AN ABSTRACT FOR THE THESIS OF

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Alzheimer's disease (AD) is the most common neurodegenerative disease and the main cause of geriatric dementia. A key event in AD is the accumulation of the 1-42 amino acid fragment of β-amyloid ($A\beta_{1-42}$). Clinical studies indicate that both 17-β-estradiol and IGF-I (when used separately) improve cognition and can protect cells from AD. Both hormones decrease in plasma with age, more so in women where 17-β-estradiol circulation falls to about 1% of pre-menopausal levels. I wanted to determine if combinatorial hormone replacement therapies of steroid and peptide hormones would lead to a synergistic protective effect against AD compared to single hormone replacement therapy. Hippocampal hybridoma HT22 cells were exposed to $A\beta_{1-42}$ or nontoxic controls ($A\beta_{40-1}$or 0.01% dimethylsulfoxide (DMSO)) and were additionally treated with different combinations and concentrations of hormones. After six days, survival of HT22 cells was measured by MTT (3-(4, 5-dimethylthiazol-2-y)-2, 4-diphenyltetrazolium bromide) assay and the results were expressed as percentage of the untreated control. $A\beta_{1-42}$ significantly reduced cell survival ($P < 0.0001$, ANOVA, $N=16$) compared to $A\beta_{40-1}$ or DMSO controls regardless of IGF-I dose (0 μM to 1 μM), estradiol dose (0 μM-10 μM) or any combination of the two hormones. IGF-I contributed to significantly higher HT22 cell survival ($P < 0.0001$, ANOVA) for all combinations of

peptides ($A\beta_{1-42}$, $A\beta_{40-1}$, and DMSO) and estradiol (0 μM to 10 μM). Estradiol did not contribute to any significant improvement of cell survival ($P > 0.05$, ANOVA) and a higher dose was deleterious ($P < 0.004$, ANOVA). No positive interaction between IGF-I and estradiol at any of the combinations of hormones was found for $A\beta_{1-42}$, $A\beta_{40-1}$ or DMSO treated cells ($P > 0.05$, ANOVA). In conclusion, IGF-I is able to protect HT22 cells from $A\beta_{1-42}$ toxicity while estrogen did little to protect the cultured neurons from $A\beta_{1-42}$. The failure of estrogen to protect HT22 neurons from β-amyloid could be due to lack of astrocytes that appear to mediate estrogen's effect by producing IGF-I.

HORMONAL REPLACEMENT THERAPY REDUCES B-AMYLOID TOXICITY: AN ALZHEIMER'S DISEASE TREATMENT MODEL.

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PREFACE

This thesis was prepared following the publication style of Journal of Neuroscience.

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INTRODUCTION

Alzheimer's disease (AD) was first described about 100 years ago by neurologist Alois Alzheimer in Munich (Ingram, 2003). The first patient, 57 years old female Auguste D., presented symptoms that are the hallmark of this disease: regressive memory loss, dementia and aggressiveness. Postmortem histology of the brain of Auguste D. showed the characteristic development of neuritic plaques and loss of neurons (Ingram, 2003). AD is now the most common neurodegenerative disease directly affecting about 11,000,000 people in the U.S. and costing the U.S. economy more than $ 65,000,000 annually (Taylor et al., 2002). AD is also the main cause of geriatric dementia. This disease progresses from loss of short-term memories to the complete loss of memory as the region of the brain that is responsible for forming new memories is destroyed (Selkoe, 2002).

The most common form of AD appears in patients who are in their 70s and 80s. This form of AD progresses slowly over the course of 15-25 years (Ingram, 2003). Although AD is typically an age-related disease, there have been rare cases where the victim was in mid-life leading to the suggestion that it may have some genetic link (Taylor et al., 2002). This inherited link may be due to a mutant allele or an altered gene locus that is passed from generation to generation. These individuals usually have an early onset of AD in their late 30s and early 40s and their conditions deteriorate rapidly (Petit and St. George-Hyslop, 2005; Tanzi and Bertram, 2001; Cruts and Broeckhoven, 1998; Rochhi et al., 2003; Goate, 1997). Another genetic clue to the inherited from of AD comes from victims of Down's syndrome. Down's syndrome patients have an extra copy of chromosome 21 which increases their risk for AD. This increased risk is because the

gene that encodes for amyloid precursor protein (APP) is believed to be located on chromosome 21 (Ingram, 2003).

AD causes both structural and functional changes in the brain. It affects primarily the cortex and leads to the formation of two types of abnormalities identified histologically in post-mortem brain tissues. These abnormalities are:

1. Extracellular senile neuritic plaques found between brain cells and
2. Intracellular tangles found within the cells (Taylor et al., 2002; Felician and Thomas., 1998; Ingram, 2003). The extracellular neuritic plaques are made up of a toxic protein fragment, β-amyloid. The ability of β-amyloid to cause neuritic plaque formation is dependent on the length of the primary amino acid chain. The 1-40 and 1-42 β-amyloid forms are the most hazardous (25-35 is the lethal active site of the peptide) (Ingram, 2003; Taylor et al., 2002; Haars et al., 2000; Lu et al., 2001; Villa et al., 2002). These amyloid plaques appear first in the hippocampus, a region in the brain that is responsible for memory encoding and emotional stability (Selkoe, 2002). As the disease progresses and the amyloid plaques accumulate in the cortex, the hippocampal cells become damaged and undergo apoptosis.

The toxic protein fragment, β-amyloid, is cleaved from a normal cell membrane protein termed the APP (Ingram, 2003; Selkoe, 2002; Taylor et al., 2002; Kopcho et al., 2003). APP is a transmembrane protein that is expressed in many cells including neurons. It is a receptor protein that binds directly to a light chain on the tail of kinesin-1 and is proposed to be a transmembrane motor protein receptor molecule in nerve cell axons (Alberts et al., 2002). APP has three cleavage sites targeted by three normal body enzymes; α, β, and γ secretases. Normal processing of APP by α secretase eliminates the

hazardous β-amyloid peptide and yields several important non-hazardous proteins (Sherwood, 2001). One of the peptides that is secreted when α secretase cleaves APP is sAPPα. The sAPPα protein has many functions in the body including: 1. Promotion of long term neuronal survival, 2. Promotion of neurite outgrowth and synaptogenesis, 3. Modulation of neuronal excitability and synaptic plasticity, 4. Control of gene expression (nuclear factor kappa B expression) and 5. Act as a ligand for the class A scavenger receptors (Selkoe, 1994; Greenfield et al., 2000; Barger and Basile, 2001; Barger and Mattson, 1996; Luo et al., 2001). In the development of AD, APP is cleaved within its transmembrane segment by β and γ secretases leading to two peptides. One of the peptides is deposited into the cytosol of the neurons and the other one into the extracellular space of the brain. The length of the extracellular fragment is variable. The predominant fragment has a length of 40 amino acids from the N-terminus but a minor fragment with two additional hydrophobic amino acids is also produced (Ingram, 2003). These extracellular toxic fragments then aggregate and can lead aggressively to the dysfunction of the nearby brain cells and their consequent death (Ingram, 2003). The presence of these aggregates causes a calcium ion influx that disrupts cellular processes and eventually leads to an apoptotic event (Ingram, 2003).

Experimental models

AD processes can be studied in the cell culture system (Selkoe, 2002; Dore et al., 1997; Haars et al., 2002; Murphy and Andrews, 2000; Lu et al., 2001; Xia et al., 1998; Liang et al., 2001; Villa et al., 2002; Zhao et al., 2002; Manthey et al., 2001; Hosoda et al., 2001). In this study, I examined the rescuing effects of estradiol and IGF-I on cells

that have been exposed to the toxic β-amyloid by using cultured immortalized hippocampal hybridoma cells. I used hybridoma cultured cells because neurons, the functional units of the brain, cannot replicate after embryogenesis.

Hormones in human physiology

Hormones play a major role in the regulation of day to day activities of the human body. As people age, production of some of these vital hormones is reduced (Sherwood, 2001, Carro et al., 2002). In this research, I examined the rescuing effects of two major hormones during amyloid exposure: 17-β-estradiol and insulin-like growth factor 1(IGF-I).

17-β-Estradiol

Estrogen is a large group of steroids that includes estriol, estrone, and estradiol (Norris, 1997). The estrogen that I used in this study was 17-β-estradiol. Like androgens, estrogens are ultimately derived from cholesterol (Norris, 1997). Most of the cholesterol that is used in synthesizing steroid hormones is obtained from food or is synthesized in the liver and released into the blood (Norris, 1997). The main function of the estrogens is to stimulate differentiation and proliferation of the uterine lining and maintain secondary sexual characteristics of the female (Norris, 1997). Estrogen affects areas in the brain related to reproduction and also affects brain regions involved in learning and memory (Dohanich and Daniel, 2001). Menopause marks the end of a woman's reproductive life and is accompanied by a decrease in estrogen levels to about 1% of pre-menopausal levels (Sherwood, 2001; Wise, 2003). During the last century, the average lifespan of

women has increased to 78 years but the average age of menopause has remained at 51 years (Wise, 2003). This means that many women will live in an estrogen-deprived condition for over 20 years. Because memory loss is one of the symptoms of AD, it suggests that a decrease in estrogen with age predisposes the victim to AD (Murphy and Andrews, 2000) and indeed, compared to men, postmenopausal women have a four-fold increased risk of AD (Gibbs, 1999).

The importance of estrogen in AD is reflected by hormone replacement therapy (HRT) (Schoenknecht et al., 2002). Experiments with female AD victims show that estradiol therapy improves cognitive function (Zheng, 2002; Zandi et al., 2002; Wise, 2003). In one of the clinical studies, the geriatric female AD patients who received estradiol in HRT had fewer symptoms of AD (Zheng, 2002). This suggests that estradiol was able to protect cells against the toxicity of $A\beta_{1-42}$ peptide of AD in clinical studies.

Insulin-like growth factors

Insulin-like growth factors (IGF-I and IGF-II) are hormones synthesized and secreted by the liver and other peripheral tissues (Norris, 1997). Their main function is not well understood but they are known to mediate the effects of growth hormone which are: Stimulate absorption of amino acids and protein synthesis, especially epiphyseal plates of bones (Norris, 1997). These peptides are called insulin-like because they are structurally similar to insulin (40 % homologous to insulin) and have insulin-like activities (i.e. activation of the Glut-4 glucose transmembrane transporter). IGF-I receptors are similar to those of insulin in that the IGF-I and insulin receptors are both dimers composed of two α chains (containing the binding site) and two β chains

(containing the tyrosine kinase activity). In contrast, the IGF-II receptor is a single transmembrane protein. These receptors (IGF-I, IGF-II, and insulin) bind specifically to their ligands but they can recognize and bind the other two ligands with lower affinity (Nissey et al., 1985; Dore et al., 1997). IGF-I and IGF-II receptors are uniquely concentrated in the hippocampus (Dore et al., 1997) which is the area that is used in memory and is greatly affected in AD patients. Levels of serum and brain IGF-I and the IGF-I binding proteins decrease with age (Carro et al., 2002). This decrease is more pronounced in people with neurodegenerative diseases such as AD (Carro et al., 2002). IGF-I not only prevents, but also rescues (pre-exposed up to 4 days) hippocampal neurons from β-amyloid induced toxicity (Dore et al., 1997). Other trophic factors such as nerve growth factors, basic fibroblast growth factors, and transforming growth factor lack this rescuing effect (Dore et al., 1997).

Rationale

I wanted to examine if IGF-I and estradiol would increase the survival of cultured brain cells which have been exposed to a lethal protein fragment ($A\beta_{1-42}$) of AD patients. I hypothesized that combinatorial hormone replacement therapy of steroid and peptide hormones would increase cell survival against this lethal protein fragment. These combinations were between different concentrations of estradiol and IGF-I. Therefore, I wanted to determine the effects of estradiol and IGF-I on cell survival during $A\beta_{1-42}$ exposure.

METHODOLOGY

Cell culture

The cells that I used in my experiment were a gift from Dr. Pam Maher of the Scripps Research Institute (La Jolla, CA). This hybridoma cell line (HT-22) was made by fusing mouse hippocampal brain cells with a human cervical cancer cell line (HT-3). The hybridoma that was formed maintained properties of mouse hippocampal brain cells and continuously replicated. This cell line is easily grown in culture. My preliminary research showed that these cells exhibited reduced metabolic activity associated with AB_{1-42} while the non-toxic control reverse sequence β-amyloid, AB_{40-1}, did not affect the cells.

The cell culture work was done aseptically in a vertical laminar flow hood (Nuaire, Nu-425-600, Plymouth, MN). The cells (HT-22) were grown in Dulbecco's modified eagle medium (DMEM) (Gibco, Grand Island, NY) that was supplemented with fetal bovine serum (10 %) (Hyclone, Logan, UT) and gentamicin (100 μg/ml of DMEM) (Gibco). The cells were grown in a CO_2 incubator (Revco Scientific, RCO3000TABA, Asheville, NC) at 37^0C and 5% CO_2. Every three days, the DMEM was removed and replaced with fresh media.

Chemicals for experimentation

IGF-I, trypsin, dimethylsulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-y)-2, 4-diphenyltetrazolium bromide (MTT), and 17-β-estradiol were obtained from Sigma-Aldrich Chemicals Co. (St. Louis, MO). The toxic β-amyloid (AB_{1-42}) and the non-toxic β-amyloid (AB_{40-1}) were obtained from Bachem Bioscience Inc.

(King of Prussia, PA). I used microtiter 96 well plates and T25 culture flasks obtained from Costar (Corning, NY) and sterile transfer pipettes and micropipeter from Fisher Scientific (Pittsburgh, PA). Toxic and non-toxic amyloid were dissolved in DMSO then diluted into culture media. DMSO control treatment involved culture media supplemented with 0.01% DMSO. A final DMSO concentration in all experiments was maintained at less than 0.01% to avoid cytotoxicity.

Experimental design

On day one, cells were dissociated from T25 flasks with trypsin and phosphate buffered saline (PBS) (recipe: 0.0027 M KCl , 0.0015 M KH_2PO_4, 0.14 M NaCl, 0.0081 M $Na_2PO_4 \cdot 7H_2O$, sterile filtered at 0.2 μM) (Freshney, 1987). I counted the number of cells in a drop of suspended cells with a hemocytometer. Cells were diluted to a final concentration of 10,000 cells/ml and transferred (1000 cells/well) to each well of the 96 well culture plates. The cells were incubated in the CO_2 incubator at 37^0C and 5% CO_2. On day two, the cells were treated with combinations of β-amyloid and hormones. Each well was exposed to toxic $A\beta_{1-42}$, non-toxic $A\beta_{40-1}$, or DMSO then treated with different combinations and concentrations of hormones (Fig. 1). Measurements of cell survival were performed after six days.

Measurement of cell survival

The method for measuring HT22 survival was modified from Dore, at el., (1997). I used two different measures of survival with 16 replicates for each measure of survival. In the first method, the cells were dissociated from the 96 well culture plate and

suspended in 100 µl of media. The suspension was placed on a hemocytometer and examined with a compound microscope. The suspended cells were counted and the number of cells recorded. In the second measurement of cell survival, I used the MTT colorimetric assay. MTT is a proposed indicator of mitochondrial activity in living cells (Mosmann, 1983; Freshney, 1987). The mitochondria of cells convert MTT into an insoluble blue-black product, formozan, in proportion to the amount of glucose that has been metabolized. After 6 days of exposure to Aβ related peptides ($A\beta_{1-42}$, $A\beta_{40-1}$), DMSO control and hormones, the media in the wells of the 96 well culture plates was removed and replaced with DMEM mixed (9:1) with MTT stock (5 mg/ml MTT in phosphate buffered saline). After three hours of exposure to the MTT mixture, acidified isopropyl alcohol (isopropanol with 0.1M HCl) was applied to each well. I used a multi-well plate reader (Bio-Tek Instruments, Mo, EL 800, Winooski, Vermont) to measure absorbance of the MTT product at 550 nm. If the mitochondria are not functional (cell is morbid or moribund), the change in color will not occur. Survival via MTT assay was reported as a percentage relative to DMSO control cells that were not exposed to Aβ related peptide, IGF-I or estradiol. All experiments were repeated 16 times and the means were analyzed using two-way ANOVA via SAS software (Cary, NC). Results are given as the mean ± standard deviation with $P \leq 0.05$ considered significant.

RESULTS

Growth curve of HT22 cells

I examined the normal growth rate, in terms of MTT absorbance values, of HT22 cells. There was an increase in cell numbers from day 1 (Figs. 2, 4) to day 6 (Figs. 3, 4) by which the cells reached confluency. The MTT absorbance values followed an exponential growth curve (almost doubling every day from day 1) (Fig. 4).

Neuronal toxicity induced by $A\beta_{1-42}$

I found that HT22 cells were susceptible to $A\beta_{1-42}$. $A\beta_{1-42}$ caused a dose-dependent decrease in MTT absorbance values (Fig. 5). Results of an ANOVA on the $A\beta_{1-42}$ dose response indicated that there was a significant difference in the effects of the different doses of $A\beta_{1-42}$ ($P < 0.0001$, $N = 16$). There was no significant difference among the MTT absorbance values for 0 μM, 1 μM and 2 μM doses of $A\beta_{1-42}$ while higher concentrations (5 μM and 10 μM) of $A\beta_{1-42}$ significantly slowed growth more than lower concentrations, (0 μM-2 μM), but no difference existed between 5 μM and 10 μM ($P < 0.0001$, ANOVA-LSMA) (Fig. 5). A 3.3 μM $A\beta_{1-42}$ dose was chosen for the reminder of the trials to simulate levels of $A\beta_{1-42}$ during early stages of AD.

I also found that the $A\beta_{40-1}$ and DMSO controls did not affect HT22 cell survival. $A\beta_{1-42}$ (3.3 μM dose) yielded an MTT absorbance value that was significantly lower than $A\beta_{40-1}$, DMSO and untreated controls ($P < 0.0001$, ANOVA-LSMA, $N = 16$). There was no significant difference among the three controls: $A\beta_{40-1}$, DMSO and untreated cells ($P < 0.0001$, ANOVA-LSMA, $N=16$). The positive control for amyloid toxicity ($A\beta_{40-1}$) and the negative control (DMSO) did not affect the survival of HT22 cells (Fig. 6).

Effects of hormones on $A\beta_{1-42}$ toxicity

I sought to determine whether hormone replacement therapy using IGF-I, 17-β-estradiol, or combinations of the two hormones would improve cell survival during exposure of the HT22 cells to $A\beta_{1-42}$. $A\beta_{1-42}$ significantly reduced cell survival ($P < 0.0001$, ANOVA-LSMA, N=16) compared to $A\beta_{40-1}$ or DMSO controls regardless of IGF-I dose (0 μM to 1 μM), estradiol dose (0 μM-10 μM) or any combination of the two hormones (Fig. 7). Interestingly, the positive control ($A\beta_{40-1}$) produced a significantly higher MTT percentage score than $A\beta_{1-42}$ ($P < 0.0001$, ANOVA-LSMA, N=16) but was significantly lower than the DMSO negative control for all hormonal treatments ($P < 0.0254$, ANOVA-LSMA, N=16) (Fig. 7).

IGF-I hormonal treatment

A fairly consistent pattern of MTT absorbance results were obtained for IGF-I treatments during exposure of HT22 cells to the amyloid peptides ($A\beta_{1-42}$, $A\beta_{40-1}$ and DMSO) and estradiol (0 μM-10 μM). IGF-I contributed to significantly higher HT22 cell survival ($P < 0.0001$, ANOVA-LSMA, N=16) at all combinations of peptide ($A\beta_{1-42}$, $A\beta_{40-1}$, and DMSO) and estradiol (0 μM to 10 μM) (Fig. 7A-E). Interestingly, the lower doses of IGF-I (0.1 μM and 0.2 μM IGF-I) produced the best cell survival (Fig. 7A-E) at all peptide and hormone combinations ($P < 0.0001$, ANOVA-LSMA, N=16). IGF-I at 0.1 μM increased cell survival of $A\beta_{1-42}$-treated cells from (60%) to (80%) of untreated controls at all estradiol doses (Fig. 7) while 0.2 μM IGF-I elevated from (60%) to (75) of untreated controls at all estradiol doses (Fig. 7). For the cells exposed to $A\beta_{40-1}$ or DMSO, IGF-I at 0.2 μM produced the best cell survival at all estradiol doses but 0.2 μM

IGF-I was significantly different, but higher, from 0.1 μM ($P < 0.2932$, ANOVA-LSVA, N=16) or 0.5 μM IGF-I ($P < 0.2207$, ANOVA-LSMA, N=16). Higher application rates of IGF-I (0.5 μM and 1.0 μM) did not contribute to improved HT22 cell survival ($P > 0.05$, ANOVA-LSVA, N=16).

Estradiol hormonal treatment

Estradiol application at any dosage failed to improve HT22 cells survival ($P > 0.05$, ANOVA-LSMA, N=16) (Fig. 8). The MTT percentage scores appeared to be consistent at each estradiol dose for combinations of $A\beta_{1-42}$, $A\beta_{40-1}$, or DMSO application and each IGF-I treatment (Fig. 8A-E). The least-squares means analysis of the results found that the highest dose of estradiol (10μM) produced a significantly lower cell survival for all peptides ($A\beta_{1-42}$, $A\beta_{40-1}$, DMSO) and IGF-I applications ($P < 0.004$, ANOVA-LSMA, N=16).

Combinations of IGF-I and estradiol

No positive interaction between IGF-I and estradiol at any of the applied doses of hormones was found for $A\beta_{1-42}$, $A\beta_{40-1}$ or DMSO treated cells ($P > 0.05$, ANOVA-LSMA, N=16).

Effects of IGF-I on $A\beta_{1-42}$ toxicity using both MTT assay and hemocytometer cell counts

I sought to determine if the increase in MTT percentage scores associated with higher IGF-I concentrations during $A\beta_{1-42}$ exposure was due to increase in HT22 cell

numbers or was due to increase in cell mitochondria activity. The MTT assay is a proposed indicator of mitochondrial activity in living cells (Mosmann, 1983).

MTT assay results

$A\beta_{1-42}$ significantly reduced cell survival ($P < 0.0001$, ANOVA-LSMA, N=53) to 79% compared to 104% for $A\beta_{40-1}$ or 100% for DMSO at 0 uM IGF-I (Fig. 9A). At higher IGF-I doses (0.1 μM and 1.0 μM), $A\beta_{1-42}$ reduced cell survival to 99% for 0.1μM IGF-I and 85% for 1μM IGF-I compared to 131% for 0.1 μM IGF-I and 118% for 1 μM IGF-I DMSO or 125% for 0.1 μM IGF-I and 119% for 1 μM IGF-I $A\beta_{40-1}$ (Fig. 9A). IGF-I contributed to significantly higher HT22 cell survival ($P < 0.0001$, ANOVA-LSMA, N=16) at all combinations of peptide ($A\beta_{1-42}$, $A\beta_{40-1}$, and DMSO) (Fig. 9A). The lower dose of IGF-I (0.1 μM IGF-I) produced the best cell survival at all Aβ-peptides and DMSO (Fig. 9A, ($P < 0.0042$, ANOVA-LSMA, N=16). The 0.1 μM IGF-I dose increased cell survival of $A\beta_{1-42}$-treated cells from (79%) to (98%) of untreated controls (Fig. 7A). The higher dose of IGF-I (1.0 μM) did not contribute to further improvement of HT22 cell survival (Fig. 9A).

Hemocytometer cell count

$A\beta_{1-42}$ significantly reduced HT22 cell numbers ($P < 0.0001$, ANOVA-LSMA, N=16) compared to $A\beta_{40-1}$ or DMSO controls at all IGF-I doses (0, 0.1 and 1 μM) (Fig. 9B). At 0 μM IGF-I, $A\beta_{1-42}$ significantly reduced HT22 cell numbers ($P < 0.0003$, ANOVA-LSMA, N=16) to 83,000 ± 10,600 cells compared to $A\beta_{40-1}$ (96,000 ± 26,000 cells) or DMSO (111,000 ± 13,600 cells) (Fig. 9B). The 0.1 μM IGF-I dose increased

$A\beta_{1-42}$ treated cells numbers from 83,000 ± 10,600 cells to 93,000 ± 10,600 cells and $A\beta_{40-1}$ treated cells number from 96,000 ± 26,000 cells to 116,000 ± 4,5000 cells. It also increase DMSO treated cells from 111,000 ± 13,600 cells to 129,000 ± 19,800 cells (Fig. 9B). The 0.1 μM IGF-I dose had the highest protective effect in all the treatments ($A\beta_{1-42}$, $A\beta_{40-1}$ and DMSO) ($P < 0.0467$, ANOVA-LSMA, N=16). The highest dose of IGF-I (1.0 uM) did not have a greater protective effect (Fig. 9B).

Comparing MTT assay and hemocytometer cell count

MTT assay and hemocytometer cell count methods yielded results (for IGF-I, $A\beta_{1-42}$, $A\beta_{40-1}$, DMSO) that were similar (Fig. 9). In both methods, $A\beta_{1-42}$ reduced cell survival compared to $A\beta_{40-1}$ and DMSO at all IGF-I doses (Fig. 9A and B). IGF-I contributed both to a significantly higher HT22 cell number and for a higher MTT percentage score with 0.1and 0.2 μM IGF-I being the more effective IGF-I doses (Fig. 9A and B). I concluded that the increase in MTT percentage scores was due to an increase in cell number but not an increase in mitochondria activity within each cell.

DISCUSSION

My data show that IGF-I but not estradiol, is able to protect HT22 hybridoma cells from β-amyloid toxicity. HT22 cells were significantly affected by $A\beta_{1-42}$ but not by $A\beta_{40-1}$ or DMSO (Fig. 6). Compared to estradiol, IGF-I increased cell survival, with the greatest protection provided by 0.1 μM IGF-I (Fig. 7). Total cell numbers (Fig. 9) showed that IGF-I increased cell numbers after HT22 cells were exposed to $A\beta_{1-42}$ with 0.1 μM IGF-I being more effective than higher IGF-I concentrations. Even though estradiol has been shown to be protective in both clinical and cell culture studies, estradiol did not protect HT22 cells from $A\beta_{1-42}$.

Alzheimer's disease

AD is a multifactorial disease with both genetic (familial) and non-genetic (sporadic) causes (Petit and St. George-Hyslop, 2005). Over 100 rare but highly penetrant mutations have been described in three different genes for early-onset familial AD (Tanzi and Bertram, 2001). These three genes are: the APP gene, the presenilin-1, and the presenilin-2 genes. Together, these mutations are responsible for 30-50% of the cases of autosomal dominant AD, and for 5% of AD in general (Cruts and Broeckhoven, 1998). The remaining 95% of AD patients are mostly sporadic late-onset cases, with a complex etiology due to interactions between environmental conditions and genetic features of the individual (Rochhi et al., 2003). Additionally, association studies have shown that the epsilon 4 allele of the apolipoprotein E gene increases risk of AD in a dose-dependent manner in both familial and sporadic forms of AD (Goate, 1997). The APOE epsilon 4 story is not as firm as the APP/Presenilin genetic story since 50% of sporadic AD cases

have no APOE epsilon 4 allele. Therefore, it is likely that there are additional unidentified AD risk factors. Moreover, there are families that have Mendelian-like inheritance of AD in which there are no mutations in any of the known AD genes (Goate, 1997).

One of the risk factors that could affect AD development could be the decrease in IGF-I or estradiol levels, or both of them. Research has shown that both hormones decrease with age (Carro et al., 2002; Sherwood, 2001) and both hormones have neuroprotective abilities (Dore et al., 1997; Asthana et al., 2001; Zhang et al., 2004). Therefore, depression of estradiol and IGF-I has negative effects on the cerebral cells. Dore et al. (1997) demonstrated that IGF-I can protect pre-incubated cells from β-amyloid toxicity. Dore also found an increase in IGF-I receptors on hippocampal cells that were exposed to $A\beta_{1-42}$. This increase in IGF-I receptors is presumably a super-sensitivity response due to decrease in IGF-I levels. In agreement with Dore's study, our results show that IGF-I is able to protect HT22 cells from β-amyloid. These results show that a decrease in IGF-I with age could be a contributing factor to onset and progress in sporadic AD.

IGF-I

Previous studies with IGF-I show that this hormone is able to protect cells from different kinds of insults. First, IGF-I was able to protect rat hippocampal neuronal cultures from $A\beta_{1-42}$ induced apoptosis (Dore et al., 1997). Second, IGF-I was also able to protect R 28 cells (neural cell line from the neonatal rat retina) from a different lethal challenge: serum-deprived-induced apoptosis (Barber et al., 2001). Dore et al. (1997)

demonstrated that IGF-I rescues neurons previously exposed to $A\beta_{1-42}$. In addition to IGF-I, other trophic factors and hormones protect different cell types against various types of insults *in vitro*. One such hormone with protective actions is insulin. Barber et al. (2001) showed that insulin in addition to IGF-I is able to protect R 28 cells from serum-deprived-induced apoptosis. Another growth factor with protective effect is nerve growth factor (NGF) which protects PC-12 cells from apoptosis induced by tumor necrosis factor *in vitro* (Haviv and Stein, 1999). In addition to insulin and NGF, brain-derived neurotrophic factor prevents retinal ganglion cell death after axotomy (Nakazawa et al., 2002). Even though all these trophic factors and hormones have neuroprotective abilities, none of them is as effective as IGF-I. Moreover, none of these other trophic factors rescues neurons from $A\beta_{1-42}$ (Dore et al., 1997).

The mechanism involved in IGF-I protection is not well known but it has been speculated that IGF-I offers its protection through the IGF-I –induced phosphorylation and inhibition of a pro-apoptotic transcription factor, the fork head transcription factor 1(FKHRL1) (Zheng et al., 2002). The IGF-I receptor is a dimer that is inserted in the cell membrane. When IGF-I binds to its receptor, it leads to autophosphorylation of the cytoplasmic domain of the receptor dimer. This leads to the activation of several signaling pathways, including the phosphotidylinositol 3-kinase (PI3K)/Protein kinase B (Akt) pathway (Zheng et al., 2002) and the mitogen-activated protein kinase (MAPK) pathway. Activation of the phosphotidylinositol 3-kinase (PI3K)/Protein kinase B (Akt) pathway by the IGF-I receptor leads to reduced apoptosis (Zheng et al., 2002). Protein kinase B is a serine/threonine kinase and is a downstream target of PI3K. Protein kinase B is involved in improved cell survival that is induced by various growth factors such as

transforming growth factor (Dudek et al., 1997; Dhandapani et al., 2005) and IGF-I (Zheng et al., 2002). When IGF-I binds to its receptor, it activates the tyrosine kinase subunit of the IGF-I receptor which recruits and activates PI3K. The activated PI3K then activates protein kinase B (Dudek et al., 1997). Other peptide hormones such as brain-derived neurotrophic factor (Zheng and Quirion, 2004; Kim et al., 2004) and insulin also activate the PI3K/Protein kinase B pathway in retinal neurons (Barber et al., 2001). Phosphorylated protein kinase B in turn phosphorylates and inhibits several pro-apoptotic proteins such as Bad (del Peso et al., 1997), caspase-9 (Cardone et al., 1998) and FKHRL 1 (Zheng et al., 2000) (Fig. 10). Inhibiting pro-apoptotic activities of these proteins leads to cell survival (Datta et al., 1999).

Another pathway that is triggered when IGF-I binds to its receptor is the activation of MAPK pathway. Binding of IGF-I to its receptor leads to an increase in the transcription of specific genes (e.g., c-jun) that are involved in the growth response (Zheng et al., 2002) (Fig. 10).

17-β-Estradiol

Estradiol protects different kinds of cells from apoptosis (Asthana et al., 2001; Zhang et al., 2004). Clinical studies demonstrate that women who take estrogen in hormone replacement therapy (HRT) have fewer symptoms of AD (Zheng, 2002). Female AD victims also perform better in cognition tests during estrogen replacement therapy (Zheng, 2002; Zandi et al., 2002; Wise, 2003; Duka et al., 2000; Asthana et al., 2001; Baker et al., 2003).

In vitro studies have shown that estradiol can protect some types of cultures of acutely dissociated neurons from apoptosis (Hosoday et al., 2001; Botao et al., 2004; Pike, 1999; Hawkins et al., 1999). The exact mechanism involved in preventing apoptosis or ameliorating AD symptoms is not well known but estradiol increases transcription of IGF-I in the frontal cortex of ovariectomized rhesus monkeys (Cheng at el., 2001) and in rat hypothalamic neurons (Garcia-Segura et al., 1996). Estradiol also increases transcription of transforming growth factor (Dhandapani and Brann, 2002). Estradiol, a steroid hormone, can easily cross the plasma membrane and travel to the nucleus of cells where it attaches to the estrogen receptor and initiates transcription. Examples of gene products activated in cells by the estrogen-nuclear receptor complex include growth hormone (GH) (Tulipano et al., 2004; Wilson, 2005) and IGF-I (Cardona-Gomez et al., 2001; Cheng et al., 2001; Quesada and Micevych, 2004; Kamanga-sollo et al., 2004; Kanbur-Oksuz et al., 2004; Shingo and Kito, 2003). Furthermore, estradiol replacement therapy increases GH secretion in postmenapausal women and in women with gonadal dysgenesis (Wilson, 2005). The GH that is produced can either go to the liver where it causes IGF-I production (Xu et al., 1995; Wilson, 2005; Kanbur-Oksuz et al., 2004) or cause a paracrine / autocrine IGF-I production by the astrocytes, a class of glial cells in brain (Velasco et al., 2005). Hepatic IGF-I is then transported by insulin-like growth factor binding protein in plasma to the brain where IGF-I can exert its effects. Even though IGF-I is a peptide hormone and thus cannot diffuse across membranes, there is evidence that IGF-I is transported across the blood-brain barrier through transcytosis (Schneider et al., 2003). In addition to hepatic IGF-I, a relevant amount of IGF-I is also produced in the brain in response to estradiol. This production of IGF-I is demonstrated

by the presence of increased levels of IGF-I mRNA in glial cells (Shingo and Kito, 2003; Shamanga-Sollo et al., 2004; Schneider et al., 2003; Cheng et al., 2001). Furthermore, both GH and IGF-I receptors occur in the brain, with the highest density in the hippocampal formation (Dore et al., 1997).

The failure of estradiol to protect neurons in my study contradicts results from both clinical and culture studies. This failure of estradiol could be due to differences in the cell cultures that were employed. Dore et al. (1997) and other researchers used acutely dissociated cells. Such acute cultures of nervous tissue harvested from fresh brain tissues are typically contaminated with glial (predominantly astrocyte) and endothelial cells. Glia account for almost 90% of all brain cells (Dhandapani and Brann, 2002). The main targets of estradiol are astrocytes (Dhandapani et al., 2005; Dhandapani et al., 2003; Sortino et al., 2005). In my study, the HT22 cells were not contaminated with glial cells.

Astrocytes express estrogen receptors α and β (Sortino et al., 2005). The culture of HT22 cells lacked glial or endothelial cells to produce IGF-I locally in the culture. This lack of glial contamination of the HT22 culture could explain why estradiol protected neurons in acutely dissociated cultures (Ashana et al., 2001; Zheng, 2002) but not in my study. Interestingly, the lack of neuroprotective effect of estradiol has been reported in pure cortical neuron cultures challenged with toxic insults such as hypoxia/ischemia, oxidative damage, β-amyloid and glutamate excitotoxicity (Sortino et al., 2005). Estradiol failed to show protective effects in other highly purified primary neuronal cultures (Dhandapani and Brann, 2002) even with physiological concentrations of estradiol (Dhandapani et al., 2005). Evidence supporting the hypothesis that astrocytes are the main target for and mediator of estrogen action in the brain includes; 1)

astrocytes are the most abundant type of glial cells in the brain (outnumbering neurons by a 10:1 ratio) 2) astrocytes are localized in juxtaposition to neurons 3) estrogen increases glial cell proliferation (Dhandapani and Brann, 2002) 4) estrogen receptors α and β have been identified in rat cortical and hippocampal astrocytes in vitro (Dhandapani and Brann, 2002; Sortino et al., 2005) and 5) estradiol increases transcription of IGF-I mRNA in glial cells (Shingo and Kito, 2003; Shamanga-Sollo et al., 2004). In addition to its neuroprotection against β-amyloid, estradiol has been implicated in the reduction of neuronal death following stresses such as excitotocixity and oxidative stress (Dhandapani et al., 2003).

Large scale randomized HRT trials have shown a modest increase in breast cancer risk with long-term use (>15 years) of estradiol in HRT (Marsden, 2002; Seeger et al., 2004; Lippert et al., 2003; Lippert et al., 2002; Isaksson et al., 1999). In one study, low concentrations of estradiol (serum concentrations) had a proliferative effect on MCF-7 cells (a human breast cancer cell model) (Lippert et al., 2002). High concentrations of estradiol had no effect on MCF-7 cell or endothelial cell proliferation (Lippert et al., 2002). This increase in cell proliferation after estradiol treatment is through estradiol metabolites that are involved in carcinogenesis (Seeger et al., 2004). Not all estradiol metabolites lead to carcinogenesis, some have proliferating and others have anti-proliferating properties. Therefore, even though some endogenous estradiol metabolites stimulate breast cancer cell proliferation, others (i.e. 4-hydroxyestradiol 1, 2-methoxyestradiol 1 and 2-methoxyestradiol 2) may be suitable for breast cancer treatment when used in high dosages, since they inhibit cancer cell growth and neoangiogenesis (Seeger et al., 2004). Since some metabolites exhibit no proliferative activity, they can be

used in clinical studies of chemoprotection and adjuvant therapy of breast cancer (Seeger et al., 2004).

Further studies with IGF-I, estradiol and acutely dissociated cells (with intentional glial contamination) would help determine if combinatorial hormone replacement therapy with both a peptide and a steroid hormone has a greater effect in protecting neurons from β-amyloid. We did not test our cells for glial contamination but there should not have been glial contamination because our culture was a pure hybridoma culture not an acutely dissociated culture.

Conclusion

IGF-I was able to protect HT22 cells from $A\beta_{1-42}$ toxicity while estrogen did nothing to protect HT22 cultured hybridoma neurons from $A\beta_{1-42}$. Even though a combinatory effect of IGF-I and estradiol is more effective in protecting other cell cultures such as osteocytes of aged ovariectomized rats (Verhaeghe et al., 1996) and nigrostriatal dopamine neurons in Parkinson's disease (Quesada and Micevych, 2004) than individual hormones, estradiol was not effective in our study. The failure of estrogen to protect HT22 neurons from β-amyloid was likely due to lack of glial cells that mediate estrogen's effect.

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Figure 1. Treatment paradigm for $A\beta_{1-42}$, $A\beta_{40-1}$ and DMSO.

$A\beta_{1-42}$(3.3 μM), $A\beta_{40-1}$(3.3 μM) or DMSO

IGF -1 (μM)

17-β-Estradiol (μM)

	1.0	0.5	0.2	0.1	0
10					
5					
2					
1					

Figure 2. Photomicrograph of day two HT22 cells. Cells were grown in DMEM media supplemented with fetal bovine serum (10%) and gentamicin (100 μg/ml of DMEM).

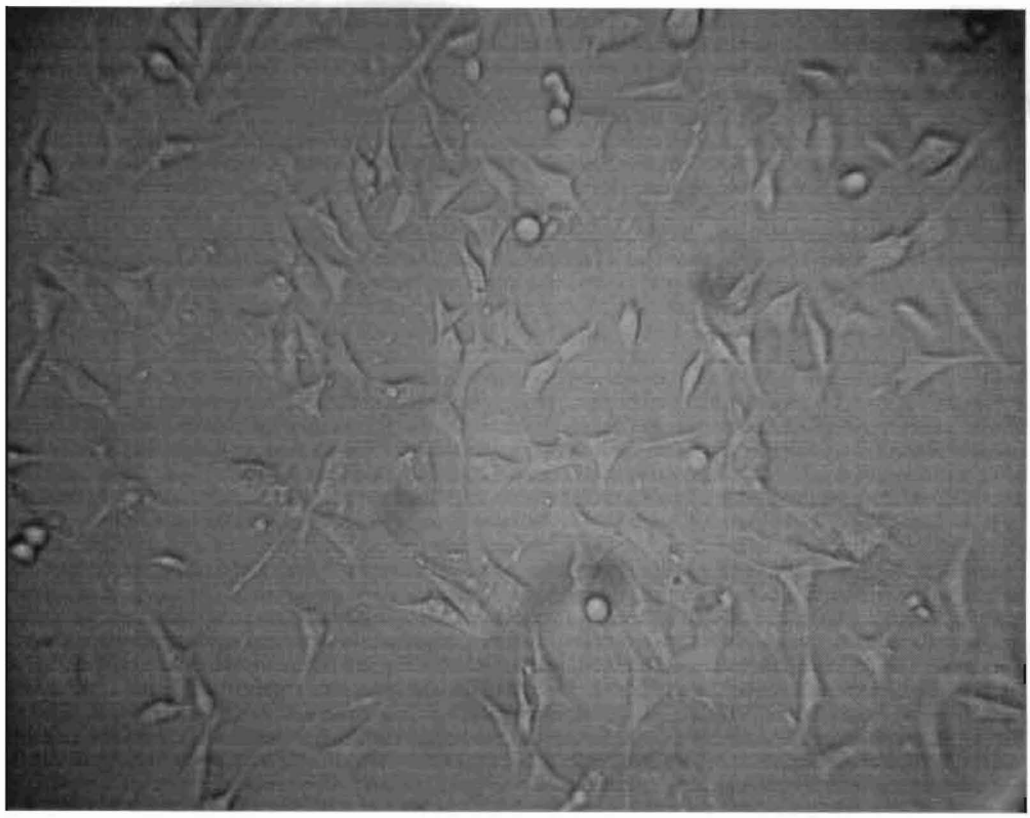


Figure 3. Photomicrograph of day six HT22 cells. Cells were grown to confluency in DMEM media supplemented with fetal bovine serum (10%) and gentamicin (100 μg/ml of DMEM).

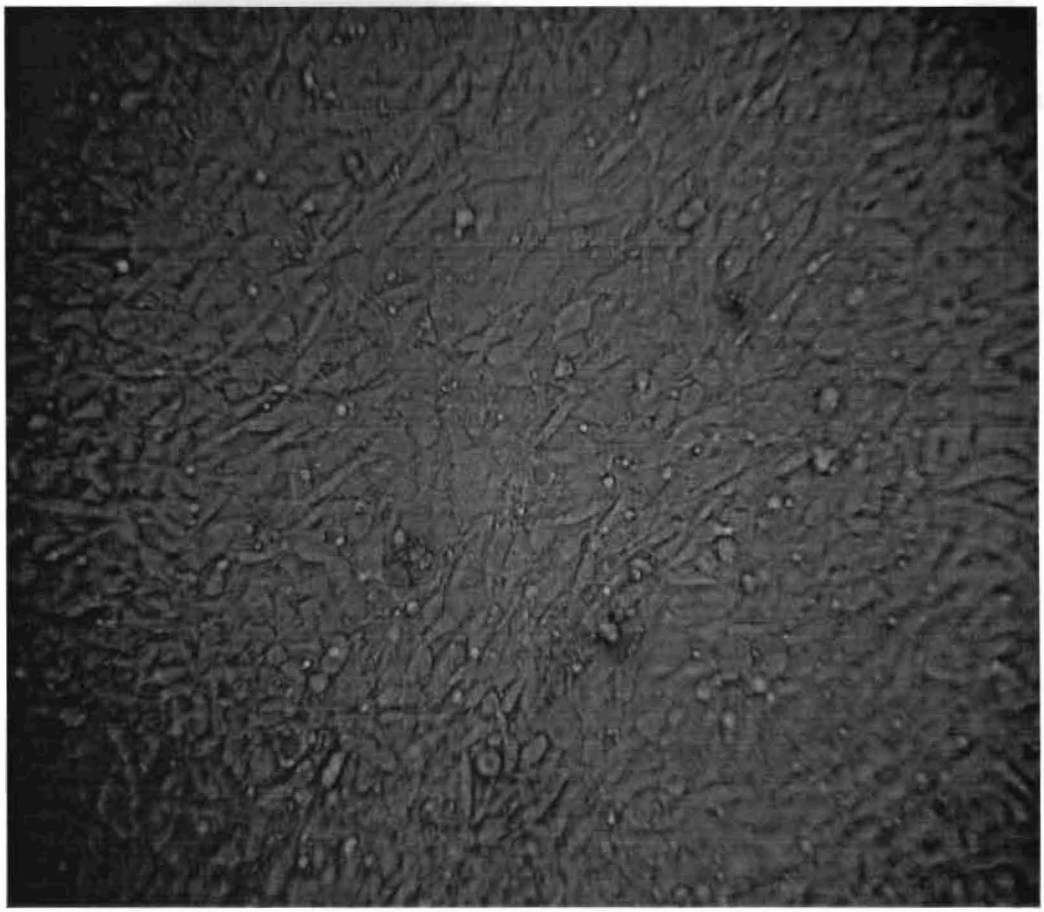


Figure 4. Growth curve for untreated HT22 cells. HT22 cells were plated and then MTT absorbance was measured daily beginning the second day of the experiment using MTT assay. The MTT A_{550} results followed an exponential growth curve. Data points are mean ± SD, N=12. $y = 0.031666 \cdot e^{(0.63941 \times)}$ (where x is in days), R = 0.99621.

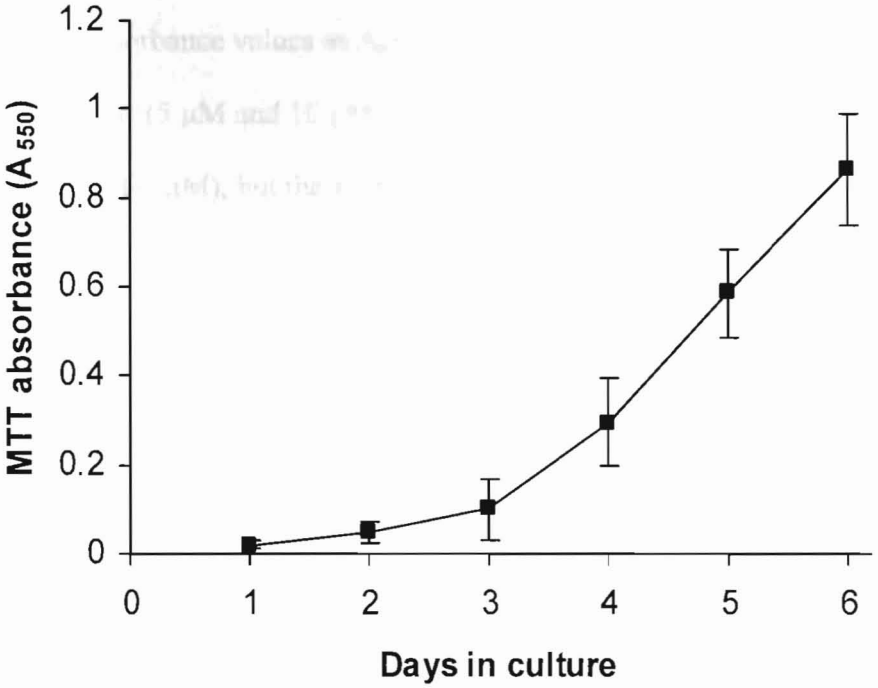

MTT absorbance (A550)
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
6
Days in culture

Figure 5. Neuronal toxicity induced by different concentrations of $A\beta_{1-42}$. There was a decrease in MTT absorbance values as $A\beta_{1-42}$ concentrations increased from 0 to 10 uM. Higher concentrations (5 μM and 10 μM) of $A\beta_{1-42}$ were more toxic than lower concentrations (0 μM-2 μM), but the higher concentrations were not significantly different from each other ($P < 0.0001$, ANOVA-LSMA, N=16). Data points are mean ± SD.

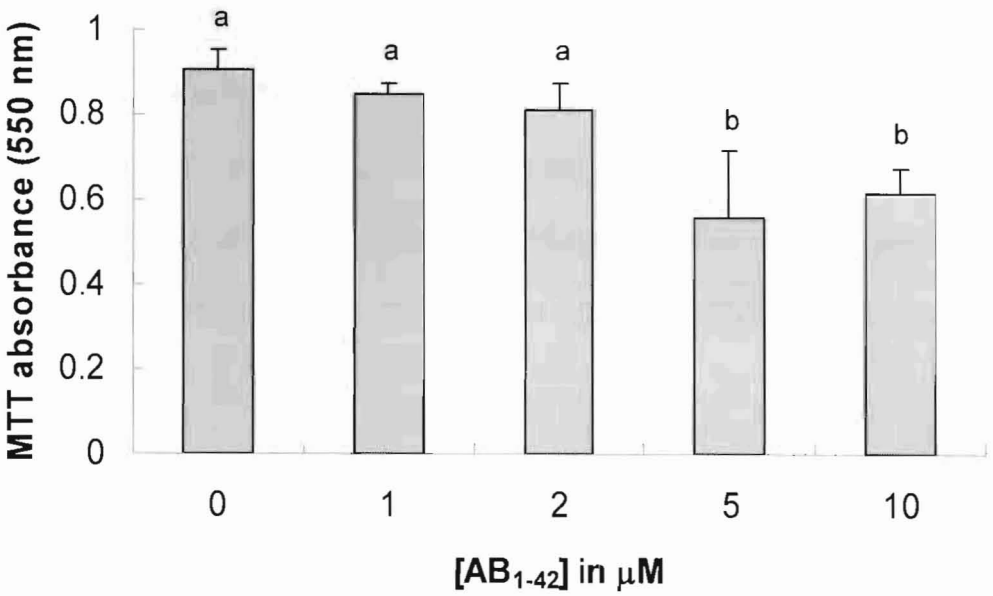

MTT absorbance (550 nm)
1
0.8
0.6
0.4
0.2
0
a
a
a
b
b
0
1
2
5
10
[AB1-42] in μM

Figure 6. Comparison of $A\beta_{1-42}$ toxicity to DMSO, $A\beta_{40-1}$ and untreated controls. Cultured HT22 hybridoma cells were treated for 6 days with $A\beta_{1-42}$ (3.3 μM), $A\beta_{40-1}$ (reversed sequence control), and DMSO or untreated (UTD controls). $A\beta_{1-42}$ reduced MTT absorbance values to 60% of untreated control but the two controls did not have a significant effect on HT22 cells. $A\beta_{1-42}$ was significantly different from the three controls ($A\beta_{40-1}$, DMSO and UTD) ($P < 0.0001$, ANOVA-LSMA, N=16) but the three controls were not significantly different from each other ($P < 0.0001$, ANOVA-LSMA). Data points are mean ± SD.

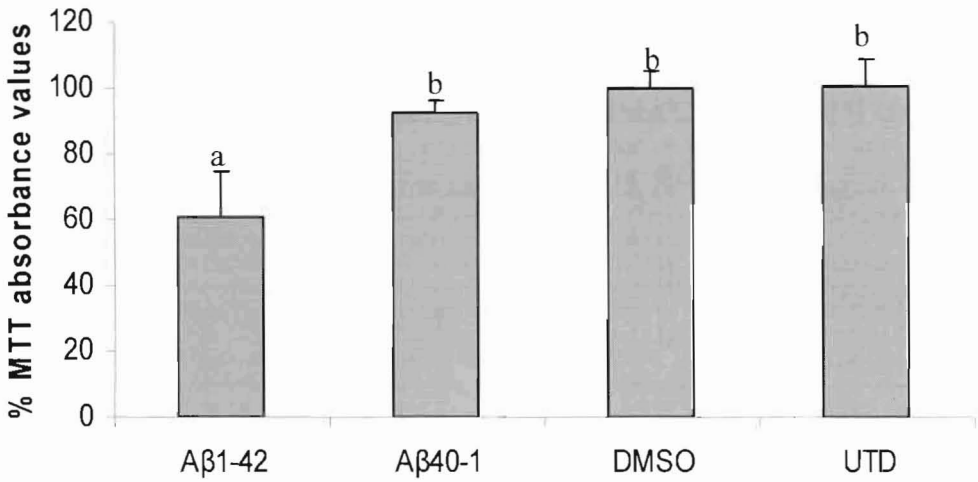
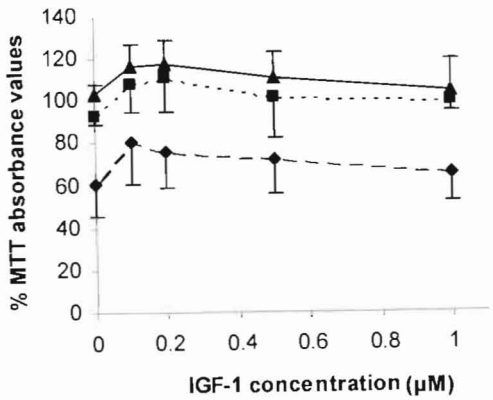
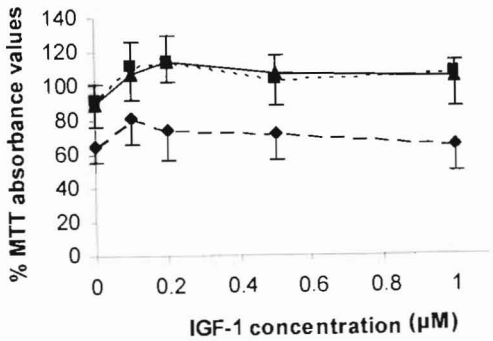

% MTT absorbance values
120
100
80
60
40
20
0
a
b
b
b
Aβ1-42
Aβ40-1
DMSO
UTD

Figure 7. Examination of neuroprotective effects of IGF-I against $A\beta_{1-42}$ induced toxicity in HT22 cells. **A** Cells exposed to 0 μM estradiol, **B** Cells exposed to 1 μM estradiol, **C** Cells exposed to 2 μM estradiol, **D** Cells exposed to 5 μM estradiol and **E** Cells exposed to 10 μM estradiol. $A\beta_{1-42}$ (— —♦— —) alone reduced cell survival to 60% of maximal cell survival in UTD controls compared to both $A\beta_{40-1}$ (reversed sequence) (---■---) and DMSO (——▲——) controls (A-E) ($P < 0.0001$, ANOVA-LSMA, N=16). IGF-I clearly protected HT22 cells from $A\beta_{1-42}$. Data points are mean ± SD, N=16.

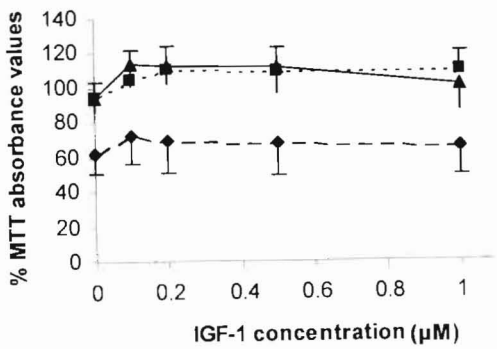
A


% MTT absorbance values
140
120
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
IGF-1 concentration (μM)

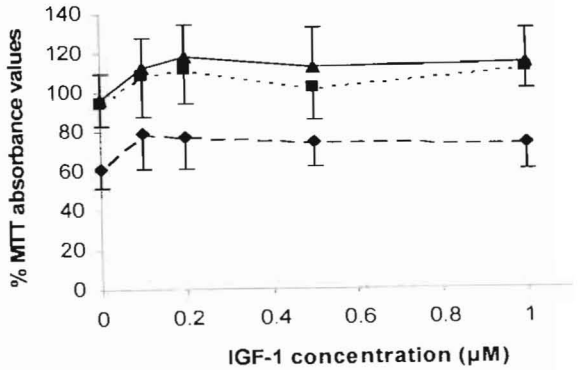
B


% MTT absorbance values
140
120
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
IGF-1 concentration (μM)

C


% MTT absorbance values
140
120
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
IGF-1 concentration (μM)

D


% MTT absorbance values
140
120
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
IGF-1 concentration (μM)

E

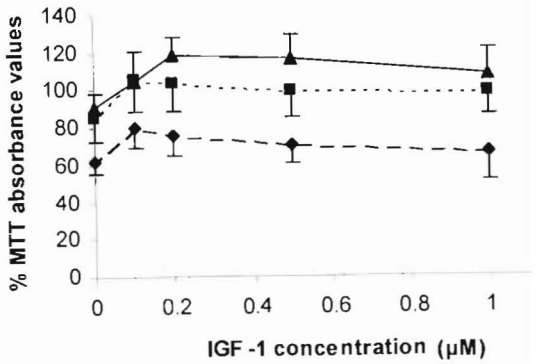
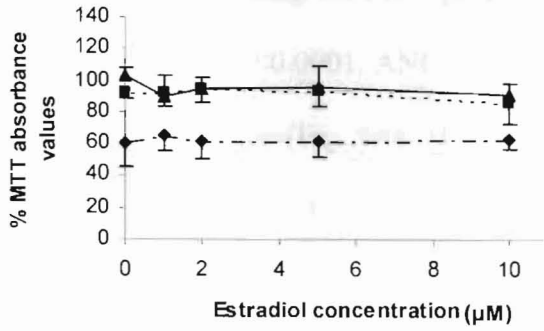
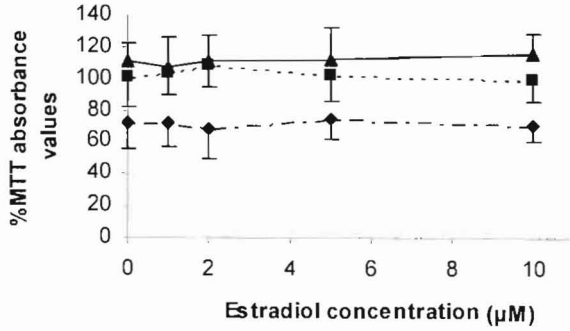

% MTT absorbance values
140
120
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
IGF -1 concentration (μM)

Figure 8. Examination of neuroprotective effects of estradiol against $A\beta_{1-42}$ induced toxicity in HT22 cells. The data in Figure 7 are replotted to illustrate estradiol effects. **A** Cells exposed to 0 μM IGF-I, **B** Cells exposed to 1 μM IGF-I, **C** Cells exposed to 2 μM IGF-I, **D** Cells exposed to 5 μM IGF-I and **E** Cells exposed to 10 μM IGF-I. $A\beta_{1-42}$ (— —♦— —) alone reduced cell survival to 60% of maximal cell survival in UTD controls compared to both $A\beta_{40-1}$ (reversed sequence) (------■-----) and DMSO (—▲—) controls (A-E) ($P < 0.0001$, ANOVA-LSMA, N=16). The results demonstrate the lack of protective effect of estradiol doses for $A\beta_{1-42}$, $A\beta_{40-1}$ or DMSO treated cells regardless of IGF-I application rate. Data points are mean ± SD, N=16.

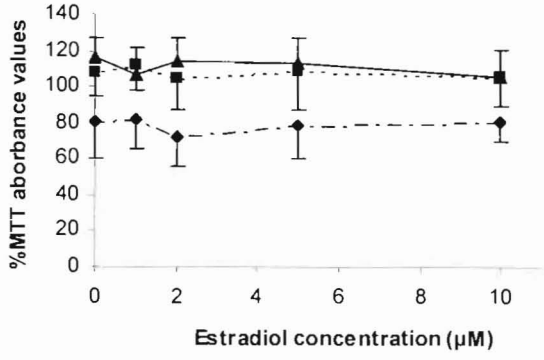
A


% MTT absorbance values
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Estradiol concentration (µM)

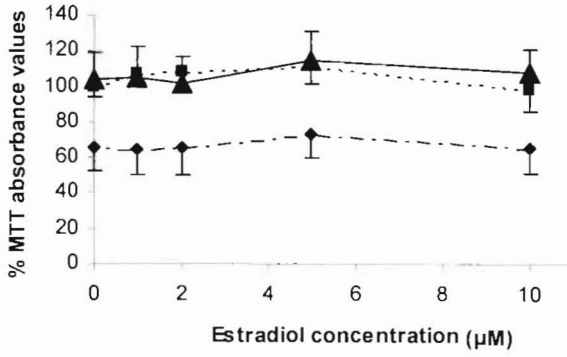
D


%MTT absorbance values
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Estradiol concentration (µM)

B


%MTT aborbance values
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Estradiol concentration (µM)

E


% MTT absorbance values
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Estradiol concentration (µM)

C

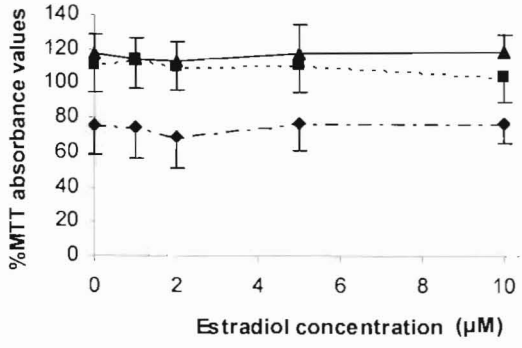
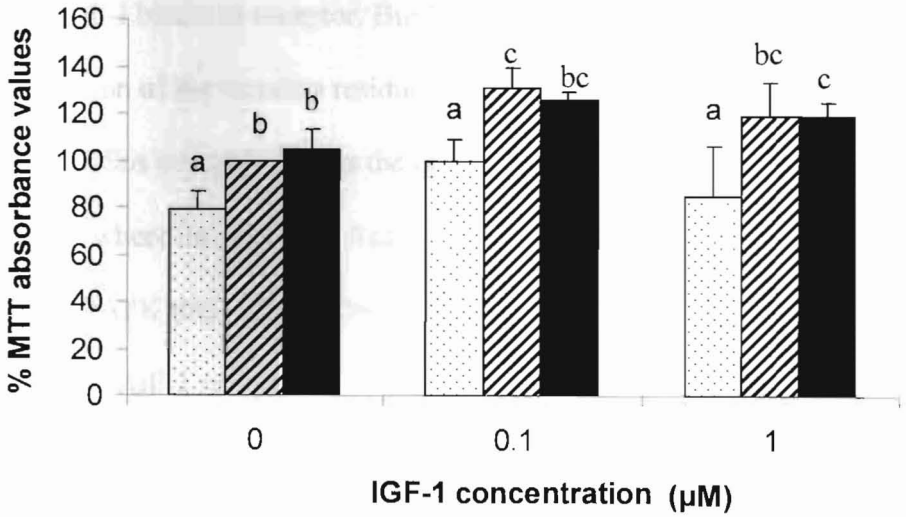

%MTT absorbance values
140
120
100
80
60
40
20
0
0
2
4
6
8
10
Estradiol concentration (µM)

Figure 9. Effects of IGF-I on $A\beta_{1-42}$ toxicity using both MTT assay and hemocytometer cell counts. A. MTT assay results. $A\beta_{1-42}$ (dotted bars) significantly reduced MTT absorbance values ($P < 0.0001$, ANOVA-LSMA, N=16) compared to $A\beta_{40-1}$(black bars) or DMSO (striped bars) (Fig. 9A). MTT absorbance values also showed that IGF-I contributed to a significantly higher HT22 cell survival ($P < 0.0001$, ANOVA-LSMA, N=16) at all combinations of peptides ($A\beta_{1-42}$, $A\beta_{40-1}$) and DMSO (Fig. 9A).

B. Hemocytometer cell count results. $A\beta_{1-42}$ significantly reduced HT22 cell numbers ($P < 0.0003$, ANOVA-LSMA, N=16) compared to $A\beta_{40-1}$ or DMSO (Fig. 9B). Cell count results also showed that 0.1 μM IGF-I contributed to a significantly higher HT22 cell survival ($P < 0.0467$, ANOVA-LSMA, N=16) at all combinations of peptide ($A\beta_{1-42}$, $A\beta_{40-1}$, and DMSO).

A


% MTT absorbance values
160
140
120
100
80
60
40
20
0
a
b
b
a
c
bc
a
bc
c
0
0.1
1
IGF-1 concentration (μM)

B

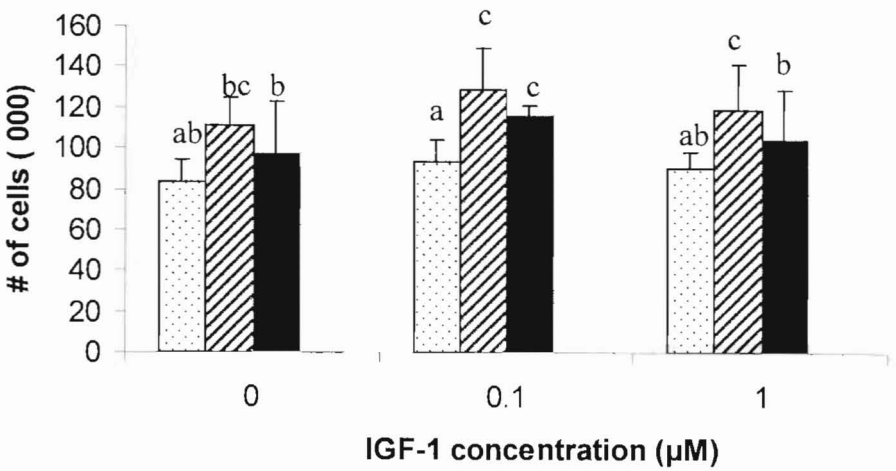
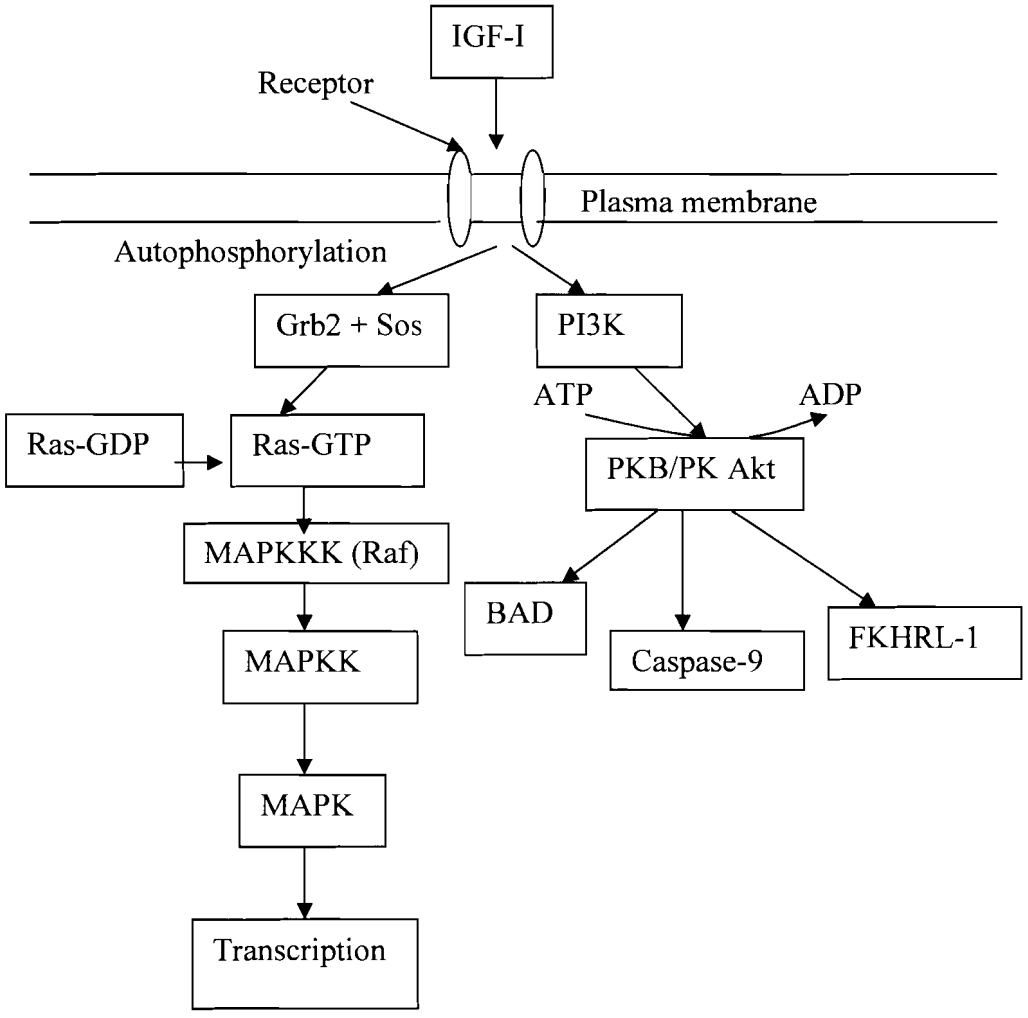

# of cells (000)
160
140
120
100
80
60
40
20
0
ab
bc
b
a
c
c
ab
c
b
0
0.1
1
IGF-1 concentration (μM)

Figure 10. Steps in the activation of mitogen-activated protein (MAP) and protein kinase B (PKB) after IGF-I binds its receptor. Binding of IGF-I to its dimer receptor leads to autophosphorylation of the tyrosine residue of the receptor and recruitment of the Grb-Sos proteins. Grb-Sos complex causes the GTP-GDP exchange of Ras which recruits Raf to the membrane where its activated. Raf phosphorylates MAPKK which in turn phosphorylates MAPK that activates transcription factors. Autophosphorylation of tyrosine residue of IGF-1 receptor also leads to activation of PI3K. PI3K in turn phorphorylates PKB that phosphorylates and inhibits several pro-apoptotic proteins such as BAD, caspase and FKHRL-1.


IGF-I
Receptor
Plasma membrane
Autophosphorylation
Grb2 + Sos
PI3K
ATP
ADP
Ras-GDP
Ras-GTP
PKB/PK Akt
MAPKKK (Raf)
BAD
Caspase-9
FKHRL-1
MAPKK
MAPK
Transcription

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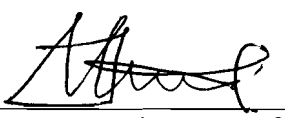

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