PARTIAL RESTORATION OF CELL POPULATION SURVIVAL BY A HUMAN EPENDYMIN MIMETIC PEPTIDE IN AN IN VITRO ALZHEIMER'S DISEASE MODEL

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APPROVED:

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder that currently affects an estimated 4.2 million to 5.8 million Americans. Although the cause of AD is not fully known, the current working model proposes that amyloid precursor protein (APP) is unnaturally cleaved by beta and gamma secretases to form the highly neurotoxic peptide beta-amyloid (Aβ) which engages cell surface receptors to cause cell death through a series of events involving 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 Aβ residues 25-35 that strongly binds receptor. Treatment of SHSY cells with 20 μM Yankner peptide strongly induces cellular apoptosis.

Synthetic peptide human ependymin-1 (hEPN-1) is a derivative of a naturally occurring protein within the human brain, previously shown by our laboratory to upregulate antioxidative enzymes in SHSY cells, and AP-1 transcription factor associated with long-term memory formation. Since hEPN-1 has anti-oxidative potential as a therapeutic, we hypothesized that hEPN-1 can reverse the neurotoxic effects of Yankner peptide treatment of cultured human SHSY neuronal cells. Microtiter dishes were plated with SHSY cells under control conditions (no Yankner peptide), in the presence of 20 μM Yankner peptide, or in the presence of Yankner peptide plus various concentrations of hEPN-1 therapeutic, then cultured for 3 days to 80% confluency. Unattached dying cells were gently washed away, then the residual cells were monitored by measuring cell number, cell viability (Trypan blue exclusion), LDH activity per mg protein (an indirect measure of cell viability), and nuclear blebbing (a measure of apoptosis). Statistical significance was determined using a One Way ANOVA under the LSD stringency,

using SPSS.   In three independent trials, average cell numbers per microtiter well decreased 44.7% (from 3.11×10^5 to 1.72×10^5) in the presence of 20 µM Yankner peptide ($p < 0.05$ compared to control), were 2.73×10^5 when 75 µM hEPN-1 was added simultaneously with Yankner ($p < 0.05$ compared to Yankner), and were 2.96×10^5 when 75 µM hEPN-1 was added 24 hrs post-Yankner ($p < 0.05$ relative to Yankner alone). The control mean was not statistically distinguishable from either of the hEPN-1-treated samples ($p = 0.220$ and $p = 0.671$, respectively).

With respect to the trypan blue data, in three independent trials, the mean percent viable cells (excluding trypan blue) decreased 41.0% (from 68.7% to 40.5%) in the presence of 20 µM Yankner peptide ($p < 0.001$ relative to control), was 60.7% when 75 µM hEPN-1 was added simultaneously with Yankner ($p < 0.001$ relative to Yankner alone), and was 61.4% when 75 µM hEPN-1 was added 24 hrs post-Yankner ($p < 0.001$ relative to Yankner alone). The control mean was not statistically distinguishable from either of the hEPN-1-treated samples ($p = 0.013$ and 0.03, respectively).

In the LDH activity experiments, in four independent trials, the average LDH OD decreased 80.8% (from 0.47 to 0.09) in the presence of 20 µM Yankner peptide ($p < 0.001$ relative to control), was 0.47 when 75 µM hEPN-1 was added simultaneously with Yankner ($p < 0.001$ relative to Yankner alone), and was 0.48 when 75 µM hEPN-1 was added 24 hrs post-Yankner ($p < 0.001$ relative to Yankner alone). The control mean was not statistically distinguishable from either of the hEPN-1-treated samples ($p = 0.174$ and 0.479, respectively). Although previous reports in the literature indicated LDH expression is constitutive in SHSY cells (thus its activity is an indirect measure of cell numbers or viability), it was possible the hEPN-1 treatments upregulated LDH activity. So to ensure our observed changes in LDH

activity levels did not represent changes per unit protein, the LDH activity values were divided by the mg of protein present in the sample, and all four experimental samples were statistically indistinguishable (p values = 0.184, 0.995, 0.872, respectively, relative to control).

In the nuclear blebbing experiments, in five independent trials, the mean percent blebbed nuclei (a measure of apoptosis) doubled from 7.5% to 16.0% in the presence of 20 µM Yankner peptide (p < 0.001 relative to control), was 6.7% when 75 µM hEPN-1 was added simultaneously with Yankner (p < 0.001 relative to Yankner alone), and was 6.5% when 75 µM hEPN-1 was added 24 hrs post-Yankner (p < 0.001 relative to Yankner alone). The decreased apoptosis observed in the hEPN-1-treated samples was however, not statistically significant (p = 0.381 and 0.279, respectively).

Overall, the data suggest that hEPN-1 can protect human neuronal cells from Yankner-induced cell death, whether added simultaneous to the insult, or 24 hrs post. Because the therapeutic can act 24 hrs post-insult, it may interfere with a late-stage apoptotic event. As there is currently no known drug that blocks Yankner-induced toxicity, the hEPN-1 therapeutic shows potential in combating the underlying apoptosis of Alzheimer's disease.

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ACKNOWLEDGEMENTS

First and foremost, I give thanks to the Lord Jesus Christ, without whom nothing is possible. I want to thank Dr. David Adams, who has not only been my advisor, but a great teacher. I have learned so much working in his lab, not only the science, but about myself as a scientist. Thanks to him, I could not have a sincerer appreciation for the ability to troubleshoot problems, and I consider myself very fortunate to have been given the opportunity to work in his lab. In addition, I owe thanks to my committee members Dr. Dan Gibson and Dr. Eric Overstrom. Their advice and encouragement was greatly appreciated throughout the project. In addition to his general thesis support, all of Dr. Gibson's help with the immunofluoresence and microscopy was absolutely invaluable. A great amount of time was taken with that portion of the project, during which, Dr. Gibson always made himself available. Furthermore, I want to express my thanks toward my former lab mate, Erica Hirsch. Without Erica's "crash course" in cell culture technique, this thesis would have been significantly hindered. Also, I owe an endless debt of gratitude toward my family. You have all supported me in every aspect possible throughout my education, which I am very grateful for. I have no doubt I wouldn't be half the person I am today without all of you. From the encouragement and support, to the little extra motivation, I wouldn't have made it this far on my own.

BACKGROUND

According to the Alzheimer's Foundation of America (2006), Alzheimer's disease (AD) currently affects an estimated 4.2 million to 5.8 million Americans and that one in ten persons over age 65, and nearly half of those 85 or older have the disease. It is projected that the number of Americans with AD could reach a high of 16 million by 2050. Prior to its discovery, AD was viewed simply as another form of dementia, considered common for aging individuals. However, in 1906, German physician Alois Alzheimer 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. One hundred years later, these same two observations still serve as hallmark lesions for the proper diagnosis of the disease.
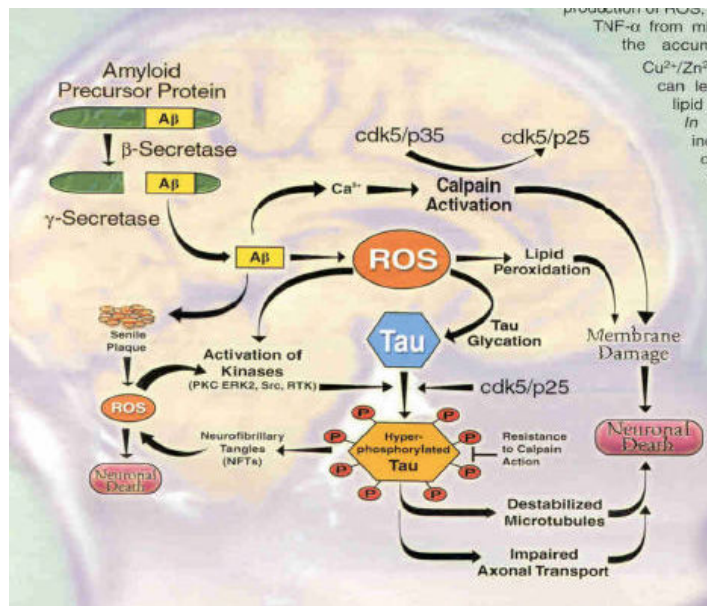
Alzheimer's Disease Mechanism

The cause of AD is unknown, but within the past 10 years a great deal has been learned about key events. The current working model for AD is often termed the "amyloid cascade hypothesis" (for reviews see Yuan and Yankner, 2000; Armstrong, 2006). This model is summarized in Figure-1. Amyloid precursor protein (APP) (shown in the upper left corner of the diagram) is a naturally occurring neuronal cell receptor that functions in the transport of β-secretase and presenillin-1. During AD, APP undergoes improper cleavage by beta and gamma-secretases to release a 42 aa length portion of the APP, known as β-amyloid (Aβ, aa 672-713) (shown as a yellow box in the diagram). Aβ monomers assemble into dimers, trimers, and higher aggregates, all of which are highly neurotoxic (Walsh et al., 2002). The aggregates eventually form the hallmark extracellular senile plaques (left side of diagram) within the brain

parenchyma and leptomeningeal areas, as well as the cortical vascular walls (Chaney et al., 2005). Although the presence of visible senile plaques has historically been thought to be responsible for the progression of the disease, the plaque precursors (Aβ monomers, dimers, trimers, and aggregates) are currently thought to serve as the neurotoxin. Of these, the dimers and trimers are thought to be the most neurotoxic (Walsh et al., 2002).

Aβ engages a variety of neuronal cell surface receptors (including the receptor for advanced glycation end products (RAGE), the NGF receptor p75, and TNFR (discussed below), to generate reactive oxidative stress (ROS), increase Ca^{++} flow into the cell, increase caspase activation, and cellular apoptosis. ROS also leads to the hyperphosphorylation of Tau protein (center of the diagram) that destabilizes microtubules. This process eventually forms intracellular neurofibrillary tangles, the second observable hallmark pathology of AD.

Figure 1 - Schematic of Alzheimer's Disease. The abnormal cleavage of Aβ from APP (upper left of diagram) is thought to initiate cellular events leading to neuronal apoptosis (Calbiochem, 2000).

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Aβ Signal Transduction

The interaction of Aβ with a variety of cell surface receptors is thought to be the key trigger initiating signal transduction events leading to neuronal apoptosis. Aβ has been shown to engage receptors with high affinity, such as the receptor for advanced glycation end products (RAGE), p75, and TNF-R (Figure 2).
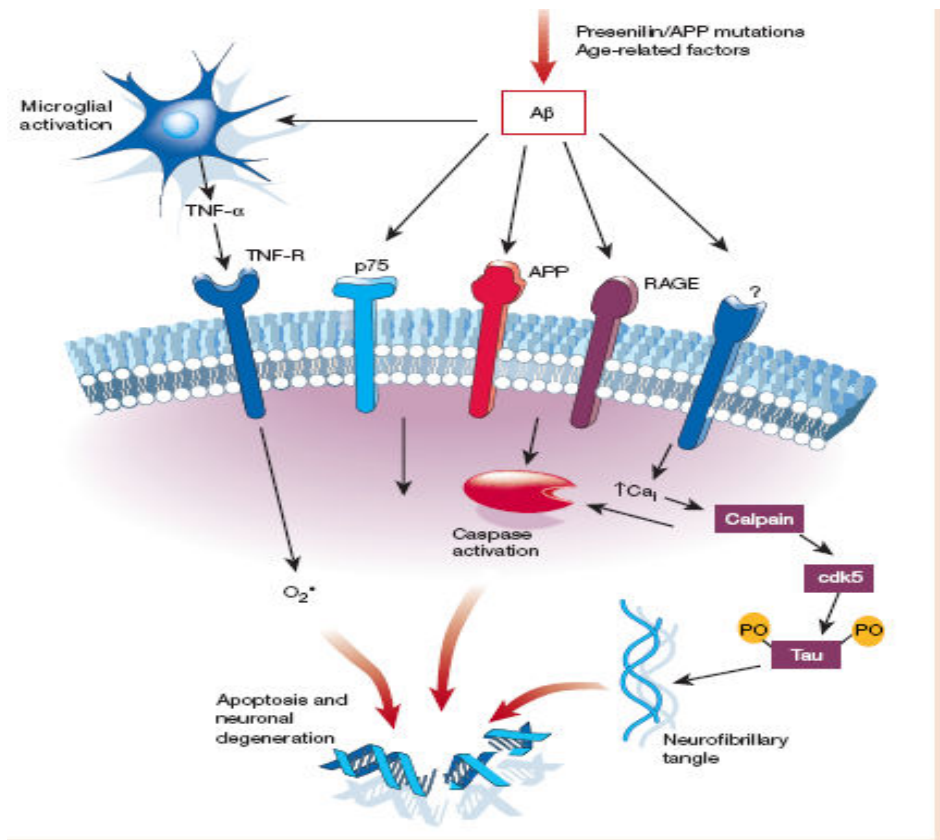
Figure 2 – Engagement of Receptors by Aβ. Aβ (upper center of diagram) has been shown to have high affinity for a variety of cell surface receptors that induces signal transduction events leading to neuronal apoptosis (Yuan and Yankner, 2000).

The interaction with the RAGE receptor has been especially well characterized. RAGE is a 404 aa long member of the immunoglobulin (Ig) superfamily of cell surface molecules that contains a single transmembrane domain and an N-terminal signal sequence of 22

aa (Bucciarelli et al., 2002). The receptor shows a high similarity with neural cell adhesion molecules (NCAMs) and induces Aβ-mediated cell adhesion and the induction of oxidative stress in microglia (Yan et al., 1996; Mackie et al., 1998). As Aβ engages RAGE, reactive oxidative stress (ROS) increases both inside the cell and within the cell membrane (Mattson and Rydel, 1996). When the ROS occurs within the cell, proteins, lipids, and DNA undergo damage that eventually leads to cell death. Inside the cell membrane itself, the ROS causes sodium and calcium ions to leave the cell, leading in turn to a depolarization of the membrane. Calcium sequentially floods the cell across the glutamate receptor, eventually causing additional damage to the DNA, proteins, and lipids. When Aβ activates microglia, it induces cytokine secretion that has cytotoxic proinflammatory effects (Chaney et al., 2005). The Aβ/RAGE interaction has further been shown to "elicit neuronal NF-κB 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.

The oxidative stress caused by Aβ activates the extrinsic p38/c-Jun N-terminal kinase (JNK) pathway, which culminates in the activation of transcription factor NF-κB and apoptosis (Morishima et al., 2001; Marques et al., 2003; Onyango et al., 2005). Antibodies against RAGE block the Aβ-induced activation of the p38/JNK pathway (Onyango et al., 2005). $Aβ_{17-42}$ (a peptide that includes the 25-35 toxic portion of Aβ has also been shown to activate initiator caspase 8, a key component of the extrinsic apoptotic pathway (Wei et al., 2002). In an elegant study, caspase 12-deficient cortical neurons were shown to be deficient in Aβ-induced apoptosis (Nakagawa et al., 2000), but not by staurosporine treatment or trophic factor deprivation. Thus

Aβ-induced apoptosis involves a variety of caspase enzymes. Further elucidation of the steps involved in Aβ-induced neuronal cell death will eventually provide more points for potential therapeutic intervention in Alzheimer's disease.

Yankner Peptide Aβ Mimetic

Various portions of the 42aa Aβ peptide have been tested for receptor-binding activity and neurotoxicity. An 11 aa portion $A\beta_{25-35}$ (more commonly termed the "Yankner peptide" after its discoverer Bruce Yankner of Harvard University) is the known minimal neurotoxic portion of Aβ (Yankner et al., 1990) (Figure-3). When added to cultured cells, the Yankner peptide has been shown to induce neuronal cell death with the same specific activity as Aβ (Yan et al., 1996; Misiti et al., 2005). PC12 neuronal cells treated with Yankner peptide show an increased cytosolic calcium concentration and a marked decrease in key metabolic enzyme activities (Bielarczyk et al., 2003; Ba et al., 2004). In human primary midbrain astrocytes, the Yankner peptide upregulates Cox 2 and increases the cellular release of inflammatory prostaglandin-2 via a protein kinase-C (PKC) pathway (Hull et al., 2006).
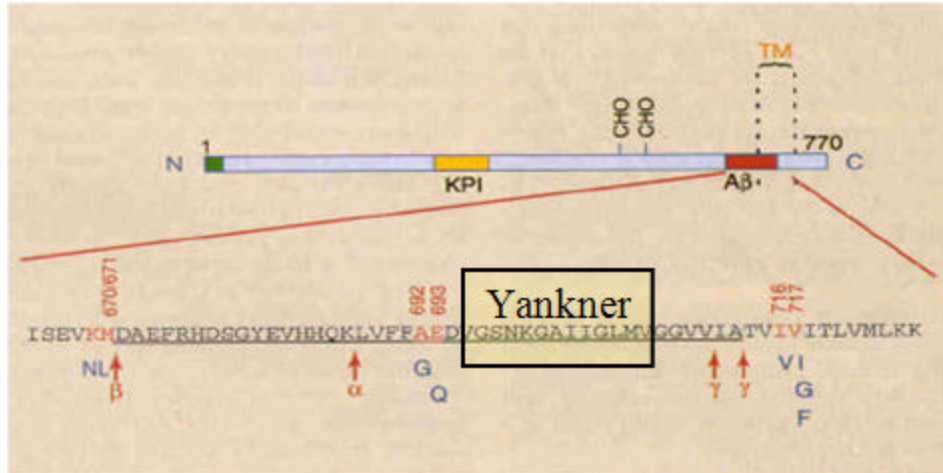
Figure 3 – The Yankner Peptide. The upper horizontal line denotes the full-length APP protein; the Aβ portion is denoted by a red box. The lower horizontal line denotes a portion of the APP amino acid sequence. $A\beta_{25-35}$ (Yankner peptide) is delineated by a box. The sequence also shows the cut sites for α, β, and γ secretases.

Cellular Apoptosis

Uncovering the various steps of Aβ-induced apoptosis is critical for providing steps for therapeutic intervention in Alzheimer's disease. Because these steps are still being elucidated, a comparison to what is generally known about cellular apoptosis may provide a background for expanding our knowledge of Aβ signal transduction. Apoptosis is a type of programmed cell death exhibiting a distinct set of morphological and biochemical features, such as: cell shrinkage, caspase activation, chromatin condensation, nuclear breakdown followed by DNA fragmentation (Kerr et al., 1972). 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).

The regulation of apoptosis has been extensively investigated, and is incredibly complex, however it usually occurs by two distinct converging pathways: the cell extrinsic pathway, and the cell intrinsic pathway (Green and Evan, 2002). In the former pathway, activation of cell

surface receptors such as the tumor necrosis factor receptor (TNFR) superfamily causes the activation of death "initiator" caspases 8 and 10, but can also stimulate survival factors such as nuclear factor kappa-B (NF-κB). If the balance of death signals overrides the survival signals, "effector" caspases (caspase 3, 6, and 7) become activated, causing chromosome condensation, DNA fragmentation, cell shrinkage, membrane blebbing, and cell death.

In either pathway, caspase enzymes are the critical mediators of cell death (Cohen, 1997; Nakagawa et al., 2000). 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 8 and 10) (extrinsic pathway) (Muzio et al., 1996), mediation of apoptotic signals after mitochondrial damage (caspase 9) (intrinsic pathway) (Li et al., 1997), and mediation of apoptosis through ER stress (caspase 12) (ER pathway).

In the intrinsic apoptotic pathway, cellular stress such as oncogene activation causes activation of the tumor suppressor p53 (Ryan et al., 2001), which results in the upregulation of pro-apoptotic proteins (e.g. Bax) or the inhibition of anti-apoptotic proteins (e.g. Bcl-2 family) causing changes in the mitochondrial membrane potential. These membrane changes cause the release of pro-apoptotic cytochrome-C, SMAC (Diablo), and apoptosis inducing factor (AIF). The pro-apoptotic factors induce cell death by activating caspase 9, or apoptotic protease-activating factor-1 (APAF-1), or by inhibiting inhibitor of apoptosis proteins (IAP's). The intrinsic pathway is regulated in part by a balance of signals coming from receptor tyrosine kinases (RTKs). Not all cell death situations use the classic extrinsic and intrinsic pathways, for example increases in endoplasmic reticulum stress uses neither (Trapani and Smyth, 2002).

Ependymin

Neurotrophic factors (NTFs) are proteins that stimulate neuronal growth and regeneration, thus they may have therapeutic applications for Alzheimer's disease. First discovered in the goldfish brain in the "zona ependyma" (Shashoua and Benowitz, 1977), ependymin (EPN) is a 216 aa neurotrophic factor (shown to function in long term memory potentiation and neuronal regeneration) that is secreted in both the extracellular fluid and cerebrospinal fluid. Existing in two forms, a β and γ version (molecular weights 37,000 and 31,000), the β form is glycosylated, whereas the γ form is not (Shashoua, 1976; Shashoua, 1985; Konigstorfer et al., 1989; Shashoua et al., 1990). The Adams and Shashoua labs have collaborated for over 15 years on projects to develop small synthetic peptide mimetics to neurotrophic factors that, unlike the parent protein, can cross the highly impenetrable blood brain barrier if delivered intravenously.

Peptide 8933 is an 8 amino acid synthetic portion of full-length goldfish ependymin, with the sequence KKETLQFR. Peptide hEPN-1 is a 14 amino acid synthetic portion of human ependymin, containing the entire analogous 8933 location plus flanking sequence, with the sequence KQCSKMTLTQPWDP. These synthetic peptides have been shown to mimic many of the signal transduction events of full-length ependymin, as shown in Figure-4. Peptide 8933 (shown as a green circle in the upper part of the figure) added to cultured neuronal cells activates the JNK pathway (middle part of the figure) and transcription factor AP-1 (a key mediator of long-term memory formation) (Adams et al., 2003). JNK pathway activation was shown to include an increase in JNK enzymatic activity, an increase in JNK phosphorylation, an increase in c-Jun phosphorylation, and an increase the cellular titers of c-Jun and c-Fos mRNAs (Ibid.). Peptide 8933 was also shown to upregulate antioxidative enzymes superoxide dismutase (SOD),

catalase (CAT), and glutathione peroxidase (GPX) (lower part of the diagram) (Shashoua et al.,

2004). Activation of AP-1 was shown to be a required step for the upregulation of SOD by 8933

(Saif, 2004). Full-length ependymin has also been shown to activate the JNK pathway (Kaska,

2003), and has been shown to engage a putative EPN receptor located on the neuronal membrane

(unpublished data).   Human peptide hEPN-1 has been shown to upregulate SOD and growth

related mRNAs for L-19, EF-2, ATP synthase in mouse neuroblastoma cells (Saif, 2004), and S-

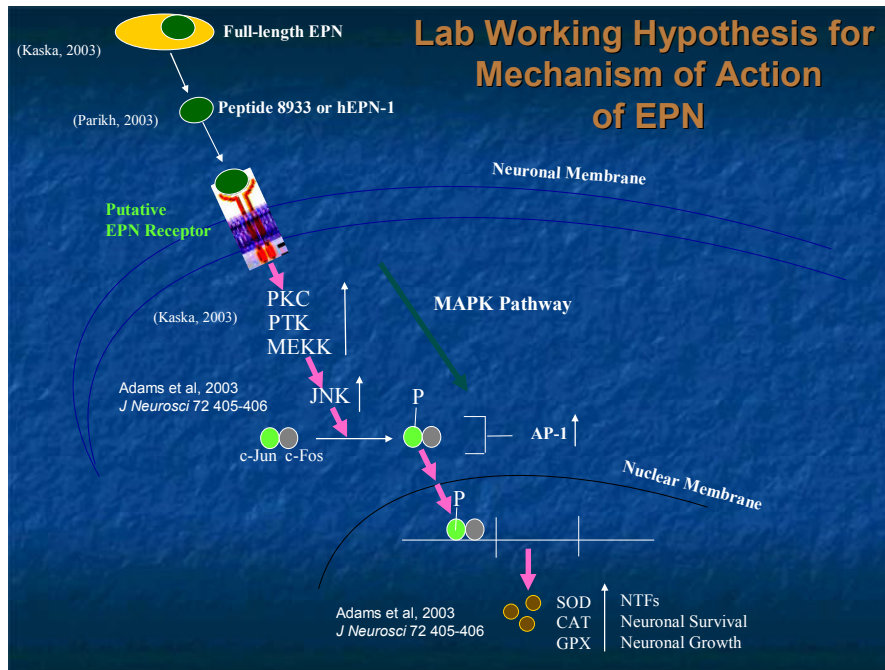19 and S-12 in human neuroblastoma cells (Arca, 2005).

Figure 4 – Our Laboratory's Proposed Mechanism of Action of Ependymin (Saif, 2004).

THESIS PURPOSE

As discussed in the Background section, the treatment of SHSY human neuronal cells with 20 µM Yankner peptide (the minimal toxic portion of Aβ) has been shown to induce neuronal oxidative stress that causes apoptosis. Data from our laboratory indicates that treatment of SHSY cells with ependymin neurotrophic factor mimetic hEPN-1 upregulates several anti-oxidative enzymes, thus it was surmised that hEPN-1 may help block Yankner-induced neuronal apoptosis. Such a therapeutic substance might help serve as an effective therapy against Alzheimer's disease. The goal of this thesis was to demonstrate that addition of hEPN-1 peptide to SHSY cells in culture can block some of the measurable neurotoxic effects of the Yankner peptide, including the induced decrease in viable cells, and an increase in nuclear blebbing as a measure of apoptosis. In addition an attempt was made to determine how late the therapy could be administered post-Yankner peptide addition to prevent apoptosis.

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 and Gentamicin were both 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, and stored at 4°C.

Upon thawing, the 1 ml ATCC sample in freezing medium was pipetted into a 15ml conical tube and brought to 10 ml with the addition of complete culture medium (see below). The sample was then centrifuged for 5 minutes at 1500 rpm to pellet the cells. After centrifugation, the supernatant was siphoned to remove any residual DMSO from the freezing medium. The cell pellet was then resuspended in 1 ml of complete medium and transferred to a T-25 flask. An additional 4 ml complete medium was added to the flask, then the flask was placed in an upright incubator and kept at 36°C and 5% CO_2. 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.

Yankner Peptide

Yankner peptide ($A\beta_{25-35}$) (Tocris Bioscience #1429, MW 1060.27) was stored at -20º C as a 1 mg vial of dry powder. The 1 mg peptide (943 nmol) was reconstituted into 0.94 ml of

filter sterilized serum-free D-MEM to create a 1 mM stock. The stock was aliquoted into several

eppendorf tubes to prevent repeated cycles of freezing and thawing with each use, then stored at

-20º C. For culture stimulations, 20 µl of 1 mM stock was added per 1 ml medium in a 24-well

microtiter dish to make a 20 µM final concentration.

hEPN-1 Peptide

The human ependymin based mimetic peptide hEPN-1 powder was received from Victor

Shashoua at Biotherapeutix (Waltham, MA), and stored at -20˚C. The peptide was reconstituted

into filter sterilized 1x PBS at a concentration of 1 mg/ml (3.75 mM), and stored at -20˚C. For

culture stimulations, 20 µl of 3.75 mM stock was added per 1 ml medium in a 24-well microtiter

dish to make a 75 µM final concentration.

Microtiter Dish Plating

After plating SHSY cells at 4×10^5 cells / ml in a 24-well microtiter dish, cells were

allowed to incubate for 3 days in a 36ºC incubator in an atmosphere of 5% CO_2. 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 plus 75

µM hEPN-1 added at the same time as the Yankner peptide), and 24 hr post (20 µM Yankner

peptide plus 75 µM hEPN-1 added 24 hours post Yankner challenge).

Cell Viability Determination

At the end of the 3-day culture incubation time, cells were trypsinized using 100 µl

Trypsin-EDTA (Invitrogen Life Technologies). Mictotiter dishes were allowed to sit for 30

seconds; the supernatant was then removed and placed in a 15ml conical tube, to which 9 ml of complete medium was added.   The tube was then centrifuged for 5 minutes at 5,000 rpm, and the supernatant was discarded. The cell pellet was resuspended in 1 ml of complete medium, and 100 µl of the cell suspension was removed and placed in a 1.5ml eppendorf tube. A 100 µl volume of Trypan Blue (Sigma-Aldrich, MW 960.81, 0.4% stock concentration) was added to the 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.

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 1x PBS, 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. As there were only viable cells remaining, the use of Trypan Blue was not employed, and the hemocytometer was loaded directly with the cell suspension after resuspending into 1 ml of complete media. Cells were viewed in the hemocytometer under 400x magnification. P-values were obtained using a One Way ANOVA test in the SPSS program with an LSD stringency parameter.

Lactate Dehydrogenase Assay

Cells were plated into a 24-well microtiter dish in the four experimental groups described above in "Mictotiter Dish Plating". At the end of the incubation period, the loosely bound dying

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cells were removed using 1x PBS washes as described above for cell count experiments. After

removing all of the PBS from the wells, 100 µl of 10% Triton X-100 was added to each well and

allowed to incubate at room temperature for 30 minutes to lyse the cells. After 30 minutes, the

100 µl of cell lysate was removed from each well and placed in 1.5ml microcentrifuge tubes.

Tubes were spun for 5 minutes at 10,000 x g. Upon completion of centrifugation, 50 µl of the

cleared supernatant from each tube was removed and placed in a second set of tubes, to which an

equal volume (50 µl) of the substrate mix (Promega #G1780, Cytotox 96® Non-Radioactive

Cytotoxicity Assay Kit) was added. After a brief mixing and centrifugation, the tubes were

allowed to incubate at room temperature for 30 minutes to complete the reaction. At the end of

the incubation period, 50 µl of stop solution (included in the Promega kit) was added to each

tube along with 850 µl of sterile dH_2O, to bring total volume to 1 ml. After a brief inversion, the

OD of the samples was read at 490nm.

Total Protein Measurement

When measuring total cellular protein, the same procedure as the LDH assay was

followed, except 2 µl of the 100 µl cleared 10% Triton X-100 cell lysate was placed in a quartz

cuvette along with 1 ml dH_2O. The OD of each sample was then read at 280 nm.

Nuclear Blebbing

Using a 24 well microtiter dish, SHSY cells were plated at $4x10^5$ cells / ml on a circular

cover slip placed in the center of the microtiter wells, using the same experimental groups as

described in the Microtiter Dish Plating section. After a three-day incubation period in a 36ºC

5% CO_2 incubator, the cells were stained for one to two minutes with 1:10,000 acridine orange in culture medium, adding approximately 4 drops per microtiter well. After allowing the staining to set in, the cover slip was removed from the microtiter dish and placed upside down on a microscope slide. Though acridine orange differentially stains RNA and DNA under a fluorescence microscope, the staining method also allows easy visualization of nuclear blebbing, a hallmark of cellular apoptosis. The stained cells were viewed with an AO Photostar microscope with mercury vapor illumination and an epifluorescence filter set appropriate for acridine orange fluorescence. The excitation wavelength was 490 nm, and the barrier filter was 520 nm, thus permitting observation of both orange and green fluorescence of RNA and DNA, respectively. In order to quantify nuclear blebbing events, 5 representative fields were chosen per slide. For each representative field, total cells and cells undergoing blebbing were quantified.

RESULTS

Microscopy

The purpose of this thesis was to determine whether the treatment of human neuronal SHSY cells with hEPN-1 peptide (a human neurotrophic factor mimetic) can block the neurotoxic effects of Yankner peptide (an Alzheimer's disease Aβ mimetic). SHSY cells were plated into a 24-well microtiter dish in medium containing no peptide addition (control), 20 µM Yankner peptide (a concentration previously shown in the literature to strongly induce SHSY apoptosis; Yankner et al., 1990), or 20 µM Yankner peptide plus 75 µM hEPN-1 peptide (a concentration chosen from our preliminary experiments, data not shown). After incubation for 3 days, dying loosely bound cells were washed off the wells, and an inverted microscope was used as an initial visual comparison between the samples (Figure-5).
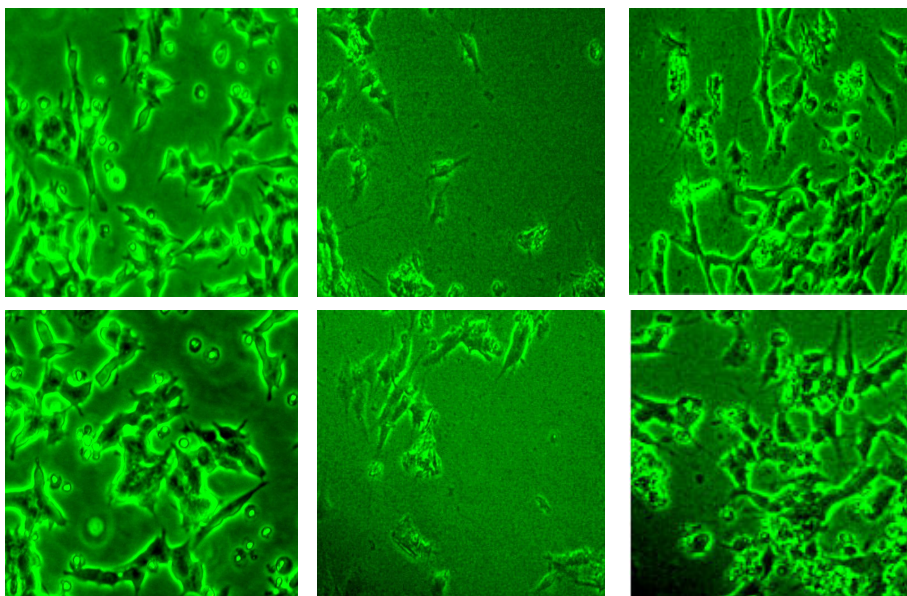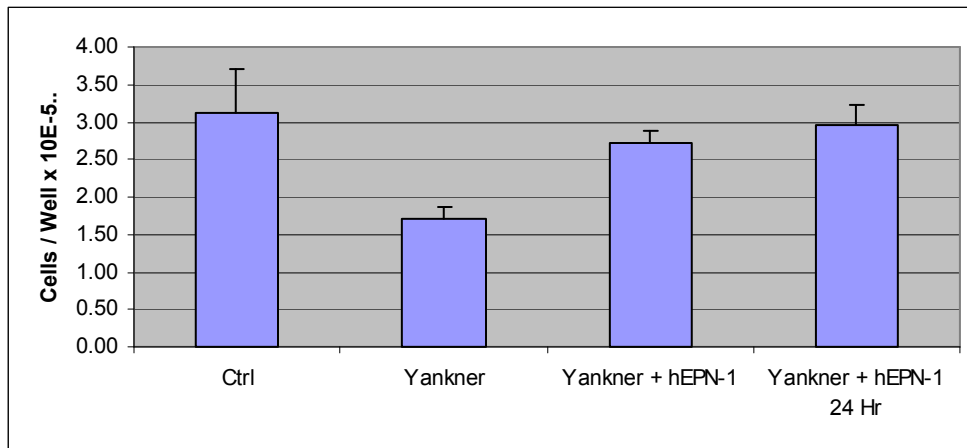

Figure 5 – Microscopy of Human SHSY Cells Treated with Yankner and/or hEPN-1 Peptides for 3 Days. Following 3 days of culture, loosely bound cells were removed by washes, then remaining cells viewed by microscopy. Upper and lower rows denote duplicate sets of representative fields. The left panels show 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.

Viewed at a magnification of 400x, two sets of representative fields (upper and lower rows) were photographed for the control cells (left panels), Yankner peptide treated (middle panels), and Yankner + hEPN-1 added simultaneously (right panels). While control cells obtained approximately 80% confluency during the 3-day culture period, the 20 µM Yankner challenged cells show a visibly reduced confluency, and the cell size was somewhat smaller. However, when hEPN-1 peptide was added simultaneously with the Yankner peptide, the cells appeared completely normal.

Measurement of Cell Numbers and Viability

Cell counts were performed on treated cultures using a standard hemocytometer (Figure 6), following a series of washes to remove dying cells. In three independent trials, average cell numbers per microtiter well decreased 44.7% (from 3.11×10^5 to 1.72×10^5) in the presence of 20 µM Yankner peptide ($p < 0.05$ compared to control), and were 2.73×10^5 when 75 µM hEPN-1 was added simultaneously with Yankner ($p < 0.05$ relative to Yankner alone). When the hEPN-1 was added 24 hrs post-Yankner (to determine whether it could be beneficial after the onset of early apoptotic signal transduction events), the cell numbers were 2.96×10^5 ($p = 0.005$ relative to Yankner alone). The control mean was not statistically distinguishable from either of the hEPN-1-treated samples ($p = 0.220$ and $p = 0.671$, respectively). 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.

Figure 6 – 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), or 20 μM Yankner peptide + 75 μM hEPN-1 peptide added 24 hrs post-Yankner (fourth histobar). Following 3 days of culture, loosely bound cells were removed by washes, and remaining cells counted in a hemocytometer. Each histobar represents the mean of three independent determinations. Error bars denote one standard deviation.

In order to determine total viable cells as a percent of the total cells present, a second set of cell counts was performed using Trypan Blue exclusion as a measure of viability (Figure-7). These experiments were performed as described above, except no cell washes were performed to remove dying cells. In three independent trials, the mean completely block the toxic effect of the Yankner peptide.
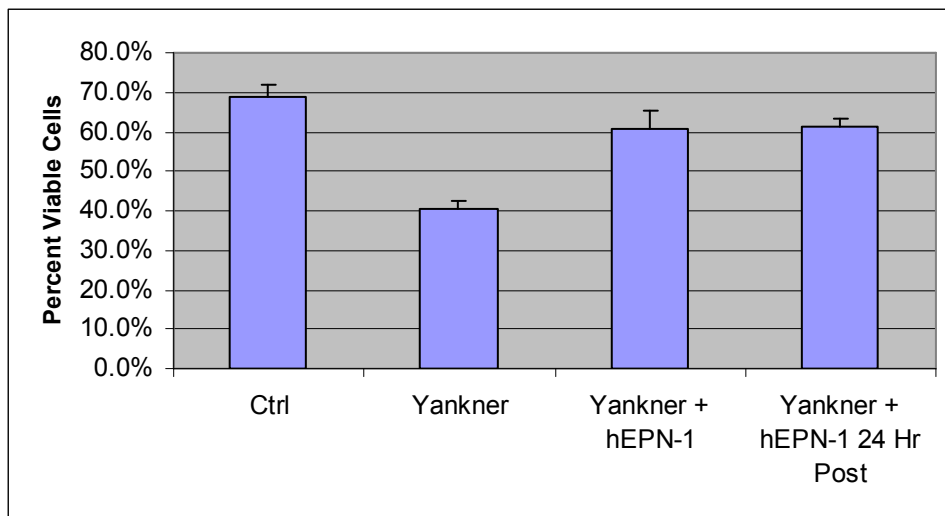
Figure 7 – Percent Viable Cells as Measured by Trypan Blue Exclusion for SHSY Cells Treated with Yankner and/or hEPN-1 Peptides for 3 Days. Cultures were treated as described in Figure-6. Following 3 days of culture, cells were stained with trypan blue, and the percent viable cells (trypan blue excluded) was determined by microscopy. Each histobar represents the mean of three independent determinations. Error bars denote one standard deviation.

Lactate Dehydrogenase Assay of Cell Viability

As an additional measure of cell viability, the enzymatic activity of Lactate Dehydrogenase (LDH) was quantified in the cells remaining attached to the wells following the washes to remove loosely bound dying cells (Figure-8). LDH is a housekeeping enzyme normally thought to be constitutively produced in all cells, including SHSY cells, and its assay has frequently been used to indirectly quantitate cell viability (Lemmen et al., 1983; Anderson et al., 2001). In four independent trials, the average LDH OD decreased 80.8% (from 0.47 to 0.09) in the presence of 20 μM Yankner peptide ($p < 0.001$ relative to control), was 0.47 when 75 μM hEPN-1 was added simultaneously with Yankner ($p < 0.001$ relative to Yankner alone), and was 0.48 when 75 μM hEPN-1 was added 24 hrs post Yankner ($p < 0.001$ relative to Yankner alone). The control mean was not statistically distinguishable from either of the hEPN-1 treated samples ($p = 0.174$ and 0.479, respectively).
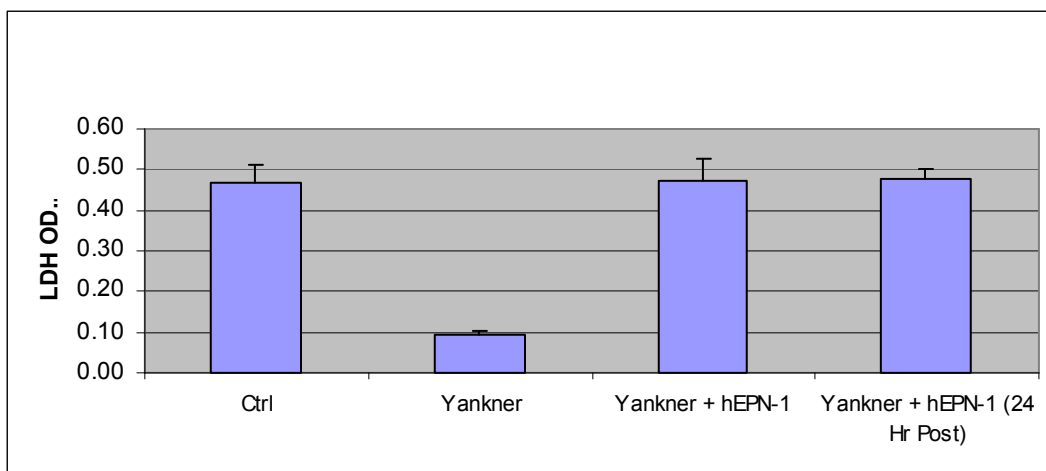
Figure 8 – LDH Activity as an Alternative Measure of Cell Viability for SHSY Cells Treated with Yankner and/or hEPN-1 Peptides for 3 Days. Cultures were treated as described in Figure-6. Following 3 days of culture, loosely bound cells were removed by washes, and cell lysates were prepared. LDH activity was measured in a spectrophotometric assay (see Methods). Each histobar represents the mean of four independent determinations. Error bars denote one standard deviation.

Although studies have shown that LDH is a constitutive enzyme not upregulated in human neuronal cells, to rule out the possibility that hEPN-1 upregulated the LDH activity in the Figure-8 experiments, total cellular protein was assayed in the same lysates used to obtain the LDH data to allow a calculation of LDH activity per unit protein. As expected, the overall histoplot profile of total cellular protein in the surviving cells (those remaining after the washes) (Figure-9) was very similar to the LDH histoplot profile. When the LDH activity value was divided by the protein value (Figure-10) for each sample, all four experimental samples were statistically indistinguishable (p values = 0.184, 0.995, 0.872, respectively, relative to control). Thus, when assaying cell viability by LDH activity, the addition of hEPN-1 peptide appears to completely block the toxic effect of the Yankner peptide.
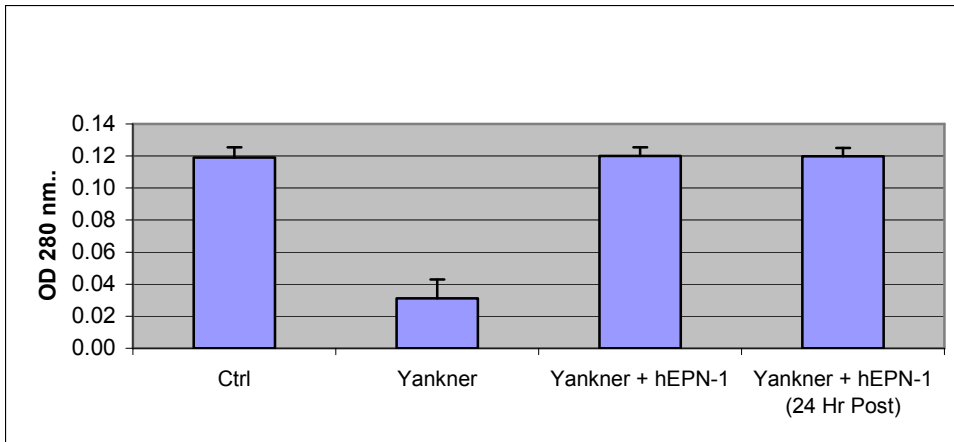
Figure 9 – Total Cellular Protein in SHSY Cells Treated with Yankner and/or hEPN-1 Peptides for 3 Days. Cultures were treated as described in Figure-6. Following 3 days of culture, the wells were washed to remove loosely bound cells, and cell lysates were prepared. Protein content was measured by UV absorbance at 280 nm. Each histobar represents the mean of four independent determinations. Error bars denote one standard deviation.
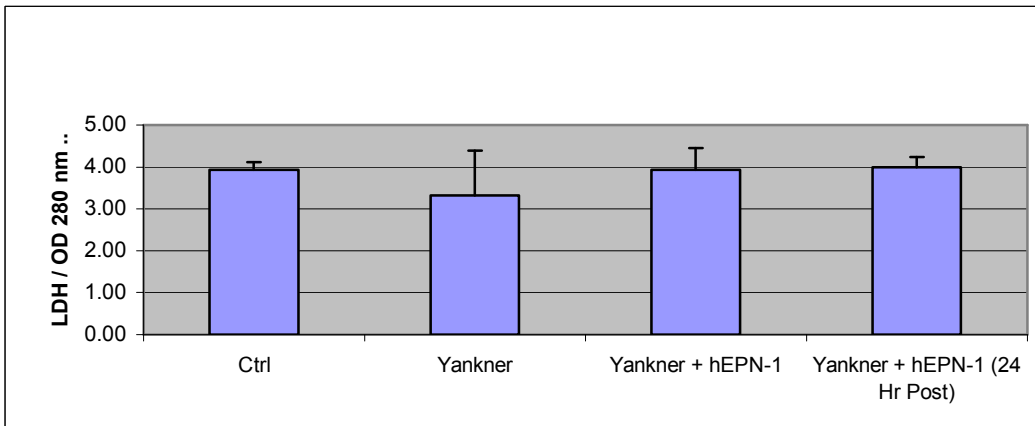
Figure 10 – LDH Activity Divided by Protein Content in SHSY Cells Treated with Yankner and/or hEPN-1 Peptides for 3 Days. The LDH data from Figure-8 was divided by the protein data from Figure-9. Each histobar represents the mean of four independent determinations. Error bars denote one standard deviation.

Quantification of Nuclear Blebbing

The therapeutic effects of hEPN-1 peptide were also tested by measuring nuclear blebbing as an indicator of apoptosis. Nuclear blebbing is one of the hallmark features of apoptosis that distinguishes it from general necrosis, and it is relatively easy to measure by microscopy (Kerr et al., 1972; Green and Evan, 2002). Cultures were treated with peptides as described in the previous experiments, then nuclear blebbing was monitored by fluorescence microscopy in acridine orange stained cells (Figure-11). In five independent trials, the mean percent blebbed nuclei doubled from 7.5% to 16% in the presence of the 20 μM Yankner peptide ($p < 0.001$ compared to control), was 6.7% when 75 μM hEPN-1 was added simultaneously with Yankner ($p = 0.001$ relative to Yankner alone), and was 6.5% when 75 μM hEPN-1 was added 24 hrs post-Yankner ($p < 0.001$ relative to Yankner alone). The decreased apoptosis relative to control observed in both hEPN-1-treated samples however, was not statistically lower than the control sample ($p = 0.381$ and 0.279, respectively). Thus, when assaying cellular apoptosis by nuclear blebbing, the addition of hEPN-1 peptide appears to completely block the toxic effect of the Yankner peptide.
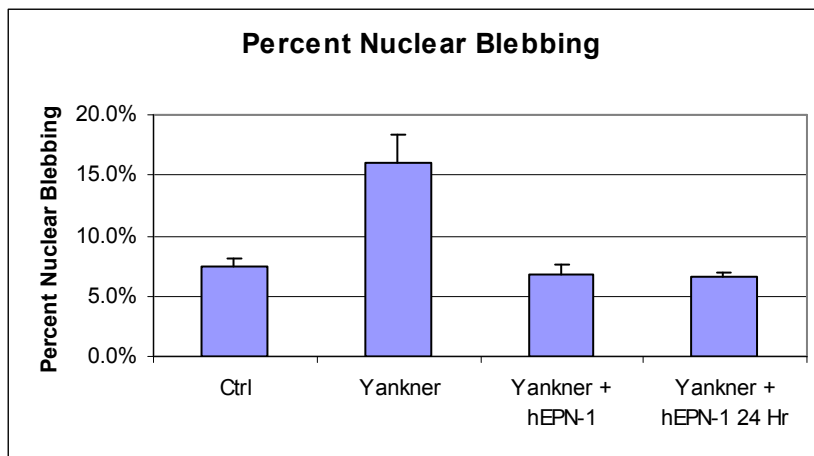
Figure 11 – Quantification of Nuclear Blebbing in SHSY Cells Treated with Yankner and/or hEPN-1 Peptides for 3 Days. Cells were treated with peptides as described in Figure-6, then the percent of cells showing blebbed nuclei was quantitated by fluorescence microscopy in acridine orange stained cells. Each histobar represents the mean of five independent field determinations. Error bars denote one standard deviation.

DISCUSSION

The overall goal of this thesis was to use an *in vitro* model for Alzheimer's disease (AD), cultured human SHSY neuronal cells treated with neurotoxic $A\beta_{25\text{-}35}$ (also termed the Yankner peptide) to determine whether the toxic effects of the Yankner peptide could be blocked by human ependymin mimetic hEPN-1 peptide. Our rationale was that because the treatment of SHSY cells with Yankner peptide is known to increase reactive oxidative stress (ROS) leading to apoptosis (Yan et al., 1996; Mattson and Rydel, 1996; Mackie et al., 1998; Yuan and Yankner, 2000; Armstrong, 2006), and because our lab has previously shown that treatment of these same cells with ependymin mimetics 8933 (Adams et al., 2001) and hEPN-1 (Saif, 2003) increases several anti-oxidative enzymes, perhaps an ependymin mimetic could block the toxic effect of the Yankner peptide.

Beginning with a qualitative approach via microscopy (Figure-5), cell confluency in the presence of the Yankner peptide appeared to be significantly decreased in comparison with the control. However, the cells treated with the hEPN-1 peptide simultaneously to the Yankner insult appeared to have normal cell numbers and morphology. Unlike a previous study suggesting that the SHSY neuronal cells must be differentiated with retinoic acid to respond to the Yankner peptide (Lambert et al., 1994), our studies agree with Yankner et al. (1990) and Yan et al. (1996) that growing SHSY cells fully respond to the presence of the Yankner peptide.

When cell counts were performed on the cells remaining attached to the wells after 3 days of culture with the various peptides (Figure-6), the data supported the initial microscopic observations, with a highly significant decrease in cell numbers in the Yankner treated culture, but no significant difference between the control cultures and those treated simultaneously with

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Yankner and hEPN-1. A similar protection was provided when adding hEPN-1 24 hrs post

Yankner insult, so hEPN-1 appears to interfere with a later event in apoptosis, not those events

that take place within minutes or hours, such as calcium flow. The hEPN-1 peptide not only

prevented cells from detaching from the substrate (as evident by post wash cell counts), but

prevented cells from undergoing cellular apoptosis as a result of oxidative stress, as evident by

trypan blue staining (Figure-7). Identical observations occurred when an alternative assay for

cell viability, the LDH assay, was used (Figure-8), which could not be explained by increased

expression of LDH (Figures-9 and 10).

As a direct measurement of cellular apoptosis, the percent of cells showing nuclear

blebbing was quantitated (Figure-11). Nuclear blebbing is one of the hallmark features of

apoptosis that distinguishes it from general necrosis, and it is relatively easy to measure by

microscopy (Kerr et al., 1972; Green and Evan, 2002). As expected based upon previous studies

showing that the Yankner peptide induces cellular apoptosis (Yan et al., 1996; Mattson and

Rydel, 1996; Mackie et al., 1998; Yuan and Yankner, 2000; Armstrong, 2006), the Yankner

challenged cells had twice as many nuclear blebbing events than the untreated control. The

hEPN-1 cells (either simultaneous or 24 hr post) protected the cells from the toxin (the mean

percent blebbing in five independent observations was statistically identical to control cultures),

actually scoring significantly less apoptosis than the control. Such protective effects have not

been seen by any drug currently used in Alzheimer's treatment (see below).

Although the data appears conclusive that hEPN-1 does prevent the Yankner neurotoxin

from inducing apoptotic events, additional work could further this hypothesis. One key question

remaining is exactly how late can the therapeutic act to save the cells? Such a study would seek

to differentiate between a matter of protection and regeneration. Specifically, how far beyond 24

hrs post insult can the hEPN-1 be added and still convey similar protection? As a negative control, a scrambled version of the hEPN-1 would be very useful to prove that it is indeed the sequence/shape of the hEPN-1 and not an unknown component of the peptide preparation that is blocking the effects of the Yankner toxin. Our lab previously showed that a scrambled version of ependymin mimetic 8933 was inactive, and 8933 is prepared using the same process as hEPN-1, so likely it is the sequence/shape of hEPN-1 that is active. Fluorescently-labeled hEPN-1 should bind the surface of neuronal cells if a receptor is being engaged, as is the case for all known neurotrophic factors. Ultimately, the next major step would be to extend the experiments to an *in vivo* Alzheimer's disease rodent model. Our laboratory helped create, in collaboration with the former Transgenic Sciences Inc., the first mouse model for AD (Games et al, 1995). hEPN-1 could be administered, with and without conjugation to a BBB carrier such as DHA (Shashoua et al., 2004), to explore its *in vivo* efficacy reducing astrocytosis and gliosis.

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[TM] - formerly called Reminyl®, 2001), and Memantine HCI (Namenda[TM], 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. hEPN-1 appears to block Aβ neurotoxicity, so this is a step in the right direction, although this treatment still does not address the fundamental cause of AD, the improper cleavage of APP to form Aβ.

Overall, the data suggest that hEPN-1 can protect human neuronal cells from Yankner-induced cell death, whether added simultaneous to the insult, or 24 hrs post. Because the therapeutic can act 24 hrs post-insult, it may interfere with a late-stage apoptotic event. As there is currently no known drug that blocks Yankner-induced toxicity, the hEPN-1 therapeutic shows potential in combating the underlying neural apoptosis of Alzheimer's disease.

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