease. The disease was named after the German physician Alois Alzheimer who noted extracellular formations surrounding the neurons, and tangled fibers within the neurons, while performing an autopsy on a woman who had suffered years of mental degradation. To date, the only definite way to truly diagnose AD is an autopsy to determine whether senile plaques and neurofibrillary tangles occur in the brain.

Alzheimer's Disease Mechanism

The cause of Alzheimer's disease is unknown; however, age is the most important risk factor, as the number of people with AD doubles every five years beyond the age of 65. Certain risk factor genes have also been identified for AD, including ApoE, APP (amyloid precursor protein) and Presenillin-1 and -2, each of which accelerates the production of a highly neurotoxic amyloid- β ($A\beta$) peptide from APP. Thus, amyloid is a central player in most current theories of AD (Yuan and Yankner, 2000; Hardy and Selkoe, 2002; Mattson, 2004; Armstrong, 2006; Goedert and Spillantini, 2006). APP (Figure-1) is a naturally occurring neuronal cell surface protein with unknown function. Some reports suggest APP may play a role in synaptic adhesion, and in regulating neuronal survival (Mattson, 1997). During AD, cell surface APP undergoes improper cleavage by beta and gamma-secretases to release a 42 aa length portion of the APP, known as β -amyloid ($A\beta$, aa 672-713) (yellow box in Figure-1). This 42 aa $A\beta$ fragment is capable of aggregating to form dimers, trimers, and higher order aggregates, which eventually

form extracellular senile plaques. Although senile plaques were previously thought to be the main neurotoxic agent in AD, more recent data indicate that plaque precursors (A β dimers, trimers and oligomers) are the main neurotoxic agents (Walsh *et al.*, 2002).

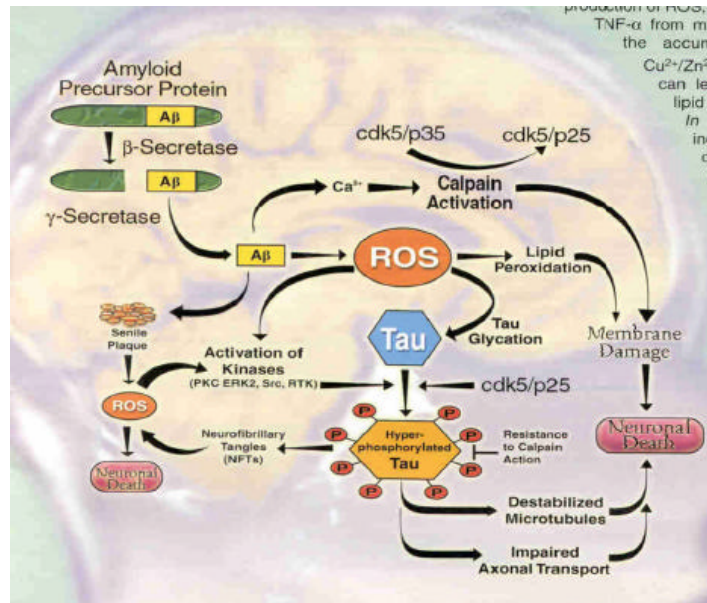


Figure 1: Schematic of Alzheimer's Disease. Improper cleavage of APP (green, upper left) by secretases leads to formation of highly neurotoxic A β (yellow). This in turn leads to a signaling cascade which involves an increase in reactive oxygen species (ROS, orange), Ca $^{++}$ influx, and Tau hyperphosphorylation (lower center) finally leading to cellular apoptosis (red). Figure from Calbiochem, 2000.

A β engages a variety of neuronal cell surface receptors (discussed in next section), to generate reactive oxidative species (ROS) (Figure-1 center), increase Ca $^{++}$ flow into the cell, increase caspase activation, finally causing cellular apoptosis (red in Figure-1). ROS and increased Ca $^{++}$ influx also leads to the hyperphosphorylation of Tau protein (Figure-1, lower center) that destabilizes microtubules (Yuan and Yankner, 2000). This process leads to the formation of intracellular neurofibrillary tangles, the second observable hallmark pathogenic lesion in AD.

A β Signal Transduction

The precise mechanism of action of A β -mediated neurotoxicity is unknown; however it has been shown that A β is capable of interacting with a number of specific neuronal cell surface receptors. A β has been shown to interact with the ‘receptor for advanced glycation end products’ (RAGE) (Yan *et al.*, 1996). RAGE is a 35 kD transmembrane receptor of the immunoglobulin superfamily first characterized in 1992 (Neeper *et al.*, 1992). RAGE is also a neuronal receptor for amphoterin (Hori *et al.*, 1995), a molecule involved in neurite outgrowth. It has been shown that interaction of A β with RAGE induces cellular oxidative stress (Yan *et al.*, 1996) (Figure 2).

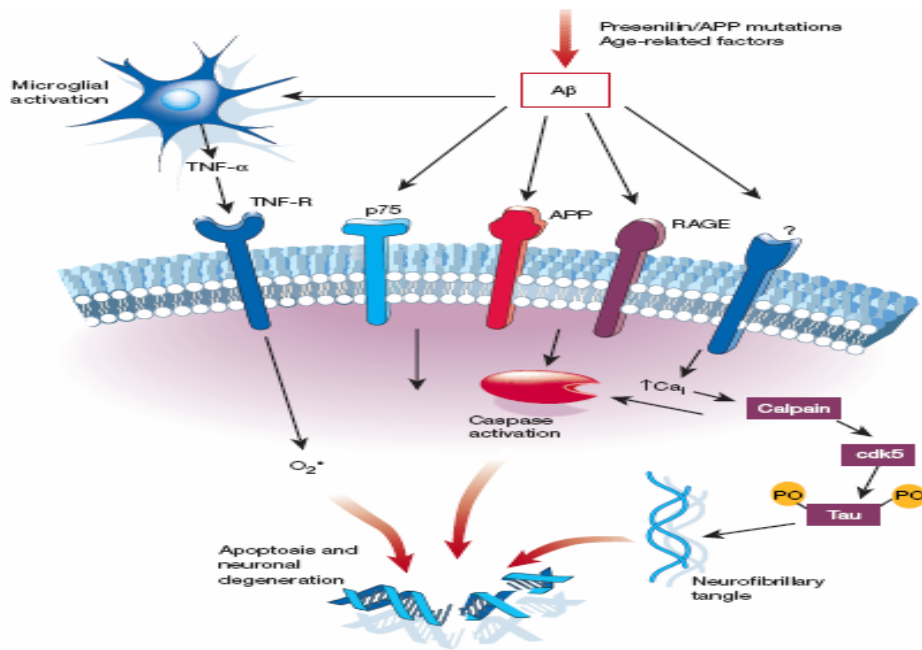


Figure-2: Engagement of Cell Surface Receptors by A β Peptide. A β peptide (red square) is capable of engaging various cell surface receptors such as RAGE, APP, and p75, and can also cause microglial activation(upper left) leading to oxidative stress, caspase activation, and finally apoptosis (lower center). (Yuan and Yankner, 2000).

The A β /RAGE interaction has further been shown to activate NF- κ B transcription factor mediated secretion of macrophage colony stimulating factor (M-CSF) (Chaney *et al.*, 2005) which interacts with its receptor C-fms on microglial surfaces causing a number of responses

including microglial chemotaxis, cell proliferation, increased scavenger receptors, and apolipoprotein-E expression, as well as oxidative stress (Guilian, 1996; Butterfield, 1997; Misonou *et al.*, 2000; Hull *et al.*, 2006; Reddy, 2006) (Figure 2). When A β activates microglia, this induces cytokine secretion that has cytotoxic proinflammatory effects (Chaney *et al.*, 2005).

In addition to RAGE, A β is also capable of interacting with p75 neurotrophin receptor, which can lead to neuronal cell death (Yaar *et al.*, 1997) (Figure 2). Interestingly, interaction of A β with APP (from which it is derived) has also been shown to induce neuronal cell death (Lorenzo *et al.*, 2000) (Figure 2). Thus the interaction of A β with a number of neuronal cell surface receptors can lead to an increase in cellular oxidative stress and induction of inflammatory signals which in turn lead to the induction of various cell-death signaling pathways (Figure-2) and caspase activation (discussed below) and apoptosis. Increasing our understanding about A β -mediated signal transduction will provide us with better therapeutic strategies to target Alzheimer's disease.

Yankner Peptide A β Mimetic

A β_{25-35} is an 11 aa long portion of the full length A β_{1-42} . This peptide (Figure-3) represents the minimal neurotoxic portion of A β_{1-42} (Yankner *et al.*, 1990), and is more commonly termed the “Yankner peptide” after its discoverer Bruce Yankner of Harvard University. When added to cultured cells, the Yankner peptide has been shown to engage the RAGE receptor and induce neuronal cell death with the same specific activity as A β (Yan *et al.*, 1996; Misiti *et al.*, 2005). The Yankner peptide has a neurotoxicity equivalent to the full length A β_{1-42} and can form fibrillar aggregates like A β_{1-42} (del Mar Martínez-Senac *et al.*, 1999). Because the Yankner peptide appears to be functionally equivalent to A β_{1-42} yet is much shorter

(less expensive to synthesize) we used the Yankner peptide to mimic AD in human SHSY cell cultures.

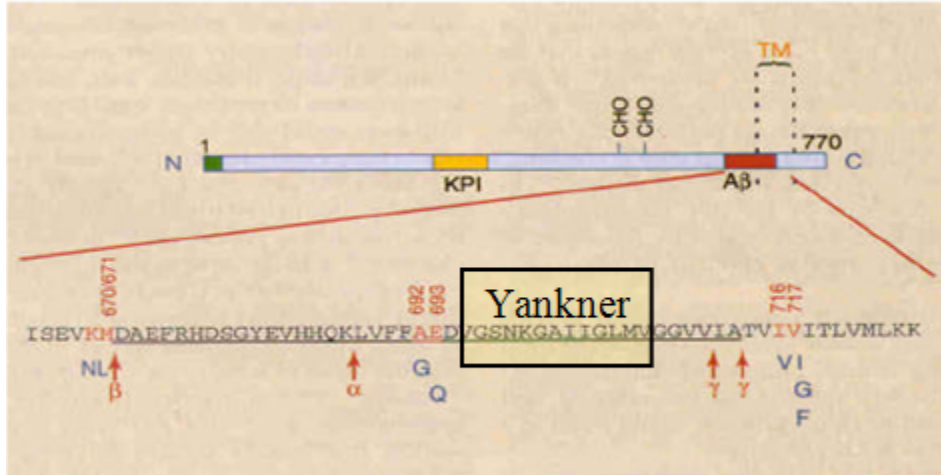


Figure 3: The Yankner Peptide. Full length amyloid precursor protein (APP) is depicted as the upper horizontal box. CHO denotes two glycosylation sites. TM denotes the transmembrane region. The red box near the C-terminal end denotes A β ₁₋₄₂. The aa sequence of key areas is shown below, with the underlined portion representing A β . Also shown are the cut sites for α , β , and γ secretases. A β ₂₅₋₃₅ (Yankner peptide) is delineated by a yellow box (Selkoe, 1999).

Caspases in Neuronal Apoptosis

Apoptosis is type of programmed cell death (PCD) which, in mammals, is regulated by the Bcl-2 family of proteins, Apaf-1 (apoptotic protease activating factor-1) and caspases (Yuan and Yankner, 2000). Caspases are the key mediators of neuronal apoptosis (Cohen, 1997; Nakagawa *et al.*, 2000). Caspases are aspartate-specific cysteine proteases initially synthesized as inactive zymogens (procaspases) which become activated via cleavage. Activation can occur via an extrinsic pathway (in which cell surface death receptors engage death ligands), or by an intrinsic pathway that involves mitochondrial or endoplasmic reticulum organelle stress (Figure-4). This family of enzymes has been shown to play a number of roles in cellular apoptosis, including: the mediation of signal transduction downstream of death receptors located on the

plasma membrane (caspases-2, -8 and -10) (extrinsic pathway) (Chen and Wang, 2002), the mediation of apoptotic signals following mitochondrial damage (caspase-9) (intrinsic mitochondrial pathway) (Li *et al.*, 1997), the mediation of apoptosis through ER stress (caspase-12) (intrinsic ER pathway) (Trapani and Smyth, 2002), and end stage effector caspases (caspases-3, -6, and -7) (Fan *et al.*, 2005) (Figure 4).

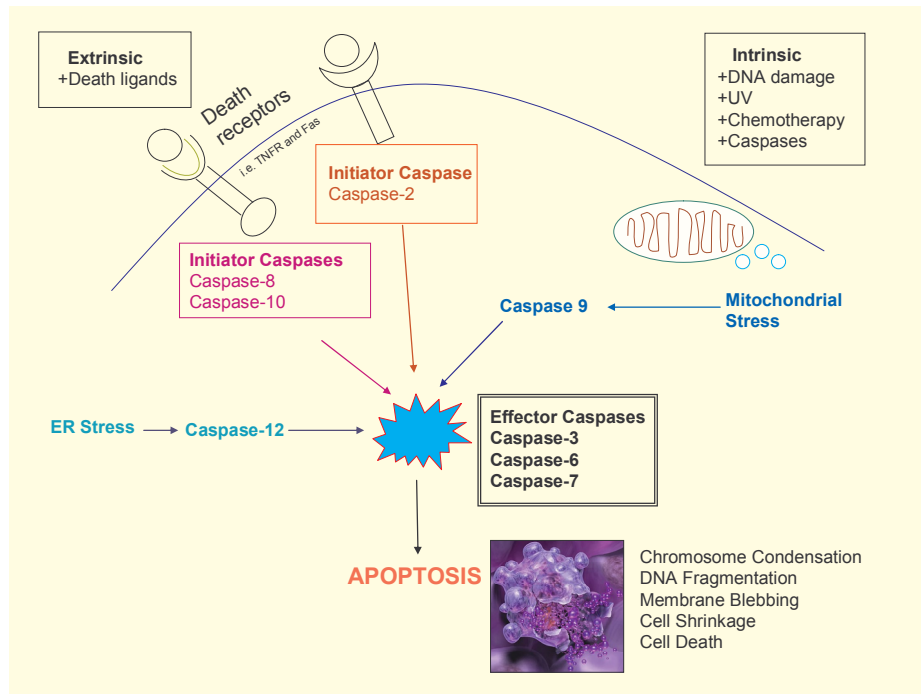


Figure 4: The Caspase Cascade Pathway During Cellular Apoptosis. Simplified depiction of the caspase cascade pathway, showing both the extrinsic (upper left) and intrinsic (upper right) caspase activation pathways which cause activation of effector caspases (center black box), finally leading to apoptosis (bottom center).

The regulation of caspase activation occurs at several different levels: a) procaspase gene transcription, b) anti-apoptotic members of the Bcl-2 family can prevent caspase activation, c) cellular inhibitors of apoptotic proteins (cIAP's) can bind and inhibit activated caspases (Earnshaw *et al.*, 1999). Based on their structure and function, caspases can be grouped into initiator caspases (caspases-2, -8, -9, -10, and -12) (Figure 4), and effector caspases (caspases-3, -6, and -7) (Fan *et al.*, 2005). The importance of caspases in neuronal apoptosis has been shown

in caspase-3 null mice which are perinatally lethal, and which show a lack of apoptosis in neuroepithelial progenitor cells during development (Kuida *et al.*, 1996). An increasing amount of evidence documents the role of apoptotic cell death in a large number of neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Holtzman and Deshmukh, 1997; Lunkes and Mandel, 1998; Namura *et al.*, 1998).

With respect to Alzheimer's disease, there is considerable evidence for the involvement of caspases in A β -induced neuronal apoptosis. In one study it was shown that caspase-2 null mice were resistant to A β_{1-42} toxicity, confirming the importance of caspase-2 in A β -induced neuronal death (Troy *et al.*, 2000). In another study, the treatment of SHSY-5Y cells with A β_{17-42} peptide caused the activation of caspase-8 and caspase-3, and the addition of selective caspase inhibitors completely blocked A β_{17-42} induced neuronal death (Wei *et al.*, 2002). In another study, a post trauma increase in A β_{1-42} production was completely abolished by caspase inhibition therapy (Abrahamson *et al.*, 2006). Thus a deeper understanding of the involvement of caspases in A β -induced neuronal apoptosis could provide a critical step for therapeutic intervention in Alzheimer's disease.

Superoxide Dismutase

Superoxide dismutase (SOD) is an anti-oxidative enzyme that catalyzes the dismutation of two superoxide anions to hydrogen peroxide and molecular oxygen (Fig. 5).

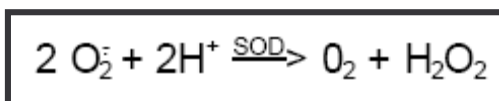


Figure 5: The Reaction Catalyzed by Superoxide Dismutase (SOD).

SOD is an important antioxidant defense in nearly all eukaryotic cells exposed to oxygen. The cytosols of virtually all eukaryotic cells contain an SOD enzyme complexed with Cu and Zn (Cu-Zn-SOD). Cu-Zn-SOD is a homodimer of molecular weight 32 kDa. Reactive oxygen species (ROS) are produced by all aerobic cells, and are widely believed to play a pivotal role in aging, as well as a number of degenerative diseases and ischemia (Venarucci *et al.*, 1999; Allen and Tresini, 2000), and their accumulation ultimately leads to oxidative stress, causing cellular damage and death. Transgenic mice overexpressing SOD show decreased infarct volumes following ischemia (Sheng *et al.*, 1999a; Sampei *et al.*, 2000) and increased resistance to neurotoxin MPTP (Przedborski *et al.*, 1992; Klivenyi *et al.*, 1998). Mice showing diminished SOD expression show increased stroke predisposition (Sheng *et al.*, 1999b; Sampei *et al.*, 2000). The cellular levels of c-Jun and c-Fos proteins increased in transgenic animals over-expressing SOD, indicating AP-1 may be involved in SOD's neuroprotection against oxidative stress (Huang *et al.*, 2001).

A β has previously been shown to increase cellular oxidative stress and the expression of HO-1 (Hemoxygenase type I) in SHSY cells (Yan *et al.*, 1995). From a therapeutic perspective, previous work performed in our lab has shown that treatment of cultured mouse neuroblastoma cells with a goldfish ependymin mimetic CMX-8933 (discussed below) can increase the gene expression of anti-oxidative genes such as SOD-1 (Parikh, 2003), catalase, and glutathione peroxidase (Shashoua *et al.*, 2004). We have also shown that the CMX-8933-induced upregulation of SOD depends on the activation of AP-1 transcription factor (Saif, 2004). Thus, the overexpression of SOD may help overcome the neurodegenerative effects of A β -induced oxidative stress.

Ependymins and Their Mimetics

Ependymins are neurotrophic factors (NTFs) that stimulate neuronal growth and regeneration. They were first discovered in the goldfish brain in the “zona ependyma” from which their name is derived (Shashoua and Benowitz, 1977), but they have since also been identified in mammals (Apostolopoulos *et al.*, 2001). Because of their potential for nerve regeneration, our laboratory, and our collaborators at Biotherapeutix Inc.(Waltham, MA), are investigating ependymins as potential therapeutics for neurodegenerative diseases and stroke. But full length ependymins when delivered systemically can not cross the blood brain barrier (BBB), so our current approach is to use small peptide mimetics of ependymins which can cross the BBB.

Peptide 8933 is an 8 amino acid synthetic portion of full-length goldfish ependymin, with the sequence KKETLQFR. Previous work done in our lab using peptide 8933 (Figure-6) has shown that when added to cultured mouse Neuro-2a cells it activates the JNK pathway and transcription factor AP-1 (a key mediator of long-term memory formation) (Adams *et al.*, 2003), and growth related proteins EF-2, L19, and S12 (Kaska, 2003). Peptide 8933 was also shown to upregulate antioxidative enzymes superoxide dismutase (SOD) (Parikh, 2003), catalase (CAT), and glutathione peroxidase (GPX) (Shashoua *et al.*, 2004) both *in vitro* in cultured Neuro-2a cells, and *in vivo* in mice (diagramed in the lower part of Figure 6). The activation of AP-1 was shown to be a required step for the upregulation of SOD by 8933 (Saif, 2004).

For this thesis project, we used peptide hEPN-1, a 14 amino acid synthetic portion of human ependymin, containing the entire analogous 8933 location plus three flanking aa on each side, with the sequence KQCSKMTLTQPWDP. Previous work in our lab showed that treatment of mouse Neuro-2a cells with hEPN-1 upregulates growth-related proteins EF-2, L19, and ATP

Synthase-C (Saif, 2004), and treatment of human SHSY cells with hEPN-1 upregulates growth related genes for S12, S19, 5.8S RNA, and 16S RNA (Arca, 2005). hEPN-1 has also shown partial restoration of cell population survival in an *in vitro* Alzheimer's model using cultured human SHSY neuroblastoma cells (Stovall, 2006). Since hEPN-1 previously showed a reversal of neuronal apoptosis in an *in vitro* Alzheimer's model, my project involved investigating the mechanism of action of this anti-apoptotic agent, while increasing our knowledge of A β -induced neuronal apoptosis.

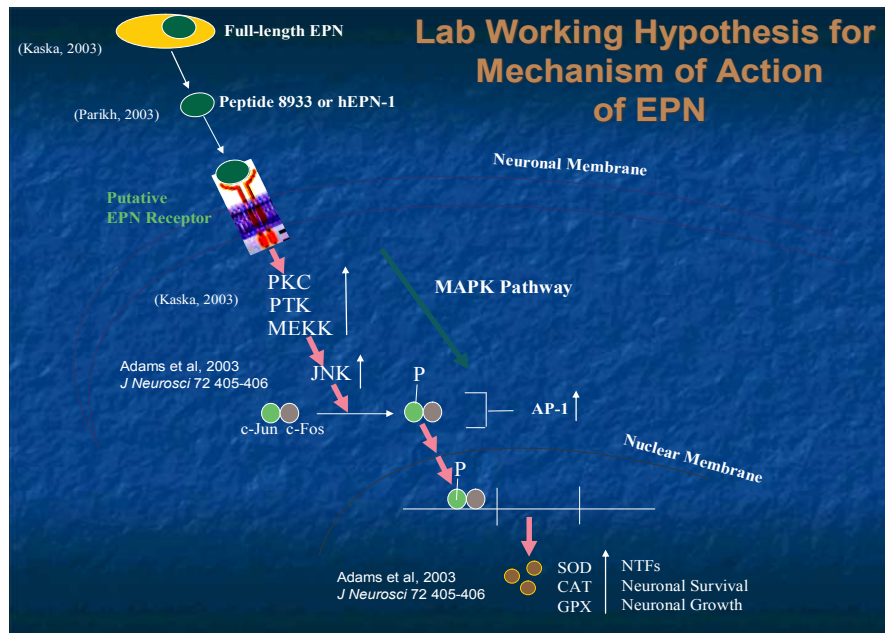


Figure 6: Proposed Mechanism of Action of Ependymin. Full length ependymin (yellow, upper part of diagram) or its receptor binding domain 8933 (dark green) binds a putative receptor on the surface of neurons which activates protein kinase-C (PKC), protein tyrosine kinases (PTK), and MEKK (diagram center) which activate JNK and AP-1. AP-1 moves into the nucleus to increase the expression of anti-oxidative genes (SOD, CAT, GPX), and neuronal growth genes (Saif, 2004).

THESIS PURPOSE

It was previously shown in our lab that human ependymin mimetic peptide hEPN-1 is capable of restoring cell survival in an *in vitro* Alzheimer's disease system (Stovall, 2006). The objective of this thesis was to begin an investigation of the mechanism of this therapeutic, while increasing our knowledge of mechanism of A β -induced neuronal death. The first part of the project was designed to investigate whether treatment of human SHSY cultured cells with Yankner peptide causes an increase in caspase protein levels and activity, and whether hEPN-1 is capable of reversing these increases. Second, since neuronal apoptosis in Alzheimer's disease involves an increase in oxidative stress, and ependymin mimetic CMX-8933 has previously been shown in our lab to cause increased expression of anti-oxidative genes, we wanted to determine whether hEPN-1 is capable of increasing SOD expression *in vivo* in AD mouse brains.

MATERIALS AND METHODS

SHSY-5Y Human Neuroblastoma Culture

Human SH-SY5Y (SHSY) neuroblastoma cells were purchased from ATCC. Complete growth medium consisted of 500 ml D-MEM / F-12 (Dulbecco's Modified Eagle's Medium, Ham's Nutrient Mixture F12, (1:1) (ATCC), 50 ml fetal bovine serum (FBS) (10% final concentration), and 0.25 ml of 10 mg/ml Gentamicin (Invitrogen Life Technologies, #15710064) (5 µg/ml final concentration). The FBS was pipetted into the 500 ml D-MEM / F-12 bottle, and swirled to mix. The mixture was then filter sterilized using a 0.2-micron filter. After filtration 0.25 ml of Gentamycin was added and the complete medium was stored at 4°C.

The 1 ml ATCC cell sample was thawed in a 37°C water bath for 30 seconds, then its contents was immediately pipetted into a T-25 flask, and the total volume was brought to 10 ml using the complete culture medium (see above). The flask was incubated at 36°C + 5% CO₂ for 24 hours. Following the 24 hr incubation, the medium containing DMSO from the freezing medium was completely removed, and fresh 5 ml complete medium was added, and the 36°C incubation was continued. Flasks were split approximately every 3-4 days, once they reached 80% confluency. Typically 1:2 splits were performed to prevent the cells from entering lag phase if the density was too low.

Yankner Peptide

Yankner peptide (Aβ₂₅₋₃₅) (Tocris Bioscience #1429, MW 1060.27) was stored at -20° C as 1 mg dry powder per vial. The 1 mg peptide (943 nmol) was reconstituted into 0.94 ml of 0.2 µm filter sterilized serum-free D-MEM to create a 1 mM stock. For this commercially available peptide, trace amounts of trifluoroacetic acid (TFA) allowed the hydrophobic peptide to dissolve

readily in the DMEM. The stock solution was aliquoted into several eppendorf tubes to prevent repeated cycles of freezing and thawing with each use, then stored at -20° C. For culture stimulations, 20 µl of 1 mM stock was added per 1 ml medium in a 24-well microtiter dish simultaneously to cell plating, or in some experiments 24 hr post-cell plating, to make a 20 µM final Yankner peptide concentration. For culture stimulations in T-25 flasks, 80 µl of 1 mM Yankner stock was added per 4 ml medium to make 20 µM final concentration.

Custom made Yankner peptide was provided by Biotherapeutix Inc. The powder was stored at -20° C as a 10 mg vial of dry powder. 1 mg peptide (943 nmol) was weighed before reconstituting in 0.94 ml of 1 mM NaHCO₃ and sonicating for 30 seconds to create a 1 mM stock. This custom made peptide contained no TFA salt as assayed by mass spectroscopy, which made its dissolution in DMEM difficult, thus sonication in sodium bicarbonate was used, with no observed loss of activity. This peptide was used at the same concentrations as the Tocris Yankner peptide.

hEPN-1 Peptide

The human endymin mimetic peptide hEPN-1 powder was received from Biotherapeutix, and stored at -20°C. The peptide was reconstituted into 0.2 µm filter sterilized serum-free D-MEM (the peptide readily dissolved without heating) at a concentration of 1 mg/ml (3.75 mM) , and stored at -20°C. For culture stimulations, 20 µl , 40 µl and 60 µl of 3.75 mM stock was added per 1 ml medium in a 24-well microtiter dish to make a 75 µM, 150 µM and 225 µM final concentrations, respectively. For culture stimulations in the T-25 flasks, 80 µl and 160 µl of the 3.75 mM stock were added per 4 ml medium to make a 75 µM and 150 µM final concentrations, respectively.

Microtiter Dish Plating

Exponentially growing SHSY cells were plated in a 24-well microtitre dish at 3×10^5 cells / ml, then the dish was incubated for 3 days in a 36°C incubator in an atmosphere of 5% CO₂. Cells were plated in 4 sets: a control (not treated with any peptide), Yankner (treated with 20 μM Yankner peptide with no hEPN-1), simultaneous therapeutic treatment (20 μM Yankner peptide + 75 μM hEPN-1 added at the same time as the Yankner peptide), and higher concentration simultaneous therapeutic treatment (20 μM Yankner peptide + 150 μM hEPN-1 added at the same time as Yankner peptide). For the hEPN-1 drug dose response experiment, an additional simultaneous treatment of hEPN-1 was also tested (20 μM Yankner peptide + 225 μM hEPN-1 added at the same time as the Yankner peptide).

Cell Viability Determination

At the end of the 3-day culture incubation time, the culture medium was removed, and the cells were trypsinized using 400 μl Trypsin-EDTA (Invitrogen Life Technologies). Microtiter dishes were allowed to sit for 1 min then gently pipeted using a micropipette to break apart cell clumps. A 100 μl volume of Trypan Blue (Sigma-Aldrich, MW 960.81, 0.4% stock concentration) was added to 100 μl of cell suspension, and the tube was inverted to mix. This sample was used to load a standard hemocytometer that was viewed at 400x magnification to perform standard cell counts of viable cells (trypan blue excluded) as a percentage of total cells. P-values were obtained using a One Way ANOVA followed by LSD post-hoc tests.

For cell count experiments in which loosely attached dying cells were not to be scored, several washes were performed post incubation. After 3 days, the microtiter dish was removed from the incubator, and the medium was removed from all wells. Using 0.2 μm filter sterilized serum-free D-MEM, 0.5 ml was pipetted into each well, and the dish was vigorously shaken back

and forth for 10 seconds. The supernatant was removed, and this procedure was repeated twice to remove all loosely bound dying cells. The remaining viable cells were trypsinized according to the same procedure detailed above.

Caspase Glo-3/7 Activity Assay

Cells were plated into microtitre plates and given different treatments as described previously. After 3 days of incubation, the medium was removed and centrifuged to collect all cells including those in the process of dying. The pellet consisting of dying and dead cells was resuspended in 200 μ l of complete SHSY medium, then added back to the microtitre plates containing the attached cells. 100 μ l of Caspase Glo 3/7 Reagent (Promega, catalog # G811C) was added to the wells containing the cells, and the contents were mixed by shaking. A blank was prepared by adding 100 μ l of Caspase Glo Reagent to 200 μ l of complete SHSY medium (no cells were added to blank). The cell suspensions were then incubated for 2 to 3 hours at room temperature. After the incubation the luminescence of each sample was read in a luminometer by exciting the samples at 485nm. The final values for activity were calculated by subtracting the background luminescence using the blank values. P-values were obtained using a One Way ANOVA followed by LSD post-hoc tests.

***In Vivo* Alzheimer's Mouse Experiments**

Whole brains of Alzheimers's mouse (strain: B6C3-Tg (APP^{swe},PSEN1^{dE9})85Dbo/J, Jackson Labs, Bar Harbor, ME) were provided to us by Biotherapeutix. The mouse handling and dosage was performed at Biotherapeutix. Mice were obtained at 3 months old. All mice except the control group were given a low dose (0.1 ml of 46 μ M) or a high dose (0.1 ml of 60 μ M)

treatment of hEPN-1 via intraperitoneal (i.p.) injections of the respective doses each day for one month. Thus at the end of treatment the mice were 4 months old when they were sacrificed.

Protein Extraction

Whole cell extracts were prepared from *in vitro* plated SHSY cells in T-25 flasks and Alzheimer's mouse brains, in order to obtain protein for immunoblots.

Cell Harvesting

The flasks were collected from the incubator, and using a plastic scraper, the cells were scraped into the medium. The suspension was placed into 15ml conical tubes, then pelleted at 500 x g for 5 minutes. The supernatant was discarded, and the cell pellet was washed once with 1 ml 1x PBS, and given a brief 15 seconds spin in the microcentrifuge. Once again the wash was discarded, then the tube was briefly inverted onto a towel to get rid of as much of the PBS as possible. Then the tube was placed on ice.

Cell Lysis

Once all the PBS wash was removed from the cell pellet, 200 μ l of Complete Lysis Buffer (20 mM HEPES pH 7.9, 10 mM KCl, 300 mM NaCl, 1 mM MgCl₂, 0.1% Triton X-100, 20% Glycerol, and freshly added 0.5 mM DTT and 0.5 mM PMSF) was added to each pellet from one T-25 cultured flask. Using a blue-tip, the cell pellet was re-suspended in the buffer and then transferred to 1.5 ml eppendorf tubes on ice. The suspension was incubated on ice for at least 10 minutes to thoroughly lyse the cells.

Lysate Clarification

The lysate was then microcentrifuged at 4°C for 5 minutes to pellet cell debris. The supernatant was placed into a 0.5 ml eppendorf tube. 5 µl of the supernatant was put into 0.5 ml of dH₂O for protein determination (1:100) as described below. The tubes were stored at -80°C.

Mouse Brain Lysates

For mouse brain lysate preparation, the whole brains were homogenized in 1 ml of complete lysis buffer (see previous section) using a small 1 ml glass dounce homogenizer. The homogenate was transferred to a 1.5ml eppendorf tube and kept in ice for 10 min, with occasional vortexing. The homogenate was then microcentrifuged at 4°C for 5 minutes to pellet cell debris. The supernatant was aliquotted into 0.5 ml eppendorf tubes (about 200 µl per tube). 1 µl of the supernatant was put into 0.5 ml of dH₂O for protein determination (1:500) (as described below). The tubes were stored at -80°C.

Protein Determination

BSA protein standards were prepared (1.25, 2.50, 5.00, 10.00, 20.00, 40.00 µg/ml), and 0.5 ml of each solution was added to 1.5ml eppendorf tubes. A blank was prepared with 0.5 ml of dH₂O. As described above, 5 µl of the sample plus 0.5 ml of dH₂O were put into 1.5ml eppendorf tubes. All tubes were then put into a water bath for 10 minutes to ensure all were at the same temperature. The tubes were briefly microcentrifuged, and 0.5 ml of Coomassie Reagent (Pierce) was added to all tubes. The tubes were then vortexed to mix. Absorbance was read on a spectrophotometer at OD 595 nm zeroed against the dH₂O blank.

Western Blot (Immunoblot) Analysis

Western blots were used to analyze the levels of caspase-3, and caspase-2 proteins in SHSY cells, and SOD and caspase-7-related protein levels in Alzheimer's mouse brain lysates after various treatments as mentioned previously. The western blots were essentially performed using the protocol described previously in our lab (Saif, 2004).

Gel Polymerization

A BRL V-16 glass plate apparatus was assembled using two non-siliconized glass plates (the front plate being 6 x 8 inches, the back plate 6 x 6 inches), and 0.8 mm thick spacers, held secure with clamps around the edges of the plates. The first gel, a lower resolving gel, was poured first as the main gel. It was composed of 7.6 ml of distilled H₂O, 6.7 ml of 30% acrylamide (2.7% cross linking), 5.1 ml of resolving gel buffer (1.5 M Tris-HCl, pH 8.0), 200 µl of 10% SDS, 400 µl of 5% ammonium persulfate, and 10 µl of TEMED (added just prior to pouring the gel as a catalyst). The solution was poured between the two plates to within about 1.5 cm from the top. A thin layer of dH₂O was overlaid on the top in order to ensure the lower gel polymerized in a straight line. The gel was then allowed to polymerize for 20 minutes.

After the lower resolving gel had fully polymerized, the water was poured off and the upper stacking gel was poured on. The upper stacking gel was composed of 5.52 ml of distilled H₂O, 1.67 ml of 30% acrylamide (2.7% crosslinking), 2.5 ml of stacking gel buffer (0.5 M Tris-HCl pH 6.8), 100 µl of 10% SDS, 200 µl of 5% ammonium persulfate, and 10 µl of TEMED (added just prior to pouring the gel as a catalyst). The upper stacking gel was poured on top of the lower resolving gel until it reached the top of the smaller back glass plate. A 20-stall comb was then carefully placed between the two plates, about 0.5 cm into the upper stacking gel, making

sure no air bubbles were near the comb teeth. The gel was then allowed to polymerize for another 20 minutes. If the gel was left overnight, the top of the plates was covered with plastic wrap to prevent desiccation.

Gel Electrophoresis

When the gel was completely polymerized, the clamps and the lower spacers were removed. After ensuring the black gaskets on the side spacers were pushed down, with no space between them and the top of the smaller plate, the plates were then mounted into the V-16 vertical electrophoresis unit, secured in place by clamps on both sides. Protein electrode buffer (25 mM Trizma Base, 0.192 M Glycine, 0.1% SDS) was then poured in the upper reservoir to check for leaks, if no leaks were present, then it was also poured in the lower reservoir until level with the electrodes. The comb was then removed, the wells were flushed out, and the gel was pre-run for one hour at 150 volts. After the pre-run, the samples (mixed with sample buffer to provide 15 µg protein per lane, in a total volume of 20 µl, unless otherwise noted) were prepared for electrophoresis by inserting them in a boiling water bath for two minutes, and then microfuging briefly. When protein markers were required, 1 µl (0.5 µg) of biotinylated broad range standard (Biorad #161-0319) was mixed with 10 µl of sample buffer, and loaded into lane-1. Samples were then loaded into each well, and electrophoresed for about 3 hours at 150 volts until the dye traversed down $\frac{3}{4}$ of the gel.

Gel Transfer

After the 3 hours of electrophoresis, the side spacers were removed, and one of the glass plates was carefully removed. A notch was made in the original right corner of the gel, as an orientation aid, so the bands could be viewed in the correct order. The gel was trimmed to contain

only the sample area, and then a nitrocellulose membrane (0.45 μm , BA-85, VWR Scientific) was cut to cover the gel (notched on the same side to match the notch in the gel). It was then surrounded on both sides by two pieces of 3 mm paper, all presoaked in transfer buffer (48 mM Trizma base, 39 mM Glycine, 0.037% SDS, and 20% Methanol) from the electroblotter. The gel/membrane sandwich was then placed in the plastic electroblot holder (with sponges on either side), fastened shut, and placed in the electroblot unit, submerged in chilled transfer buffer. The nitrocellulose membrane side must face the positive anode to ensure proper protein transfer from the gel to the membrane. The membrane was then transblotted in the refrigerator at 4°C, on a slow speed stir, for 2 hours at 50 volts.

Membrane Blocking/Antibody Incubations

Once the membrane had been transblotted, it was removed from the unit and placed in a Tupperware hybridization tray for blocking. The side that originally was in contact with the gel was marked with a felt pen, and remained face up at all times. The membrane was blocked with 30 ml of freshly made chilled blocker (1X PBS, 1% Casein powder, and 0.2% Tween-20) for one hour, mixing at low speed on the red rocker shaker. After the block, if the sample was to be tested for caspase-3, the blocker was replaced with 10 ml of fresh blocker, and the primary antibody (mouse anti-caspase-3 to Pro and Active / Cleaved Caspase-3, Imgenex, catalog # IMG-144A, main stock: 0.5 mg/ml) was added to make a final concentration of 2 $\mu\text{g/ml}$ to the liquid, not directly onto the membrane. For caspase-2 detection, the primary antibody (mouse anti-caspase 2, Imgenex, catalog #IMG-229, main stock: 0.5 $\mu\text{g/ml}$) was added to make a final concentration of 2 $\mu\text{g/ml}$ to the blocker. The membrane was then left again to mix on the Red Rocker shaker as previously described for two hours. For SOD detection, the primary antibody (rabbit anti-Superoxide Dismutase-1, Rockland immunochemicals catalog #100-4191, main stock 70 mg/ml)

was added at a dilution of 1:4000 to the blocker. The membrane was then left again to mix as previously described for two hours. For caspase-7-related protein detection, the primary antibody (rabbit anti-caspase-7, Imgenex, catalog #IMG-5560, main stock 0.5 $\mu\text{g}/\mu\text{l}$) was added to make a final concentration of 2 $\mu\text{g}/\text{ml}$ to the blocker. The membrane was then left again to mix as previously described for two hours. For loading controls, sister gels containing identical extract loads to the main immunoblot were run. For β -tubulin loading control detection, the primary antibody (rabbit anti- β -tubulin, Imgenex, catalog # IMG-5810A, main stock 0.5 $\mu\text{g}/\mu\text{l}$) was added to a final concentration of 1 $\mu\text{g}/\text{ml}$ to the blocker, with membrane incubations of two hours. For GAPDH loading control detection, the primary antibody (rabbit anti-GAPDH, Imgenex, catalog # IMG-5143A, main stock 0.5 $\mu\text{g}/\mu\text{l}$) was added to make a final concentration of 1 $\mu\text{g}/\text{ml}$ to the blocker, with membrane incubations for two hours.

After the incubation with the primary antibody, the solution was removed, and the membrane was washed twice in PBS-Tween (1X PBS, 0.05% Tween-20), 2 minutes each time, at a vigorous rate on a gyratory shaker. Next, the membrane was incubated with fresh blocker supplemented with a 1:1000 dilution of the secondary antibody (goat anti-mouse-HRP, Pierce #31430, final concentration of 0.4 $\mu\text{g}/\text{ml}$) or (goat anti-rabbit-HRP, Pierce #31460, final concentration of 0.4 $\mu\text{g}/\text{ml}$), depending on the primary antibody used, for one hour on the red rocker shaker. When a biotinylated marker had been used, 1:1000 dilution of 0.5 mg/ml streptavidin-HRP in 50% glycerol (Pierce #21126) was also added to the blocker solution to make a final concentration of 0.5 $\mu\text{g}/\text{ml}$. After the one-hour incubation with the secondary antibody, the used solution was removed, and the membrane was washed four times (3 times in PBS-Tween, then once in 1X PBS) for 2 minutes each on the gyratory shaker at vigorous speed.

Chemiluminescent Detection/X-ray Film Development

After the last membrane washes, the membrane was moved from the hybridization tray to a piece of foil on the bench top, protein side up. In a 15 ml conical tube, fresh detection solution (5 ml Luminol/Enhancer Solution plus 5 ml Stable Peroxide Solution) (Pierce #34080) was mixed. The freshly prepared solution was then poured on the membrane, and spread evenly with a glass pipette, and allowed to incubate for 5 minutes. After 5 minutes, the membrane was picked up using tweezers, and the corner was dabbed with a Kimwipe to remove excess detection solution. The membrane was placed between two sheets of clear plastic Photogene Development Folders (Gibco BRL #18195-016), and any air bubbles were removed by rubbing a Kimwipe over the plastic surface. The folder was then placed in a film cassette without a screen for transportation to the darkroom for development.

Before developing, the development tank was filled with water at a constant 25°C maintained throughout development. Kodax X-Omat AR film was exposed to the membrane for ~1 min for caspase-2 and -3, ~10 sec for SOD, and ~1 min for caspase-7. The film was then placed in the GBX developer, agitating initially to remove air bubbles, and left for 5 minutes. It was then removed, dipped briefly in the water to remove excess developer, and submerged into the fixative solution for three minutes, with intermittent agitation over the entire 3 minutes. Finally, the film was placed in the water bath for 5 minutes to remove excess fixative, and hung to air dry. Bands were quantitated using Scionimage Software (NIH). Statistical analysis was performed using one way ANOVA followed by LSD post-hoc tests (* , $p < 0.05$; ** , $p < 0.001$) .

RESULTS

Microscopy

Our laboratory previously showed that treatment of human SHSY neuronal cells with a small peptide mimetic (hEPN-1) of human ependymin neurotrophic factor can restore cell population survival in an *in vitro* model of Alzheimer's disease (Stovall, 2006). The initial experiments of this thesis focused on validating the *in vitro* model, and establishing therapeutic hEPN-1 doses, to provide a system for investigating the cellular mechanism of action of the Yankner peptide insult, and of the therapeutic. SHSY cells were plated into a 24-well microtiter dish in medium containing no peptide addition (control), 20 μ M Yankner peptide (a concentration previously shown in the literature to strongly induce SHSY apoptosis; Yankner et al., 1990; Lambert *et al.*, 1994), or 20 μ M Yankner peptide + 75 μ M hEPN-1 peptide (a concentration chosen from our preliminary experiments, data not shown). After incubation for 3 days, photomicrographs were taken at 400x as an initial visual comparison between the samples (Figure-7).

Viewed at a magnification of 400x, two sets of representative fields (upper and lower rows) were photographed for the control cells (left panels) and simultaneous Yankner + hEPN-1 treated cells (right panels), and two sets of selected fields were chosen to show Yankner peptide treated apoptotic cells (middle panels). While control cells obtained approximately 80% confluency during the 3-day culture period and show a typical stellate shape with numerous intercellular projections, the 20 μ M Yankner challenged cells show a visibly reduced confluency, and a rounded morphology with few intercellular projections and visible signs of apoptosis. However, when hEPN-1 peptide was added simultaneously with the Yankner peptide, the cells appeared completely normal.

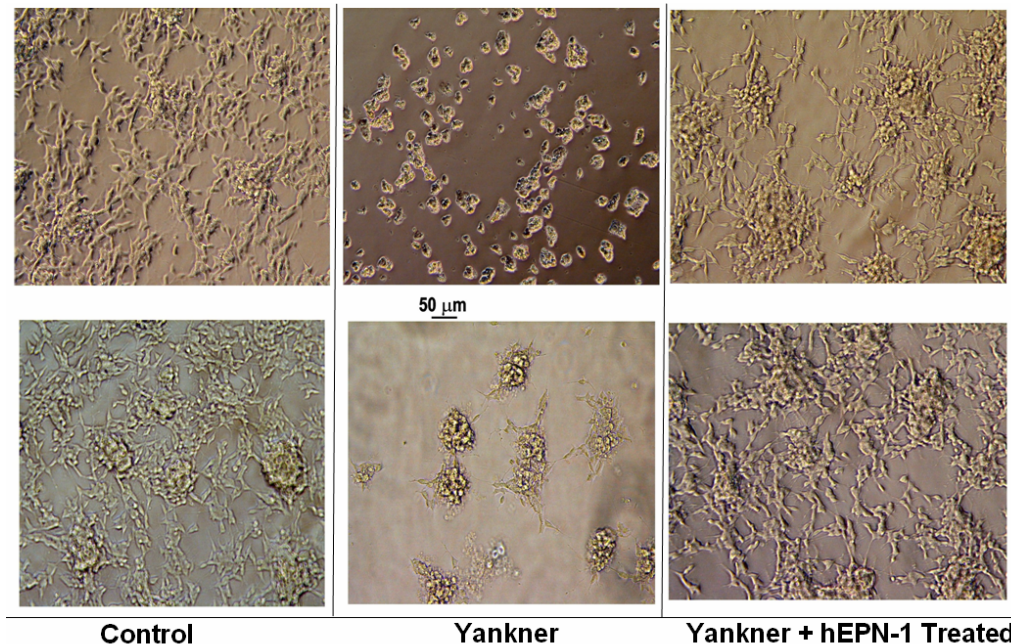


Figure 7: Microscopy of Human SHSY Cells Treated with Yankner and/or hEPN-1 Peptides for 3 Days. Following 3 days of culture, cells were viewed by inverted phase contrast microscopy: Upper and lower rows denote duplicate sets of representative fields. The left panels show control cells. The center panels show cells treated with 20 μM Yankner peptide. Right panels show cells treated with 20 μM Yankner peptide + 75 μM hEPN-1 peptide added simultaneously. Panels are viewed at 400x.

Cell Numbers, Viability, and hEPN-1 Dose-Response

Cell counts were performed on treated cultures using a standard hemocytometer (Figure 8), following a series of washes to remove loose dying cells. Trypan blue exclusion was used to determine cell viability. In six independent trials, average cell numbers per microtitre well decreased 31.7% in the presence of 20 μM Yankner when compared to control ($p < 0.001$). Upon simultaneous treatment with 20 μM Yankner + 75 μM , 150 μM hEPN-1, or 225 μM hEPN-1, the average viable cell numbers increased by 37.7%, 62.5%, and 51.6%, respectively, relative to the Yankner treatment alone ($p < 0.001$, for all treatments). Statistical significance was determined using a One Way ANOVA followed by LSD post-hoc comparisons, using SPSS software. In spite of being treated with the Yankner peptide, the 75 μM and 225 μM hEPN-1 treated samples were statistically indistinguishable from the control, and the 150 μM treated sample was statistically higher ($p < 0.05$) than the control mean. Thus, when assaying cell

numbers that remain attached to the wells after the 3-day treatments, the addition of hEPN-1 peptide appears to nearly completely block the toxic effect of the Yankner peptide and increase cell survival. Moreover, results from the drug dose response data suggest that hEPN-1 at a 150 μM dose works best to protect cells from the neurotoxic effects of Yankner peptide.

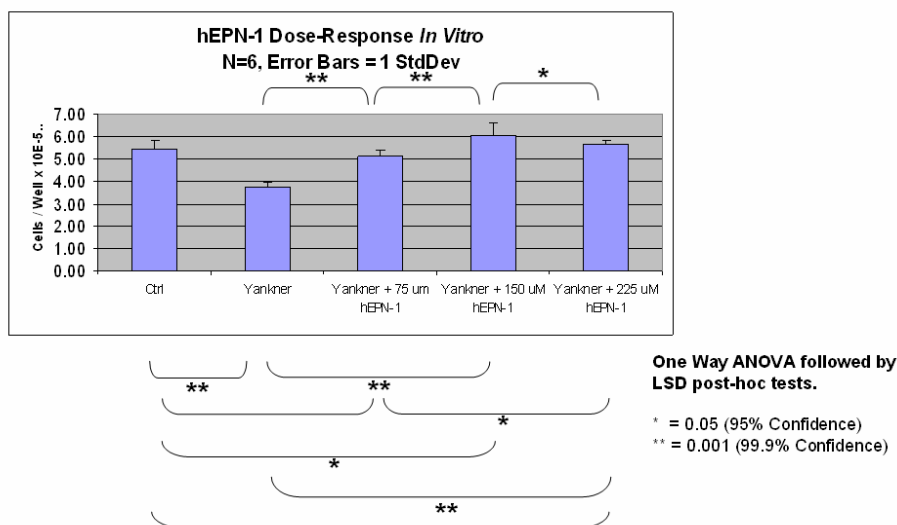


Figure 8: Viable Cell Counts of Human SHSY Cells Treated with Yankner and/or hEPN-1 Peptides for 3 Days. Cultures were treated with no peptides (control), 20 μM Yankner peptide (second histobar), 20 μM Yankner peptide + 75 μM hEPN-1 peptide added simultaneously (third histobar), 20 μM Yankner peptide + 150 μM hEPN-1 peptide added simultaneously (fourth histobar), 20 μM Yankner peptide + 225 μM hEPN-1 peptide added simultaneously (fifth histobar). Following 3 days of culture, loosely bound cells were removed by washes, and remaining cells were stained with trypan blue then counted in a hemocytometer. Only live cells (excluding trypan blue) were considered in the cell count. Each histobar represents the mean of six independent determinations. Error bars denote one standard deviation.

Cellular Levels of Procaspase-3

To initiate an investigation of the mechanism of the Yankner peptide-induced cell death, and hEPN-1's apparent ability to block the insult, we chose to monitor the cellular levels of caspase-3, a key end-stage effector caspase upon which the other cell death pathways converge, hypothesizing its cellular levels would increase with Yankner treatment, and the increase would be blocked by hEPN-1 treatment. A time-course experiment was conducted (Figure-9) to determine the optimum time for assaying caspase-3 levels. Cells were seeded in T-25 flasks and

given the usual 20 μ M dose of Yankner peptide, then incubated for various times prior to preparation of whole cell lysates for immunoblot analysis. We chose a caspase-3 antibody capable of detecting both forms of capsase-3 (inactive 32 kDa procaspase-3, and active 14-21 kDa cleaved). The results obtained showed one predominant band on the blot precisely the 32 kDa size expected for procaspase-3. The 32 kDa band disappeared in a control that lacked primary antibody (Figure-10, right panel), demonstrating excellent specificity of the caspase-3 antibody for the band. A positive control was also run with Staurosporine-treated cells (Figure-9, upper right panel), a well known stimulator of caspase-3. Cells treated with staurosporine showed increased procaspase-3 levels compared to untreated cells. The observed band was the same size as the one induced by the Yankner peptide. No band was observed in the 14-21 kDa range for active caspase-3, although we expected its appearance due to the obvious apoptotic state of the cells, so perhaps this band is too faint or transient to be observed by this assay. Faint high molecular weight bands were also seen on the blot, but they remained in the negative control lacking primary antibody, so likely they represent proteins that interact with the secondary antibody. The 20 μ M dose of Yankner peptide caused an increase in procaspase-3 levels, with a maximum expression at the 72 hour time point, while cellular levels of β -tubulin (load marker) remained unchanged. Thus, for all subsequent experiments to test the efficacy of hEPN-1 in causing a reversal of Yankner mediated upregulation of procaspase-3 levels, the 72 hour time point was chosen for analysis.

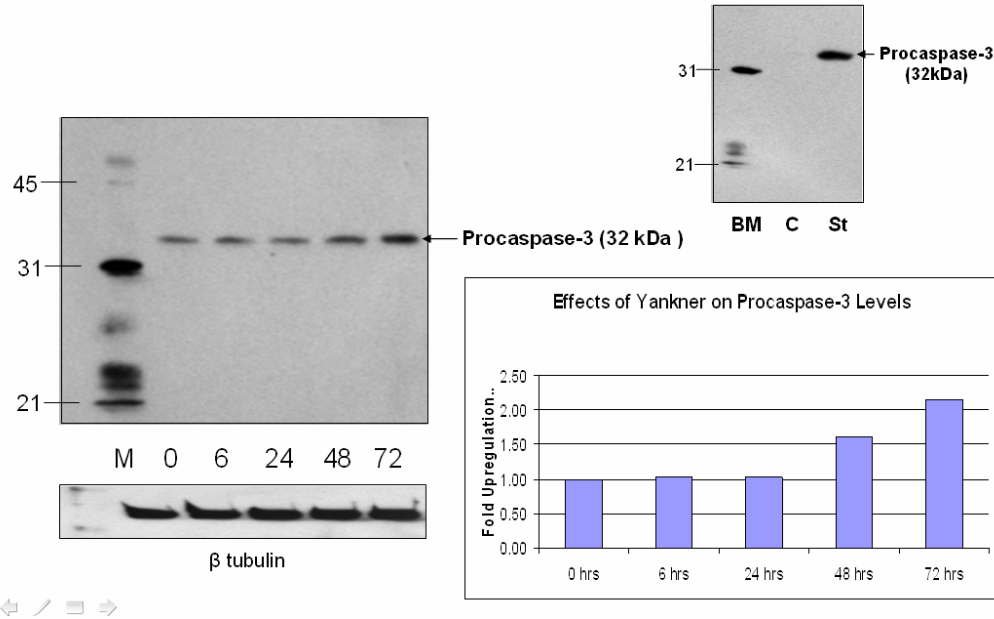


Figure 9: Time-Course for Procaspase-3. **Upper Left Panel:** Caspase-3 immuno-blot using an antibody that can detect both forms of caspase-3, inactive procaspase-3 at 32 kDa, and active caspase-3 bands at ~ 14-21 kDa. Cells were seeded in T-25 flasks and treated with 20 μ M Yankner peptide. Flasks were incubated for various times (hrs denoted under the figure), then whole cell lysates were prepared for immunoblot analysis. The arrow on the right of the blot denotes the position of 32 kDa procaspase-3. Arrows on the left denote the positions of biotinylated markers in kDa. **Lower Left Panel:** β -tubulin immunoblot as load marker, run simultaneously on sister blot with identical protein loads as the upper panel. **Upper Right Panel:** Positive control. Cells were treated with 2 mM staurosporine for 4 hours (lane St). The increase in procaspase-3 levels in presence of staurosporine relative to control (lane c) is evident. The band observed is in the expected range for procaspase-3. **Right Panel:** Quantitation of the procaspase-3 bands in the upper left immunoblot. Histograms represent the signal strength quantitated using Scion image for this single experiment.

To test the hypothesis that hEPN-1 can inhibit procaspase-3 upregulation, (Figures-10 and 11), in five independent trials 20 μ M Yankner peptide treatment caused an average 5.96 fold upregulation in caspase-3 levels compared to control ($p < 0.001$), and upon simultaneous treatment with 20 μ M Yankner peptide + 75 μ M hEPN-1, or + 150 μ M hEPN-1, there was a 13.9% and 35.5% decrease in cellular procaspase-3 levels, respectively, compared to the Yankner treatment alone. The 75 μ M treatment was not statistically distinguishable from Yankner alone, however the 150 μ M treatment was statistically lower than Yankner alone ($p < 0.05$). All treatments were significantly different than the control mean ($p < 0.001$ Yankner alone; $p < 0.001$ Yankner + 75 μ M hEPN-1; $p < 0.05$ Yankner + 150 μ M hEPN-1). Thus the caspase-3

immunoblot data indicate that Yankner peptide treatment of SHSY cells upregulates the expression of caspase-3, and simultaneous treatment with hEPN-1 can help alleviate this upregulation.

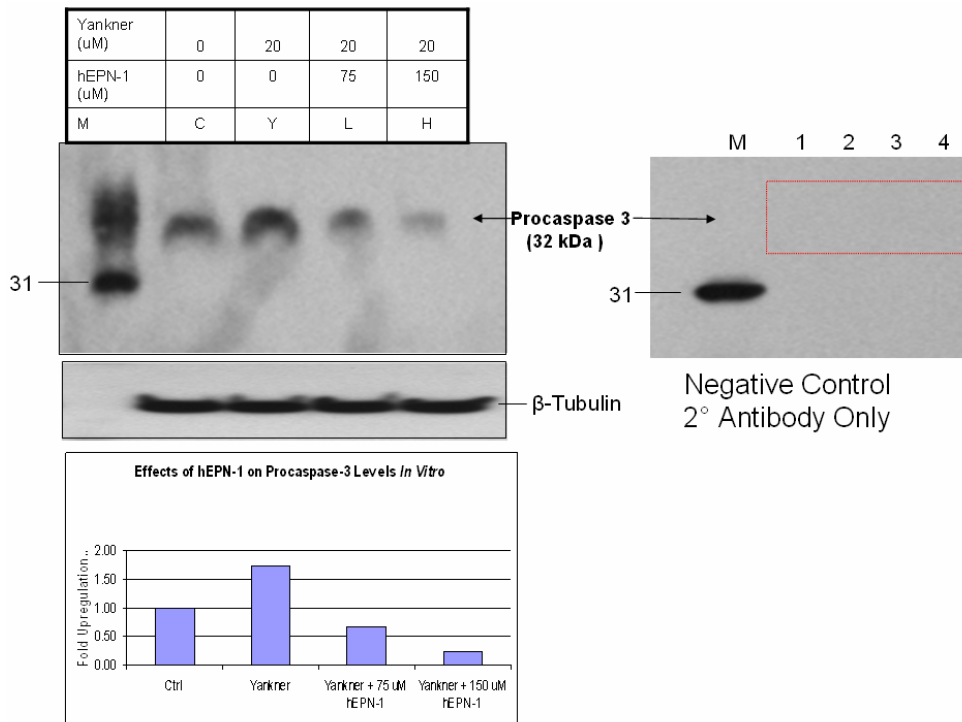


Figure-10: hEPN-1 Treatment Can Reverse the Yankner-Induced Increase in Procaspase-3. T-25 flasks were seeded with SHSY cells and treated with peptides as described previously. Whole cell lysates were prepared after 72 hours incubation, and used for caspase-3 immunoblot analysis. **Upper Left Panel:** Caspase-3 immunoblot. **Left Center Panel:** β -tubulin load marker immunoblot. **Right Panel:** Negative control immunoblot without the caspase-3 antibody, showing the disappearance of the procaspase-3 band. **Lower Panel:** Scion image quantitation of the procaspase-3 band, single experiment.

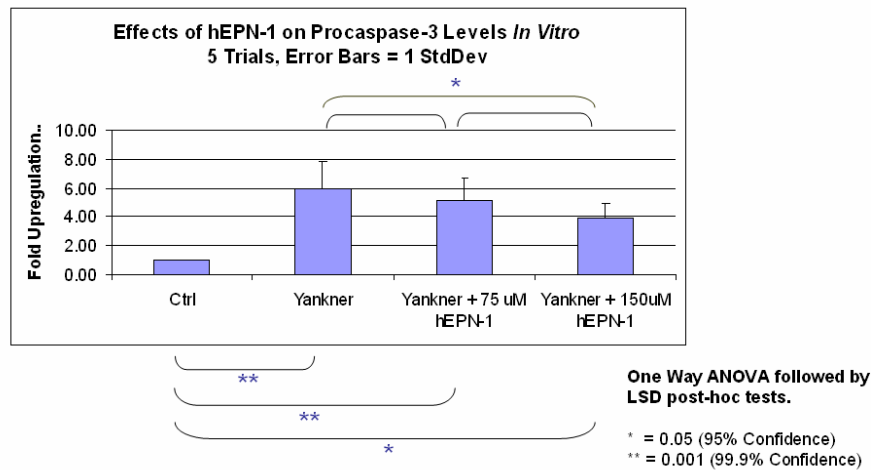


Figure-11: Mean of Five Independent Trials of the Procaspase-3 Experiment Described in the Previous Figure. Each error bar represents one standard deviation. P-values were obtained using a One Way ANOVA followed by LSD post-hoc tests.

Cellular Levels of Procaspase-2

Caspase-2 is a key initiator enzyme in the externally stimulated cell death pathway, so we tested its upregulation in our system using immunoblots to determine whether the Yankner peptide activates this pathway to induce apoptosis. As with caspase-3, a time-course experiment was performed to determine the optimum time to assay caspase-2 in the lysates (Figure-12). Cells were seeded in T-25 flasks and given various treatments as described previously. Whole cell lysates were prepared at different times for immunoblot analysis using an antibody that can only detect the inactive pro-version of the enzyme (no other caspase-2 antibodies worked well in our hands). The results obtained for a single experiment showed one strong band at 48 kDa, the expected size for procaspase-2. This band disappeared in a negative control lacking primary antibody (Figure-13, right panel) demonstrating its specificity for the primary antibody. The 20 μ M dose of Yankner peptide causes an increase in procaspase-2 levels with a maximum expression at the 72 hour time point, thus for all subsequent caspase-2 experiments this time point was chosen for analysis.

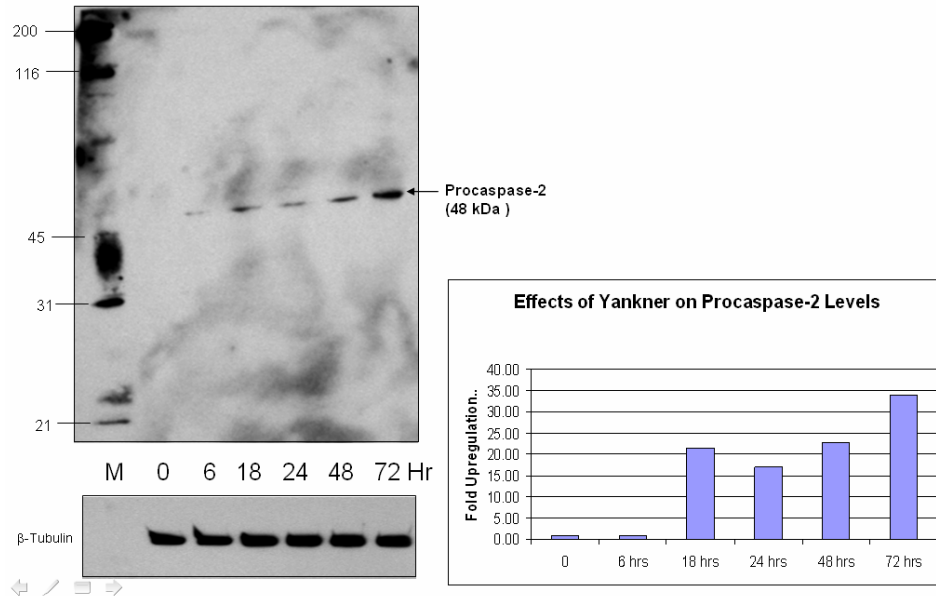


Figure 12: Time-Course of Procaspase-2 Induction by the Yankner Peptide. Cells were seeded in T-25 flasks and simultaneously treated with 20 μ M Yankner peptide. Flasks were incubated for various times (X-axis) then whole cell lysates were prepared for immunoblot analysis. **Left Panel:** Caspase-2 immunoblot. Arrow on the right denotes the 48 kDa procaspase-2 band. Arrows on the left denote the positions of biotinylated markers. **Lower Panel:** β -tubulin immunoblot as load marker, run simultaneously on a sister blot. **Right Panel:** Quantitation of procaspase-2 bands using Scion image software, single experiment.

In five independent trials (Figure-13 and 14) the 20 μ M Yankner treatment caused an average 67.21 fold upregulation in procaspase-2 levels compared to control ($p < 0.001$). Upon simultaneous treatment with 20 μ M Yankner + 75 μ M hEPN-1, or + 150 μ M hEPN-1, there was a 21.6% and 45.2% decrease in cellular procaspase-2 levels compared to the Yankner treatment alone. The 75 μ M treatment was not statistically distinguishable from the Yankner treatment alone, however the 150 μ M treatment was statistically lower than Yankner alone ($p < 0.05$). All treatments were statistically different than the control mean ($p < 0.001$ Yankner alone; $p < 0.001$ Yankner + 75 μ M hEPN-1; $p < 0.001$ Yankner + 150 μ M hEPN-1). Thus the caspase-2 data show that the Yankner peptide stimulates the expression of an external cell death pathway protein, and treatment with hEPN-1 can help alleviate this pro-caspase-2 elevation.

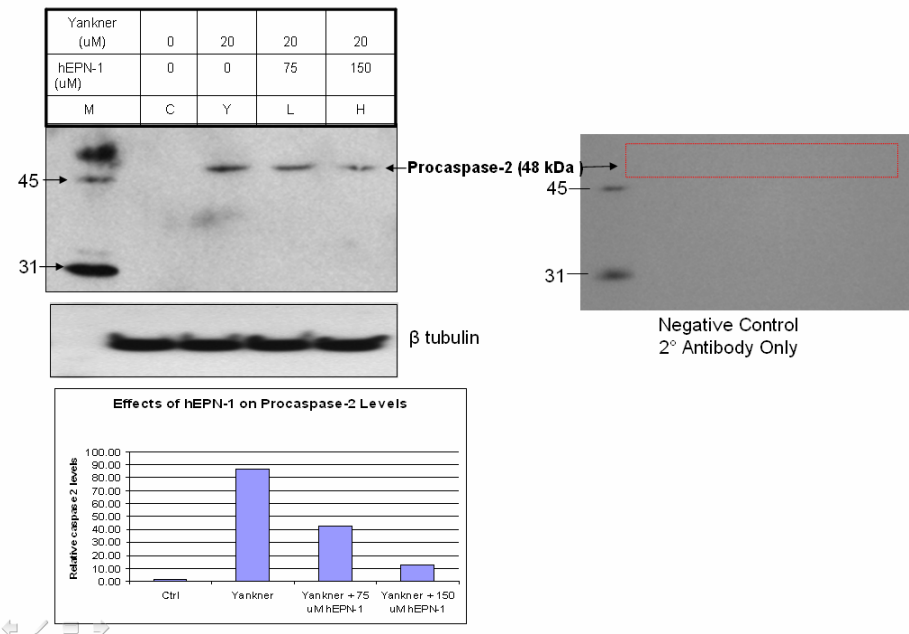


Figure 13: hEPN-1 Can Lower the Procaspase-2 Increase Induced by the Yankner Peptide. T-25 flasks were seeded with SHSY cells and treated with peptides as described previously. Flasks were incubated for 72 hrs, then whole cell lysates prepared for immunoblot analysis. **Upper Left Panel:** caspase-2 immunoblot. Right arrow denotes the position of 48 kDa procaspase-2. Left arrows denote the positions of biotinylated marker proteins. **Left Middle Panel:** β -tubulin load marker immunoblot. **Upper Right Panel:** Negative control blot without primary antibody, showing the disappearance of the procaspase-2 band. **Lower Left Panel:** Quantitation of procaspase-2 bands using Scion image software, single experiment.

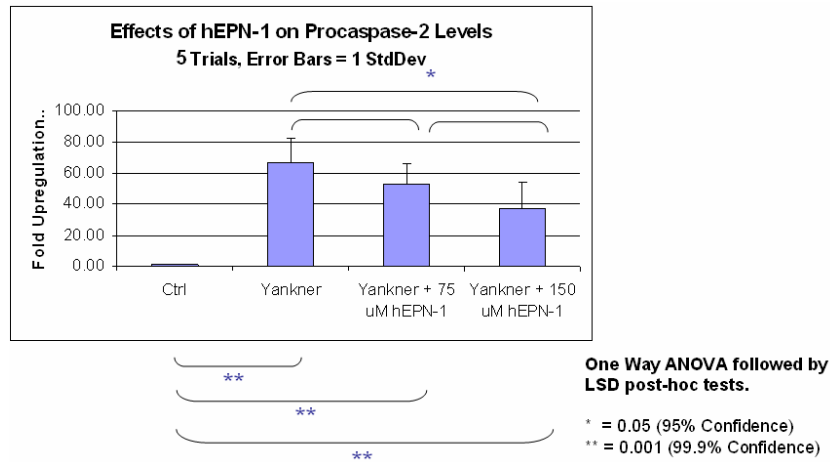


Figure-14: Mean of Five Independent Trials of the Procaspase-2 Experiment Described in the Previous Figure. Each error bar represents one standard deviation. P-values were obtained using a One Way ANOVA test followed by LSD post-hoc tests.

Caspase 3/7 Activity Assay

Because the immunoblot analyses had clearly shown the upregulation of procaspase-2 and procaspase-3 bands with the Yankner peptide treatment, but had not detected the active form of either enzyme, under conditions in which the cells were clearly morphologically apoptotic, we questioned whether the caspase activity also increased with the Yankner peptide treatment. A commercially available caspase-3/7 assay was tested. In this assay, a pro-luminescent substrate specific for caspase-3/7, and a thermostable luciferase enzyme, are mixed with the cells to make a lysate. Caspase-3 or -7 activities present in the lysate cleave the substrate to release amino-luciferin, which is cleaved by the luciferase to produce light. The amount of glow is proportional to the amount of caspase-3 or -7 activity in the lysate (Preaudat *et al.*, 2002). Although this assay can not distinguish between caspases-3 and -7, both are end stage effector caspases activated by the same pathways. The Caspase-Glo-3/7 activity assay was performed as described in the Methods section on whole cell lysates (Figure-15). In three independent trials, the 20 μ M

Yankner peptide treatment caused a 16.01 fold increase in caspase-3/7 activity compared to control ($p < 0.001$). Upon simultaneous treatment with 20 μM Yankner peptide + 75 μM hEPN-1, there was a 57.7% decrease in caspase-3/7 activity compared to the Yankner treatment alone ($p < 0.05$). The 75 μM hEPN-1 dose tested was not sufficient to completely lower the caspase activity to control levels, so the control mean was statistically lower than the Yankner + 75 μM treatment ($p < 0.05$). Due to time constraints, the 150 μM therapeutic dose was not tested here.

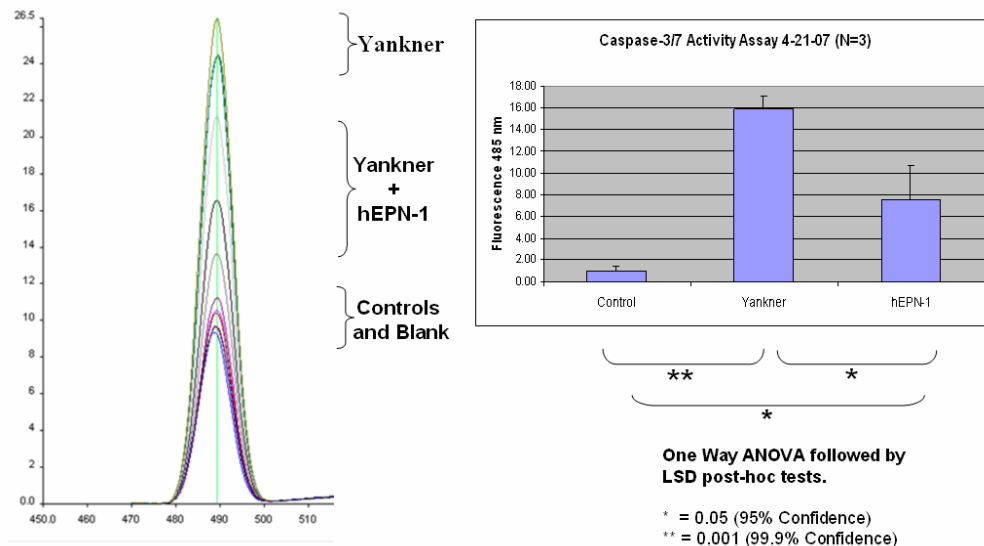


Figure 15: Caspase 3/7 Activity Assay for SHSY Cells Treated with Yankner and/or hEPN-1 Peptides for 3 Days. Cultures were treated with peptides as described previously. Following 3 days of culture, the caspase 3/7 glo reagent was added, and caspase activity was measured in a luminometer (see Methods). **a)** Luminogenic signal from different samples as a measure of caspase activity measured in a luminometer (excitation: 485nm) **b)** Quantitation of the data in panel-A for 3 independent trials. Each error bar represents one standard deviation. P-values were obtained using a One Way ANOVA followed by LSD post-hoc test in the SPSS program.

Brain Levels of SOD-1 *In Vivo*

Previous experiments in our laboratory indicated that treatment of mouse neuro-2a cells with CMX-8933 increases the cellular levels of anti-oxidative enzyme SOD-1 (Saif, 2004). Because it is well established that $\text{A}\beta$ increases oxidative stress in Alzheimer's disease, we hypothesized that hEPN-1 would increase SOD-1 expression in mouse brains, and this increase

might help alleviate the oxidative stress. A commercially available Alzheimer's mouse model (Jackson's lab) was chosen for analysis which overexpresses the A β peptide, similar to the original Alzheimer's mouse developed, in part, in our laboratory (Games *et al.*, 1995). The AD mouse shows multiple signs of AD including neural apoptosis and cognitive decline. AD mice were obtained at 3 months old. All mice except the control group were given a low dose (0.1 ml of 46 μ M) or high dose (0.1 ml of 60 μ M) treatment of hEPN-1 by intraperitoneal (i.p.) injections daily for one month until 4 months old, at which time whole brain lysates were prepared for immunoblot analysis of SOD-1 (Figures-16 and 17). One strong band was observed at 16 kDa, the expected size of SOD-1. The band disappeared in a negative control lacking the primary antibody (panel C). In five independent trials, there was a 1.4 fold, and 2.04 fold, increase in SOD-1 protein levels for the 46 μ M and 60 μ M hEPN-1 treatments, respectively. The 46 μ M treatment was not statistically different than untreated AD mice, whereas the 60 μ M hEPN-1 treatment was statistically higher ($p < 0.05$) than untreated AD mice (Figure-17). No changes in the cellular levels of housekeeper GAPDH was observed under these conditions.

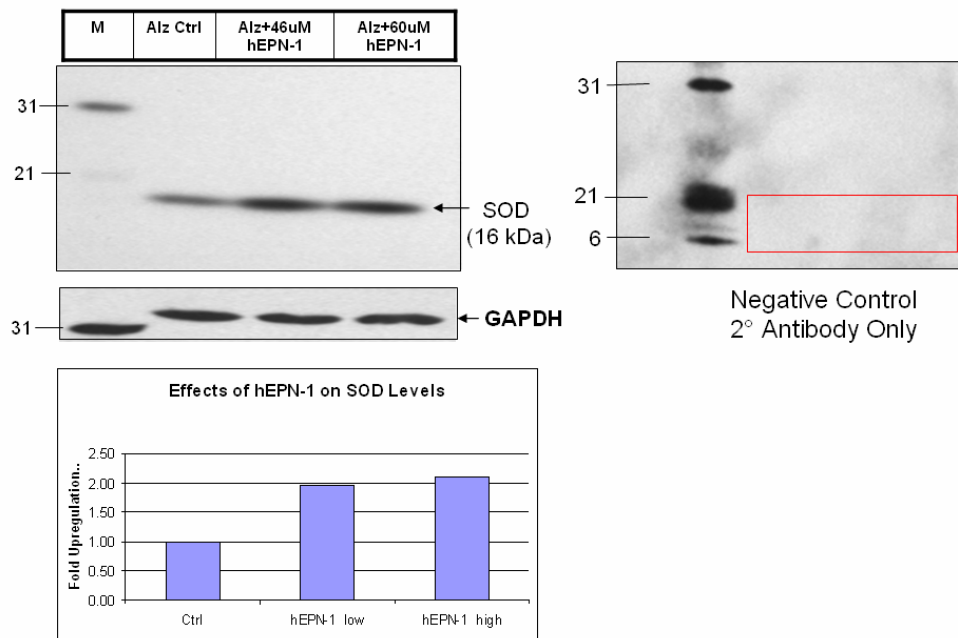


Figure-16: Increase in Brain SOD Levels in Alzheimer’s Mice by i.p. hEPN-1 Treatment. Commercially available Alzheimer’s mice were treated as described in the text, then whole brain lysates were prepared for SOD-1 immunoblot analysis. **a)** SOD-1 immunoblot. The arrow on the right side denotes the position of the 16kDa SOD-1. The arrows on the left denote the positions of biotinylated marker proteins. The GAPDH load marker immunoblot performed on a sister blot with identical protein load is shown underneath the SOD-1 blot. **b)** Quantitation of the data in panel A using Scion image software, for a single experiment. **c)** Negative control lacking primary antibody to demonstrate the specificity of the SOD-1 antibody.

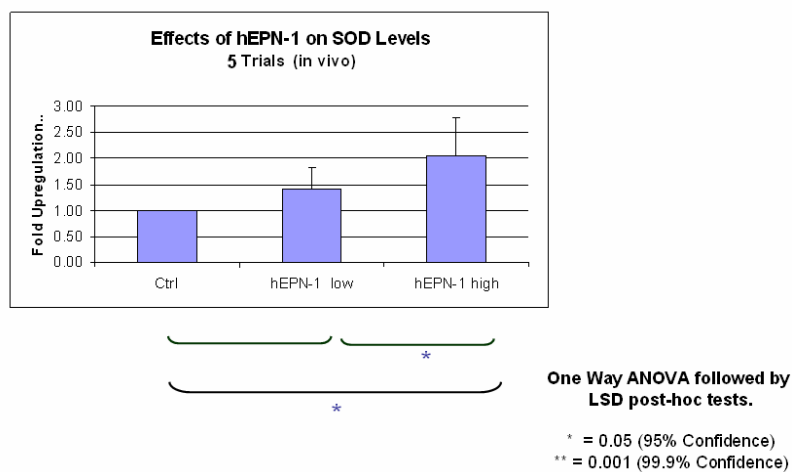


Figure-17: Mean of Five Independent Trials of the SOD-1 Data in the Previous Figure. Each error bar represents one standard deviation. P-values were obtained using ANOVA followed by LSD post-hoc tests.

Brain Levels of Caspase-7-Related Protein

During the testing of a variety of caspase antibodies for this thesis, with the exception of caspase-2 and -3, most antibodies tested gave no band whatsoever on the blot, so were not used further, and this prevents us from making any conclusions about other caspases. However, the antibody to casapase-7 produced an interesting result worth brief mention here. It produced a 90-100 kDa band much higher on the blot than the 34 kDa expected for caspase-7 (Figure-18a), which disappeared in negative controls lacking the primary antibody (Figure-18b), and behaved similarly to casapases-2 and -3 in the presence of our peptide treatments. So the band appears to be specific for the caspase-7 antibody, and we termed it caspase-7-related protein (C7RP). With respect to *in vivo* C7RP cellular levels, in six independent trials, there was a 67% and 39% decrease in caspase-7-related protein levels upon treatment of the Alzheimer's mice with 46 μ M hEPN-1 and 60 μ M hEPN-1, respectively. Both hEPN-1 treatments were significantly lower ($p < 0.001$) than untreated AD mice (Figure-19).

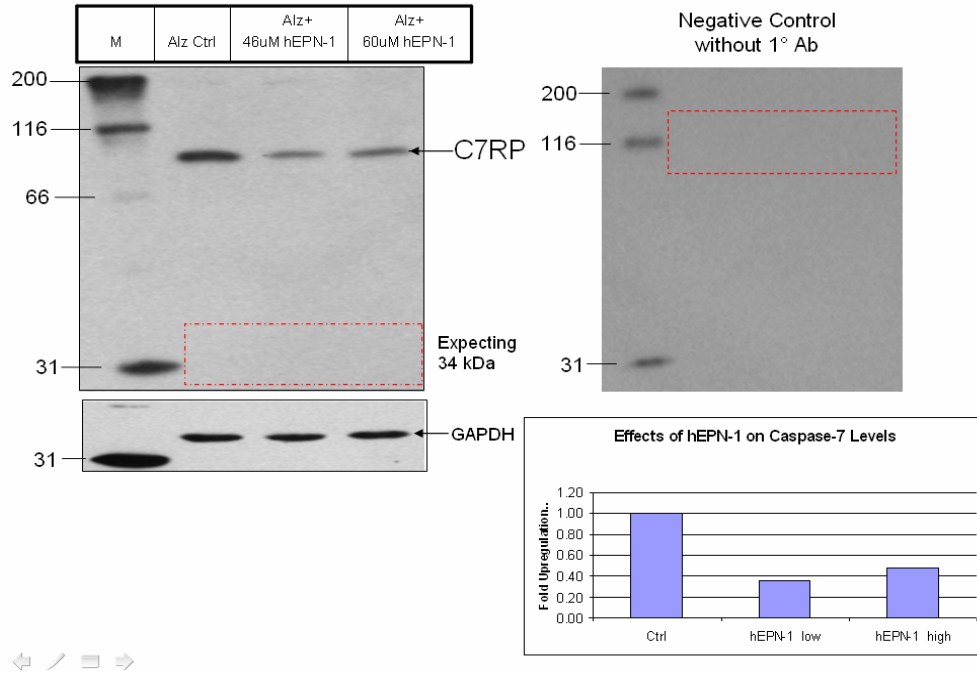


Figure-18: Decrease in Caspase-7-Related Protein Levels in Alzheimer's Mice by hEPN-1 Treatment. Commercially available Alzheimer's mice were treated with hEPN-1 as described in the text, then whole brain lysates were prepared for caspase-7 immunoblot analysis. **a)** caspase-7 immunoblot. The arrow on the right side denotes the unusual position of the caspase-7-related band. The arrows on the left denote the positions of biotinylated marker proteins. The red box denotes the expected position of 34 kDa caspase-7. The GAPDH load marker immunoblot performed on a sister blot with identical protein load is shown underneath the caspase-7 blot. **b)** Negative control lacking primary antibody to demonstrate the specificity of the caspase-7 antibody. The red box denotes the position of the caspase-7-related protein band, missing in this negative control. **c)** Quantitation of the data in panel A using Scion image software, for this single experiment.

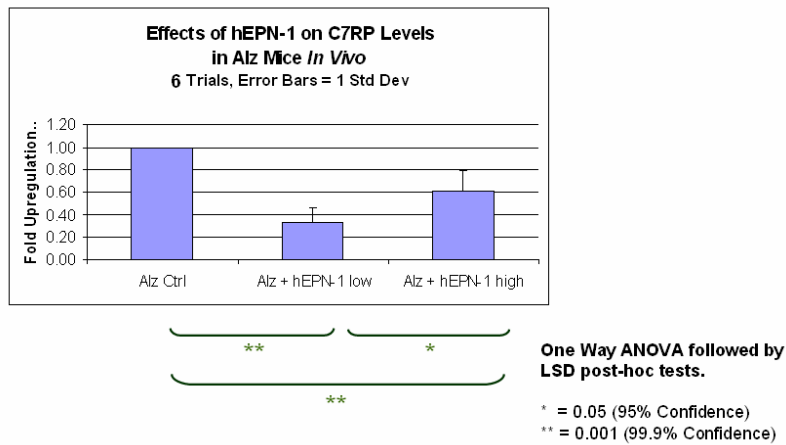


Figure-19: Mean of Six Independent Trials of the Caspase-7-Related Protein Data in the Previous Figure. Each error bar represents one standard deviation. P-values were obtained using ANOVA followed by LSD post-hoc tests.

DISCUSSION

Our lab previously showed that treatment of human SHSY cultured neuronal cells with a synthetic peptide (hEPN-1) that mimics human ependymin neurotrophic factor is capable of partial restoration of cell survival in an *in vitro* model for Alzheimer's disease in which the neuronal cells are treated with neurotoxic Yankner peptide ($A\beta_{25-35}$) (the receptor binding portion of $A\beta_{1-42}$) (Stovall, 2006). The goal of this project was to extend these previous observations to initiate an investigation of the mechanism of $A\beta$ -induced cell apoptosis, and the mechanism of hEPN-1 therapy. The rationale behind using hEPN-1 as a therapeutic was based on several previous observations in our lab: 1) hEPN-1 can upregulate growth-related mRNAs for L-19, EF-2, and ATP Synthase-C in cultured mouse neuroblastoma cells (Saif, 2004); 2) hEPN-1 can upregulate growth-related genes for S-19 and S-12, 5.8S RNA and 16 S RNA in cultured human SHSY neuroblastoma cells (Arca, 2005); 3) peptide 8933 (a mimetic to goldfish ependymin) can upregulate growth-related genes for EF-2, L19, and S12 in cultured mouse neuro-2a cells (Kaska, 2003); and 4) 8933 can upregulate the expression of antioxidative SOD expression in cultured mouse neuro-2a cells, or *in vivo* in mouse brain (Parikh, 2003). Studies on Alzheimer's disease have shown that neurodegeneration caused by $A\beta$ involves caspase activation and an increase in cellular oxidative stress (Yuan and Yankner, 2000), so we wanted to test the therapeutic efficacy of hEPN-1 against $A\beta$ -induced caspase activation and SOD expression. For the *in vivo* experiments, we used a double transgenic Alzheimer's mouse which shows increased production of highly neurotoxic $A\beta$, and abundant senile plaques, a main pathological lesion in AD.

We began with a qualitative approach via microscopy (Figure- 7) to validate the *in vitro* system established by Stovall in our lab (Stovall, 2006) and to determine useful therapeutic doses

for hEPN-1. Cell confluency significantly decreased following 3 days treatment with 20 μM Yankner peptide. While untreated SHSY cells showed stellate morphology and numerous intercellular connections, the Yankner-treated cells showed rounded morphology, fewer cellular processes, and decreased substrate attachment (clear signs of apoptosis), which agree with the original studies of Yankner et al. (1990) and Yan et al. (1996) that growing SHSY cells fully respond to the Yankner peptide. When the cells were given simultaneous Yankner peptide + hEPN-1 treatment, they showed normal cell morphology and confluency, confirming the earlier results of Stovall (2006). When viable cell counts were performed with various hEPN-1 doses (Figure-8), all hEPN-1 doses elevated the mean cell numbers, the cells treated with 75 μM and 225 μM hEPN-1 were statistically indistinguishable from the untreated cultures, and the 150 μM hEPN-1 treatment was statistically higher than the control. Higher doses did not improve cell survival any further, and may actually have slightly decreased it, perhaps indicating receptor desensitization at very high doses.

We began the mechanism of action studies by investigating cellular levels of caspases, key mediators of neuronal apoptosis (Yuan and Yankner, 2000). Caspase-3 was analyzed first because it is an end-stage effector caspase activated by several other cell death pathways (Bredesen *et al.*, 2006), so it is likely upregulated by $\text{A}\beta$. Immunoblot analysis was used to assay the cellular levels of caspase-3 using an antibody capable of detecting both inactive 32 kDa procaspase-3 and its cleaved active forms (14-21 kDa). Time-course experiments showed that 20 μM Yankner peptide induced a statistically significant increase in procaspase-3 levels, with maximum expression occurring at 72 hours (Figure-9). Thus the 72 hour time point was chosen for all subsequent caspase-3 experiments. No band was observed in the 14-21 kDa range in spite of the cells obvious apoptotic morphology, so perhaps only a small portion of procaspase-3 is cleaved to active form, and the active form is too faint to be observed in this immunoblot assay.

The 32 kDa procaspase-3 band disappeared in a negative control lacking primary antibody, demonstrating the specificity of the antibody. When cells were treated with Yankner peptide, or Yankner + various doses of hEPN-1 (Figure-10), both 75 μ M and 150 μ M hEPN-1 doses lowered procaspase-3 levels, and the 150 μ M hEPN-1 dose was statistically lower (Figure-11).

Caspase 2 is an initiator caspase belonging to the extrinsic caspase pathway (Figure-4) so we tested its upregulation in our system using immunoblots to determine whether the Yankner peptide activates this pathway to induce neuronal apoptosis. The only caspase-2 antibody that worked in our hands was one that detects only the inactive 48 kDa version of the enzyme, so no comments can be made about conversion to active caspase-2 form. As with procaspase-3, the procaspase-2 signal was maximal at 72 hrs (Figure-12), and the 150 μ M hEPN-1 dose produced a statistically significant decrease in procaspase-2 expression relative to the Yankner peptide alone (Figure-14), and the band was not visible in negative controls lacking primary antibody. Thus the Yankner peptide appears to stimulate the extrinsic cell death pathway in SHSY cells. Because no other caspase antibodies worked in our hands, at this time we are unable to draw any conclusions about whether A β or hEPN-1 affect other caspases

Because no conclusions could be drawn from the immunoblots about caspase *activation*, a commercially available caspase *activity* assay was performed (Figure-15). This assay uses a substrate recognized by end-stage caspases-3 and -7, that upon cleavage produces light that can be read in a luminometer (Préaudat *et al.*, 2002). Although the assay can not distinguish between caspase-3 and -7 activities, we reasoned this was insignificant since both are end-stage enzymes activated by the same pathways. The Yankner peptide treatment caused a significant increase in caspase-3/7 activity, while simultaneous treatment with Yankner + 75 μ M hEPN-1 treatment caused a significant decrease in the activity, although not a complete return back to normal levels. Thus hEPN-1 treatment not only decreases procaspase-3 levels but also decreases the levels of

activated caspase 3/7. Due to time constraints, the 150 μ M hEPN-1 dose was not tested in this assay, so it would be interesting in the future to see if increasing the hEPN-1 dose beyond 75 μ M can completely return the caspase-3/7 activity back to control levels.

One key point worth mentioning is that due to differences between peptide batches, both for the Yankner peptide and hEPN-1, differences were observed in the activities of the peptides. This made direct comparisons of the exact values of percent lowering, etc difficult between different experiments. However within a given experiment, conclusions could clearly be drawn. Differences in batch activities could have arisen from slight differences in storage temperatures, humidity, or number of times each batch was thawed and refrozen, or in slight contaminants present during the synthesis process.

In vivo experiments were performed using a commercially available Alzheimer's mouse (described in Methods) that overexpresses the neurotoxic A β peptide and shows a variety of Alzheimer's disease symptoms. Because an increase in neuronal oxidative stress occurs in this AD model, we tested the ability of hEPN-1 to potentially lower this stress via the upregulation of SOD. Treatment with low and high doses of hEPN-1 caused an increase in SOD levels (Figure 16), and the higher hEPN-1 dose was statistically significant (Figure-17).

Of all the caspase antibodies tested in this thesis, none produced a visible band when analyzing brain tissue, except the one for caspase-7. Caspase-7 is an effector caspase (Bredesen et al., 2006) activated essentially by the same pathways that activate caspase-3 (assayed above), so we deduced that hEPN-1 may lower its activity *in vivo*. But instead of getting the expected band of 34 kDa on the immunoblots, we got a band in the range of 80-100 kDa (Figure-18a). Because the band disappeared on a negative control lacking primary antibody (Figure-18b), we argue it interacts with the primary antibody, and so is caspase-7-related. The results of these

experiments showed that caspase-7-related protein (C7RP) levels decreased with both low and high drug doses, both statistically significant (Figure-19).

In conclusion, overall, the results presented in this thesis show that: 1) Yankner peptide-induced neuronal apoptosis appears to involve the activation (at least an increase in expression of two of its components, and an increase in activity of at least one component) of an externally stimulated (extrinsic) cell death pathway that includes caspase-2 and caspase-3, 2) that hEPN-1 can lower caspase expression and activity, and 3) that *in vivo* hEPN-1 can upregulate SOD expression, and decrease caspase-7-related protein expression.

Future experiments need to be performed to test whether the Yankner peptide activates the *intrinsic* caspase pathway (Figure-4), and whether hEPN-1 can alleviate this activation. Because the ROS generated by the extrinsic pathway often can activate the intrinsic pathway, we hypothesize the intrinsic pathway indeed is involved in A β induced apoptosis. A commercially available assay exists for caspase-9 activity (i.e. Promega G8210), which could determine whether the mitochondrial intrinsic pathway is involved. An immunoblot for caspase-12 (if a good antibody could be found) might help determine whether in ER intrinsic pathway is involved in A β signalling. It would also be interesting to determine whether known caspase inhibitors (such as Promega G7231 or G5961) can also block the effects of the Yankner peptide. Because hEPN-1 can lower caspase expression, perhaps hEPN-1 is engaging a receptor on the surface of the neuronal cells that is different than the RAGE receptor for A β . In fact, all known growth factors use cell receptors, so it would be interesting to characterize the hEPN-1 receptor if it exists. Future experiments could also be conducted to test involvement of the Bcl family of proteins, which has both pro- and anti-apoptotic members (Yuan and Yankner, 2000), and whether hEPN-1 can upregulate cellular inhibitor of apoptosis proteins (cIAPs) (Earnshaw et al., 1999). In addition, since previous work in our lab has shown that CMX-8933 goldfish

ependymin mimetic treatment causes an increase in AP-1 transcription factor (Adams *et al.*, 2003; Saif, 2004), future experiments could be conducted to test the involvement of AP-1 transcription factor in both *in vitro* and *in vivo* Alzheimer's model systems. Morishima *et al.* (2000) have shown that A β activates JNK kinase and the Fas ligand. These factors are associated with AP-1 activation, so likely A β indeed upregulates AP-1. Experiments could also be conducted to correlate the hEPN-1-mediated increase in SOD levels (shown in this thesis) with a decrease cellular levels of reactive oxidative stress using the TBARS (thiobarbituric acid reactive oxidative substrates) assay both *in vitro* and *in vivo*.

Currently there is no cure for Alzheimer's disease, but the U.S. Food and Drug Administration has to date approved five drugs for the treatment of mild to moderate AD (year of FDA approval in parentheses): Tacrine (Cognex®, 1993), Donepezil hydrochloride (Aricept®, 1996), Rivastigmine (Exelon®, 2000), Galantamine hydrobromide (Razadyne™ - formerly called Reminyl®, 2001), and Memantine HCl (Namenda™, 2003). All of these drugs inhibit acetylcholinesterase to increase the amount of acetylcholine neurotransmitter in the brain, which improves moderate symptoms temporarily. But these drugs treat only the symptoms, not the underlying neurodegeneration. Here, hEPN-1 has shown promising results in both *in vitro* and *in vivo* Alzheimer's model systems in targeting the underlying cause of Alzheimer's mediated neuronal apoptosis. Thus hEPN-1 could prove to be a therapeutic for Alzheimer's disease, and thus help millions who suffer from this disease.

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