Regulation of Tau Gene Expression by Aβ and the Amyloid Precursor Protein in Cultured Cortical Neurons

by

Benson P. Yang

Submitted to the Department of Electrical Engineering and Computer Science

in Partial Fulfillment of the Requirements for the Degrees of

Bachelor of Science in Electrical Engineering and Computer Science

and Master of Engineering in Electrical Engineering and Computer Science

at the Massachusetts Institute of Technology

May 23, 1997

Author.....
Department of Electrical Engineering and Computer Science

Certified by.....
Richard J. Wurtman, Thesis Supervisor

Accepted by.....
Arthur C. Smith, Chairman, Department Committee on Graduate Theses

Regulation of Tau Gene Expression by
Aβ and the Amyloid Precursor Protein in Cultured Cortical Neurons

by
Benson P. Yang

Submitted to the
Department of Electrical Engineering and Computer Science

May 23, 1997

in Partial Fulfillment of the Requirements for the Degrees of
Bachelor of Science in Electrical Engineering and Computer Science
and Master of Engineering in Electrical Engineering and Computer Science

ABSTRACT

The two characteristic lesions of Alzheimer's disease are intracellular neurofibrillary tangles and extracellular accumulation of amyloid plaques. Tangles are composed of abnormally hyper-phosphorylated forms of the microtubule-associated protein tau, whereas amyloid (Aβ) is derived from proteolytic processing of the amyloid precursor protein (APP). These lesions are thought to be etiologically distinct, and are spatially dissociated in postmortem brains. We show that Aβ, wild-type APP751, or familial APP751 mutations can stimulate aberrant increases in tau protein and mRNA in primary cultured cortical neurons. Neurons infected with recombinant herpes simplex virus (HSV) vectors expressing wild-type or mutated APP751 or APP695 cDNA significantly increased APP expression. Both HSV-APP751 and HSV-APP695 increased levels of 6 kb tau mRNA; however, tau protein was increased by HSV-APP751, but not HSV-APP695, suggesting that the Kunitz protease inhibitor (KPI) domain in APP751 may increase the post-translational stability of tau. There were no differences in total tau protein levels between infections with wild-type and mutated APP751. However, infections with mutated APP751 resulted in hyper-phosphorylation of tau in comparison to infection with wild-type APP751. HSV expressing $A\beta_{1-42}$ cDNA increased both APP and tau expression (mRNA and protein). In addition to 6 kb tau mRNA, HSV-$A\beta_{1-42}$ also increased a 2 kb tau transcript which codes for a nuclear tau isoform that appears to be up-regulated in Alzheimer's disease. Hence, this study indicates that APP751 and $A\beta_{1-42}$ can stimulate abnormal increases in tau expression and hyper-phosphorylation, and suggests a potential link between amyloid plaques and neurofibrillary tangles.

Thesis Supervisor: Richard J. Wurtman, M.D.
Title: Professor of Neuroscience and Cecil H. Green Distinguished Professor

Table of Contents

Abstract ii

Table of Contents iii

List of Figures iv

Acknowledgments vi

Chapter 1 - Introduction 1

Chapter 2 - Background 5
2.1 APP 5
2.2 Tau 7

Chapter 3 - Materials and Methods 9
3.1 Cell Cultures 9
3.2 Viral Vectors 10
3.3 Western Blots 11
3.4 Immunoprecipitation of Aβ Peptides 13
3.5 Northern Blots 15

Chapter 4 - Experimental Results 19
4.1 HSV-LacZ Infection 19
4.2 HSV-APP695 and HSV-APP751 Infections: Protein Expression 19
4.3 HSV-APP695 and HSV-APP751 Infections: mRNA Expression 29
4.4 HSV-$A\beta_{1-42}$ Infection: Protein and mRNA Expression 32

Chapter 5 - Discussion 38

References 44

List of Figures

Figure 1. The APP695/751 molecule with corresponding sites of mutations. 3

Figure 2. Primary cortical neurons are stained blue with X-gal after 24-hour infection with HSV-LacZ. 20

Figure 3. Uninfected primary cortical neurons stained with X-gal show no endogenous expression of β-galactosidase. 20

Figure 4. Effect of HSV-APP695/751 (wild-type or mutations) infection on APP protein expression. 22

Figure 5. Mean APP protein expression after HSV-APP751 or HSV-APP695 infection. 23

Figure 6. Effect of HSV-APP695/751 (wild-type or mutations) infection on tau protein expression. 24

Figure 7. Mean tau protein expression after HSV-APP751 or HSV-APP695 infection. 26

Figure 8. Effect of HSV-APP695/751 (wild-type or mutations) infection on tau-1 protein expresion. 27

Figure 9. Mean tau-1 protein expression after infection with HSV-APP751 mutations or with HSV-APP695 mutations. 28

Figure 10. Representative Northern blot showing the effect of HSV-APP695/751 (wild-type or "London" mutations) or HSV-$A\beta_{1-42}$ infection on tau mRNA expression. 30

Figure 11. Effect of HSV-APP695/751 (wild-type or "London" mutations) infection on levels of 6 kb tau mRNA. 31

Figure 12. Representative Northern blot showing the effect of HSV-$A\beta_{1-42}$ infection on $A\beta_{1-42}$ and APP mRNA expression. 33

Figure 13. Representative Western blot showing the effect of HSV-C100 infection on APP and tau protein expression. 34

Figure 14. Effect of HSV-APP751 595K→N/596M→L and HSV-$A\beta_{1-42}$ infection on Aβ secretion. 34

Figure 15. Effect of HSV-$A\beta_{1-42}$ infection on APP and tau protein expression. 36

Figure 16. Regulation of tau by Aβ and APP. 39

Acknowledgments

I would like to thank Dr. Richard Wurtman and Dr. Robert Lee for guiding me through my thesis, as well as helping me understand larger issues in science. Dr. Wurtman's example and stimulating discussions have been instrumental in shaping my approach to problems in neuroscience. Dr. Lee's incredible patience and consideration in mentoring me is the source of my enthusiasm for research. I am indebted to Dr. Lee for his critical comments through several revisions of this thesis. Special thanks to Dr. Rachael Neve who constructed and provided the viruses that made this study possible. I am also very grateful to Dr. Henry Querfurth, Dr. Stefan Knapp, Ingrid Richardson, Jeff Breu, and Jian-Ping Shi for their expert assistance with various technical procedures. And finally, I appreciate everyone who has supported me through my research and my years at M.I.T.

CHAPTER 1

Introduction

Alzheimer's disease (AD) is a neurodegenerative disease that results in the progressive loss of cognitive function. The two characteristic brain lesions in AD are neuritic plaques and neurofibrillary tangles. Neuritic plaques are primarily comprised of extracellular aggregates of amyloidogenic fragments, principally Aβ, derived from proteolytic processing of the amyloid precursor protein (APP; Glenner and Wong, 1984). Neurofibrillary tangles are intra-neuronal accumulations of abnormally formed paired helical filaments (PHFs), which contain the microtubule-associated protein tau (Kidd, 1963).

Increased APP gene dosage in Down's syndrome (DS)/Trisomy 21 results in AD by early adulthood (Neve *et al.*, 1988), suggesting that over-expression of the APP gene on chromosome 21 may lead to AD pathology. Indeed, over-expression of APP in neurons promotes neurodegeneration and the production of toxic amyloidogenic derivatives (Yoshikawa *et al.*, 1992). Mutations of the APP gene are responsible for familial forms of the disease (FAD), though the precise mechanisms involved are unclear. These findings strongly suggest that over-expression of or alterations in the APP gene renders the APP molecule more vulnerable to aberrant processing and leads to the formation of amyloid deposits and development of AD.

The severity and duration of AD correlate better with the formation of neurofibrillary tangles than with the accumulation of amyloid plaques (Arriagada *et al.*, 1992). Levels of tau

protein are dramatically elevated in the brain (Khatoon *et al.*, 1992) as well as in cerebrospinal fluid of AD patients (Arai *et al.*, 1995). Normally, tau is required for microtubule assembly; the main component of PHFs is hyper-phosphorylated tau, which binds less readily to microtubules and serves as nucleation centers for tangle formation by disrupting cytoskeletal integrity (Alonso *et al.*, 1996).

Although APP and tau have both been implicated in the pathology of AD, their relationship to each other is yet unclear. Generally, the formation of plaques and the accumulation of tangles have been thought to be etiologically distinct. Recent studies suggest that the development of these two lesions may not be entirely independent of each other. Neuritic plaques have been observed to precede neurofibrillary tangles in DS brains (Mann and Esiri, 1989; Murphy *et al.*, 1990). Tau mRNA as well as APP mRNA are up-regulated in normal aged brains and in DS brains, despite the fact that the two genes are located on different chromosomes (Oyama *et al.*, 1992; Oyama *et al.*, 1994). Additionally, extracellular Aβ fibril formation has been found to cause abnormal tau formation and loss of microtubule binding in AD (Busciglio *et al.*, 1995). Elucidation of the relationship between amyloid plaques and neurofibrillary tangles will represent a significant breakthrough in AD research.

This study investigates the influence of APP and $A\beta_{1-42}$ expression on the expression and phosphorylation state of tau protein and on the transcription of tau mRNA. Neurons were infected with herpes simplex virus over-expressing APP751 (HSV-APP751) or APP695 (HSV-APP695) cDNA, which differ only in the presence or absence of a Kunitz protease inhibitor (KPI) coding domain (figure 1). Our data show that increased APP751 expression and transcription results in increased tau expression and transcription. These increases can

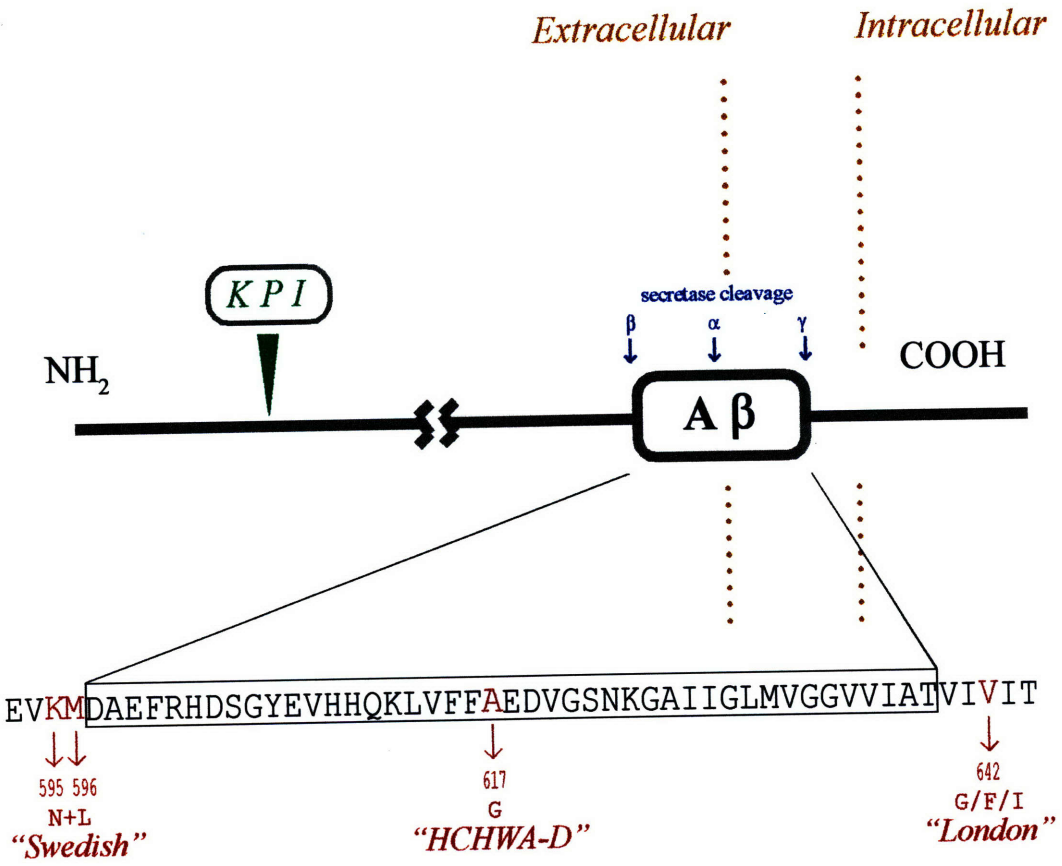


Figure 1. The APP695/751 molecule with corresponding sites of mutations (shown in red with their common names; numbered according to APP695). The N-terminus of APP is located extracellularly whereas Aβ partially resides in the membrane and is contiguous with the intracellular C-terminus. The only structural difference between APP751 and APP695 is the presence of a 56 amino acid KPI insert (shown in green) in APP751. Proteolytic cleavage of APP within the Aβ segment by α-secretase prevents Aβ formation whereas cleavage by β- and γ-secretases releases an intact Aβ peptide that is amyloidogenic.

be attributed to the KPI domain's neurite outgrowth promoting activity (Akaaboune *et al.*, 1994; Qiu *et al.*, 1995) or its stabilizing effects on tau protein (Oleson, 1994; Wang *et al.*, 1996). Five known FAD mutations were tested for their effects on APP and tau expression. In general, mutations in APP751 but not APP695, increased tau hyper-phosphorylation. Two APP proteolytic fragments, $A\beta_{1-42}$ and C100, were also tested for their effects on APP and tau expression. HSV-$A\beta_{1-42}$ increased APP and tau (protein and mRNA), suggesting that intracellular $A\beta_{1-42}$ regulates the transcription of APP and tau. Hence, our data suggest that APP751 and its associated mutations stimulate aberrant increases in tau synthesis and hyper-phosphorylation. Furthermore, abnormal increases in APP751 and tau synthesis caused by intracellular accumulation of $A\beta_{1-42}$ may also accelerate the progression of AD.

CHAPTER 2

Background

2.1 APP

The APP gene is located on the long arm of chromosome 21 and spans approximately 400 kb (Kang *et al.*, 1987; Lamb *et al.*, 1993). Alternative splicing of the APP gene generates APP mRNAs encoding several isoforms ranging from 365 to 770 amino acids in length (Kosik, 1993). APP is an integral membrane glycoprotein consisting of an extracellular domain, a single transmembrane domain, and a short cytoplasmic tail. A 39- to 43-residue Aβ segment bridges the outer membrane layer and is the precursor of amyloid plaques in AD pathology. Two major APP isoforms that encode Aβ measure 695 and 751 amino acid residues (referred to as APP695 and APP751). APP751 also contains a 56-amino acid domain with approximately 50% homology to the Kunitz-type serine protease inhibitors (KPI; Kitaguchi *et al.*, 1988; Ponte *et al.*, 1988) that is absent in APP695. APP695 is expressed at particularly high levels in neurons, whereas APP751 is expressed at higher levels in astrocytes and microglia. However, APP751 is reportedly up-regulated in neurons of AD brains and may serve as a molecular marker for neuritic plaque density (Johnson *et al.*, 1990).

APP can undergo several processing pathways to produce Aβ which can lead to the formation of plaques within the neuropil and in the walls of cerebral blood vessels. In the constitutive secretory pathway, APP is cleaved by α-secretase within the Aβ region to produce non-amyloidogenic fragments (Esch *et al.*, 1990; Sisodia *et al.*, 1990). However,

secretory cleavage may also occur in the regions flanking Aβ by β- and γ-secretases (figure 1), leading to the generation of an intact Aβ peptide (Seubert *et al.*, 1993). In FAD, aberrant cleavage of APP leads to elevated secretion (Citron *et al.*, 1992; Cai *et al.*, 1993; Suzuki *et al.*, 1994; Haass *et al.*, 1994) and intracellular accumulation (De Strooper *et al.*, 1995) of Aβ and other amyloidogenic fragments. Alternatively, APP can be internalized from the cell surface and transported to the endosomal/lysosomal compartment, where potentially amyloidogenic and neurotoxic fragments are generated (Golde *et al.*, 1992; Turner *et al.*, 1996).

Although almost all mammalian cells express APP holoprotein, the functions of APP and Aβ remain uncertain. APP has been implicated as a growth promoting or autocrine molecule (Saitoh *et al.*, 1989), as a mediator of cell-cell or cell-substrate adhesion and neurite outgrowth (Schubert *et al.*, 1989; Milward *et al.*, 1992), and as a neuroprotective down-regulator of intra-neuronal calcium levels (Mattson *et al.*, 1993). The secretory form of the KPI-containing APP751 (protease nexin II), in addition to being a serine protease inhibitor, is thought to be neurotrophic (Akaaboune *et al.*, 1994). Indeed, cell-surface KPI-containing isoforms of APP are more effective in stimulating neurite outgrowth than non-KPI-containing isoforms (Qiu *et al.*, 1995). Aβ is produced and secreted by all cells under normal metabolic conditions (Haass *et al.*, 1992; Seubert *et al.*, 1992). There has been some discordant evidence that Aβ is neurotoxic (Yankner *et al.*, 1990; Pike *et al.*, 1991); however, the Aβ peptide has also been shown to exhibit neurotrophic activity (Yankner *et al.*, 1990; Koo *et al.*, 1993). Additionally, extracellular Aβ may stimulate second messenger systems to

regulate the transcription of several proteins, including APP (Le *et al.*, 1995; Pena *et al.*, 1995). Intracellular Aβ may more directly regulate transcription (Kaltschmidt *et al.*, 1997).

Tau

Tau protein is a major component of neurons and of PHFs (Wischik *et al.*, 1988). The human tau gene, located on chromosome 17, gives rise to all known isoforms of tau, ranging from 352 to 441 amino acids in length (Neve *et al.*, 1986). Alternative splicing of tau mRNA in different neuronal types results in the expression of six different isoforms (Goedert *et al.*, 1989). Two tau transcripts measuring 2 kb and 6 kb have been observed; the former seems to be up-regulated in AD (Goedert *et al.*, 1988; Kosik *et al.*, 1989). Four 18 amino acid repeats, located near the C-terminus of tau, constitute the microtubule-binding domain (Ennulat *et al.*, 1989). The linker region between the first and second repeats is also important in microtuble assembly (Goode and Feinstein, 1994).

Tau is normally an abundant protein in both the central and peripheral nervous systems. Tau was first found to promote polymerization of tubulin (Weingarten *et al.*, 1975). Tubulin dimers form microtubules which are essential for cell structure, vesicle and organelle movement, and cell division. Tau is required for microtubule assembly and increased tau levels correlate with increased assembly, polymerization, and stabilization of microtubules (Drubin *et al.*, 1985). Another function of tau is to form short crossbridges between axonal microtubules, which enable axonal elongation and maintenance (Hirokawa *et al.*, 1988). The flexible binding domain of tau can accommodate the irregular geometries in intermediate stages of microtubule polymerization (Butner and Kirschner, 1991). During neurite

extension, tau synthesis is up-regulated prior to that of tubulin (Drubin *et al.*, 1985); hence, tau may act as an important regulator for neurite elongation (Hanemaaijer and Ginzburg, 1991).

The major pathology of AD lies in the hyper-phosphorylation of tau (Cleveland *et al.*, 1977; Grundke-Iqbal *et al.*, 1986; Ihara *et al.*, 1986). Abnormally hyper-phosphorylated tau disrupts the binding of tau to microtubules and leads to the breakdown of the microtubule system in AD (Iqbal *et al.*, 1986; Bramblett *et al.*, 1993). One common site of aberrant phosphorylation is Ser 202, the epitope for the monoclonal antibody Tau-1 (Kosik *et al.*, 1988). Phosphorylation of Ser 262, located within the second repeat, has a particularly strong influence on binding; this site can be phosphorylated by kinases present in brain tissue and is unique in AD brain (Mandelkow *et al.*, 1995). Several kinases can hyper-phosphorylate tau (Drewes *et al.*, 1992; Goedert *et al.*, 1992; Mandelkow *et al.*, 1992) while protein phosphatases can dephosphorylate tau *in vitro* (Yamamoto *et al.*, 1988; Drewes *et al.*, 1993; Harris *et al.*, 1993).

CHAPTER 3

Materials and Methods

3.1 Cell Cultures

Cortical neuronal cultures were prepared from embryonic day-17 to -18 fetal rat pups, using previously described procedures (Goslin and Banker, 1991). Briefly, pregnant rats were overdosed with ketamine before pups were removed for brain dissection. Cortices were dissected free of meninges, and placed in ice-cold Earle's Balanced Salt Solution (Sigma). The cortices were dissociated mechanically by light trituration with Pasteur pipettes and chemically with 0.25% trypsin in serum-free Neurobasal Medium (Gibco). After centrifugation, supernatant fluids were aspirated to prevent further digestion of the cell pellet. Using a flame-narrowed Pasteur pipette, the pellet was triturated and resuspended in fresh Minimum Essential Medium (MEM; Gibco) containing 10% heat-inactivated fetal calf serum. The cells were plated at a density of 10^4 cells/cm^2 on 35-mm, 60-mm, or 10-cm dishes that were precoated with poly-L-lysine (1 mg/ml in 0.1 M borate buffer) to facilitate cell adhesion. After 24 hours, when most of the cells were well attached, the medium was replaced by MEM containing 5 mM glutamine and B27 components (Gibco). Fetal bovine serum (5%), which contains a high concentration of growth factors, was added to promote neuronal growth and cytosine arabinoside (5 μM) was added to prevent replication of non-neuronal cells. The cultures were maintained for 1 to 2 weeks in a humidified incubator (5% CO_2/95% air) at 37°C; half the medium was replaced every 3 days. To facilitate infection of neurons, fresh

culture medium was added prior to adding HSV vectors. Immunocytochemical methods with cell-specific antibodies (neural cell typing set; Boehringer Mannheim) confirmed neuronal culture purity.

3.2 Viral Vectors

HSV vectors (Geller *et al.*, 1994) containing the genes of interest—HSV-LacZ, HSV-APP751 (wild-type and mutations), HSV-APP695 (wild-type and mutations), HSV-C100, and HSV-$A\beta_{1-42}$—were gifts from Dr. Rachael Neve (Harvard Medical School). Neuronal cell cultures were infected with 15 µl (1.6×10^6 infectious particles/35-mm dish of cells) of the virus.

Testing the Virus

The reliability and efficiency of the HSV vectors were assessed by infecting a set of cell cultures with HSV expressing the *lacZ* gene (HSV-LacZ). 24 hours after infection, cells were rinsed with phosphate buffered saline (PBS; pH7.5), fixed with glutaraldehyde (1% in PBS), and rinsed again with PBS. 1 mg 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) was dissolved in 20 µl dimethyl sulfoxide and added to Fe solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 0.2 mM magnesium chloride in PBS) to achieve a final concentration of 1 mg/ml X-gal. Uninfected cells and HSV-LacZ infected cells were immersed in this mixture and incubated at 37°C overnight. The *lacZ* gene codes for β-galactosidase which cleaves X-gal, a lactose analog, to form galactose and 5-bromo-4-chloro-indigo, a blue dye.

3.3 Western blots

Cell Collection and Assay

The medium in each 35-mm dish was aspirated and cells were briefly washed with ice-cold PBS. Cells were scraped after adding lysis buffer (62.5 mM tris-HCl, 4% sodium dodecyl sulfate [SDS], 20% glycerol; pH6.8) and dithiolthreitol (1 mM) to stabilize cellular contents. The lysates were sonicated to disrupt cell membranes and boiled to inhibit protease activity. Aliquots of the protein samples were assayed in a 96-well microplate using a bichinchonic acid solution/$CuSO_4$ mixture against known concentrations of bovine serum albumin (BSA). Samples were placed in a CO_2-free incubator at 37°C for 30 minutes prior to measurement in a microplate reader.

Gel Electrophoresis and Transfer

Bromophenol blue (0.1%) was added to each sample prior to loading equivalent amounts of cellular protein onto 4-20% Tris-Glycine Gels (Bio-Rad) along with standard pre-boiled samples of Rainbow Marker (Amersham) and rat brain (20 μg). Electrophoresis was conducted at 175 volts for 1 hour in running buffer (25 mM tris base, 192 mM glycine, 0.1% SDS). Proteins were transferred onto Immobilon-P membranes (Millipore) at 250 volts for 1 hour in transfer buffer (250 mM tris base, 1.92 M glycine) and fixed onto the membranes with a brief immersion in glutaraldehyde (1% in PBS). The membranes were incubated with 5% dried milk/tris-buffered saline-Tween-20 (TBS-T; Boehringer Mannheim) solution for 30 minutes to block non-specific antibody binding and washed in TBS-T. The blocked membranes were then incubated in primary antibodies overnight, rinsed with TBS-T,

incubated in secondary antibodies for 1 hour, and rinsed again with TBS-T. The secondary antibody was detected using Renaissance Western Blot Chemiluminescence Reagent (NEN) and exposed to X-Omat AR Scientific Imaging Film (Kodak) at -20°C.

Antibodies

Three different primary monoclonal antibodies (mAb) were used to quantify APP and tau on Western blots. The mAb 22C11 (Boehringer Mannheim) was produced by immunization with the fusion protein pre-$A4_{695}$ and reacts with the region between the N-terminus and the KPI domain (amino acids 1 to 289) of the human APP molecule. 22C11 is mouse IgG and was used to assess total APP in the cell pellet. The mAb 5E2 (courtesy of Dr. Ken Kosik) was raised against tau and reacts exclusively with the chemically heterogeneous tau in both phosphorylated and non-phosphorylated forms. The antibody does not react with other microtubule-associated proteins or with tubulin. 5E2 is mouse IgG and was used to assess total tau in the cell pellet. The mAb Tau-1 (Sigma) reacts with all known species of tau, but its binding is inhibited by the phosphorylation of tau at Ser 202. Reduced Tau-1 immunolabeling was used as an indicator of phosphorylated tau. The secondary antibody was horseradish peroxidase conjugated sheep Anti-Mouse IgG (Amersham).

Stripping and Reprobing

Prior to immunolabeling with subsequent antibodies, primary and secondary antibodies initially bound to membranes were removed. The membranes were incubated in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM tris-HCl; pH 6.7) at 50°C for 30

minutes and were washed with TBS-T and blocked in a 5% dried-milk/TBS-T solution for 1 hour. New primary and secondary antibodies were then applied as described in previous sections.

3.4 Immunoprecipitation of Aβ Peptides

Labeling Adherent Cells

After 24-hour infection, the medium was aspirated from 60-mm culture dishes and cells were washed with methionine-free Dulbecco's Minimum Essential Medium (D-MEM; Gibco). After adding 1.5 ml methionine-free D-MEM to starve neurons of methionine, the cell cultures were stored for 36 hours at 37°C in a humidified incubator (5% CO_2/95% air). Each culture was then metabolically labeled with 300 μCi ^{35}S-methionine and incubated for 16 hours at 37°C. Medium was collected in screw-top Eppendorf tubes and frozen at -20°C prior to immunoprecipitation of secreted proteins.

Immunoprecipitation of Secreted Proteins

The media samples were centrifuged at 14000 rpm for 10 minutes to remove cellular debris and precipitated proteins. The supernatant was removed to another screw-top Eppendorf tube and antibody was added to each sample and incubated with shaking at 4°C for 1 hour. Protein A-Sepharose CL-4B (100 mg/ml; Sigma) was added to STEN buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.2% NP-40, 20 mM PMSF, 1X protease inhibitors; pH 7.6). 25 μl of this solution was added to each sample and the samples were incubated with continuous shaking at 4°C overnight. The antigen-antibody-protein A complexes were

pelleted by centrifuging at 6000 rpm for 5 minutes. After removing the supernatant, the pellets were subjected to three separate washes for 20 minutes each at 4°C. The first wash was in 0.5 M-STEN buffer (STEN buffer, 0.5 M NaCl); the second wash was in SDS-STEN buffer (STEN buffer, 0.1% SDS); the third wash was in STEN buffer.

Gel Electrophoresis

Following the final wash, the beads were centrifuged at 6000 rpm for 5 minutes and the supernatant was removed. 15 μl 2X loading buffer (40% glycerol, 6% SDS, 6% β-mercaptoethanol, 5% bromophenol blue) was added to each sample. The samples were boiled for 5 minutes to dissociate the antigen-antibody-protein A complexes and spun at 14000 rpm for 10 minutes. The supernatant of each sample was transferred to a fresh screw-cap Eppendorf tube and boiled and centrifuged as before. Samples were loaded onto 10-20% Tris-Tricine Gels (Bio-Rad), which give good resolution for low molecular weight peptides, along with standard pre-boiled samples of Rainbow Marker. Electrophoresis was conducted in running buffer (25 mM tris base, 192 mM tricine, 0.1% SDS) at 80-100 volts until the dye front was 1 cm from the bottom of the gel.

Antibodies

Antiserum R1282 (courtesy of Dr. Dennis Selkoe; Haass *et al.*, 1992) was raised against an uncoupled synthetic Aβ protein peptide comprising residues 1 through 40 of Aβ. R1282 is rabbit IgG and was used in immunoprecipitation (1:300 dilution) to assess levels of Aβ in conditioned media of cultured cells.

Autoradiography

The SDS-PAGE gel was immersed in destaining solution (50% methanol, 18% glacial acetic acid in water) and gently agitated for 30 minutes to fix proteins to the gel. The gel was then immersed in EN^{3}HANCE (Dupont) and gently agitated for 30 minutes to enhance the autoradiographic signal of ^{35}S, a low energy β-emitter, through fluorography. Then the gel was immersed in cold water and gently agitated for 30 minutes to precipitate the fluorescent material inside the gel. Finally, the gel was immersed in Gel-Dry Drying Solution (Novel Experimental Technology) and gently agitated for 30 minutes before drying on a gel dryer. The dried gel was exposed to X-Omat AR Scientific Imaging Film against an intensifying screen for 1 week at -80°C.

3.5 Northern blots

RNA Extraction and Quantification

After aspirating the medium from 10-cm culture dishes, Tri Reagent (Molecular Research Center) was added to each plate to homogenize the cells and to isolate RNA, DNA, and proteins. The scraped cells were first drawn through a syringe (27 ½ G) and then forcibly expelled to shear the RNA/DNA/protein mixture. RNA was separated from DNA and proteins by chloroform extraction for 30 minutes and centrifuged at 12000 rpm. The RNA mixture was precipitated with isopropanol for 30 minutes and centrifuged at 12000 rpm. The pellet was washed with 70% ethanol for 15 minutes to remove residual salts and centrifuged at 7500 rpm. The final RNA pellet was solubilized in FORMAzol (Molecular Research Center) and warmed on a heating-block at 60°C for 15 minutes. The purity of each RNA

sample, as well as a rough estimation of quantity, was assessed by optical density readings at 260λ and 280λ on a diluted fraction of the samples.

Gel Electrophoresis and Transfer

Approximately 10 µg of RNA was added to 10 µl FormaZOL, 3.4 µl formaldehyde, 1 µl 20X 3-[N-Morpholino] propanesulfonic acid (MOPS) buffer (0.4 M MOPS, 0.1 M sodium acetate, 0.02 M EDTA), and enough diethylpyrocarbonate (DEPC) treated-water to achieve a final volume of 20 µl. The resulting mixture was warmed to 60°C on a heating-block and dyed with Northern dye. Samples were loaded, along with a 5 µg RNA sample designated as a marker, onto an agarose gel (1.8 g agarose, 25 ml formaldehyde, 200 ml MOPS buffer). Gel electrophoresis was conducted at 70 volts for 6 hours in MOPS buffer. The marker lane was stained in ethidium bromide (0.5 µg/ml in 0.5 M ammonium acetate) for 15 minutes and illuminated by ultraviolet light to visualize 18S and 28S rRNA measuring 2.4 kb and 6.3 kb, respectively. The sizes of other mRNA bands were estimated from their positions relative to the positions of 18S and 28S rRNA. RNA was transferred from the agarose gel onto Hybond-N nylon membranes (Amersham) via capillary transfer overnight using SSC (150 mM NaCl, 15 mM sodium citrate, 0.1% SDS) as the carrier. Dried membranes were exposed to ultraviolet light for 2 minutes to fix RNA onto the membranes.

Hybridization

Membranes were prehybridized for 1 hour at 70°C with Rapid-hyb buffer (Amersham). The hybridization probes were prepared using the Megaprime labeling kit

(Amersham). Briefly, primer solution was added to DEPC-water and the DNA template (10 ng/membrane). After denaturation by boiling for 5 minutes, labeling buffer, Klenow enzyme, and ^{32}P-dCTP were added to the mixture to enable the radiolabeled polymerase reaction. The resulting mixture was incubated at 37°C for 10 minutes and the reaction was stopped with 50 mM EDTA. The labeled DNA was boiled for 3 minutes and chilled on ice. To purify the probe of unbound nucleotides, the DNA sample was run through an Elutip-d Minicolumn (Schleicher & Schuell) prior to application to prehybridized membranes. Briefly, the DNA sample was washed with low salt buffer (0.2 M NaCl, 20 mM tris-HCl, 1.0 mM EDTA) before elution with high salt buffer (1.0 M NaCl, 20 mM tris-HCl, 1.0 mM EDTA). After verifying the specific activity of the radiolabeled DNA in a scintillation counter, the probe was reacted to the membranes overnight in a rotating incubator at 70°C. To remove unbound probes, the membranes were subjected to four washes lasting 15 minutes each: twice in 2X SSC/0.1% SDS at room temperature and twice in 0.1X SSC/0.1% SDS at 50°C in a rotating incubator. The washed membranes were allowed to dry at room temperature prior to exposure to X-Omat AR Scientific Imaging Film against an intensifying screen at -80°C.

DNA Probes

The tau probe (courtesy of Dr. Rachael Neve) is a rat tau cDNA which hybridizes with all transcripts of the tau gene (Kosik *et al.*, 1989). The APP probe (courtesy of Dr. Rachael Neve) is a full-length APP695 cDNA which hybridizes with APP695 or APP751 mRNA. Human G3PDH cDNA Control Probe (Clontech) was used to verify equal loading of each sample on Northern blots. The probe hybridizes to the ubiquitously expressed

housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (G3PDH), whose transcript levels remain relatively refractory to many cell treatments.

Stripping and Rehybridizing

Prior to hybridization with subsequent probes, ^{32}P-dCTP labeled DNA probes initially bound to membranes were removed with two 15 minute rinses in 95°C water. The membranes were allowed to dry at room temperature for 1 hour. New DNA probes were then hybridized to the membrane as described in previous sections.

CHAPTER 4

Results

4.1 HSV-LacZ Infection

HSV-LacZ was used as an assay to measure the effectiveness and efficiency of the HSV amplicon system. The *E. Coli lacZ* reporter gene codes for β-galactosidase, an enzyme which catalyzes the hydrolysis of X-gal to a blue dye. Primary cortical neurons infected with HSV-LacZ were stained with the β-galactosidase enzyme substrate X-gal 24 hours after infection to assess the efficacy of HSV infection with the *lacZ* reporter gene (figure 2). Virtually 100 % of neurons in culture were β-galactosidase-positive after HSV-LacZ infection (1×10^6 infectious particles/35-mm dish of cells) at three different cell densities (0.5×10^4, 1.0×10^4, 1.5×10^4 cells/cm^2). Infected neurons appeared to be morphologically healthy with no evident signs of viral toxicity. In contrast, all uninfected neurons were β-galactosidase-negative when stained with X-gal (figure 3). To ensure that the effects observed after viral infections were caused by the genes of interest and not by the HSV vector system, HSV-LacZ infections were included in all experiments as controls.

4.2 HSV-APP695 and HSV-APP751 Infections: Protein Expression

In all Western blots, neurons infected and not infected with HSV-LacZ did not differ with respect to APP and tau levels; therefore, these data were pooled together and designated as controls.

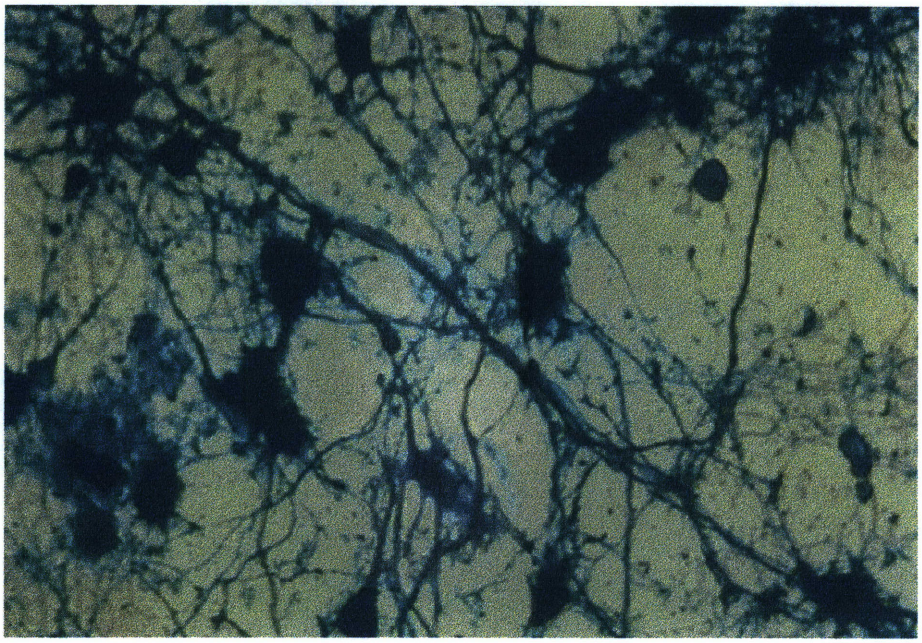


FIGURE 2. Primary cortical neurons are stained blue with X-gal after 24-hour infection with HSV-LacZ. Expression of β-galactosidase by the *lacZ* gene is evident in all cell structures. Furthermore, there is no evidence of viral toxicity.

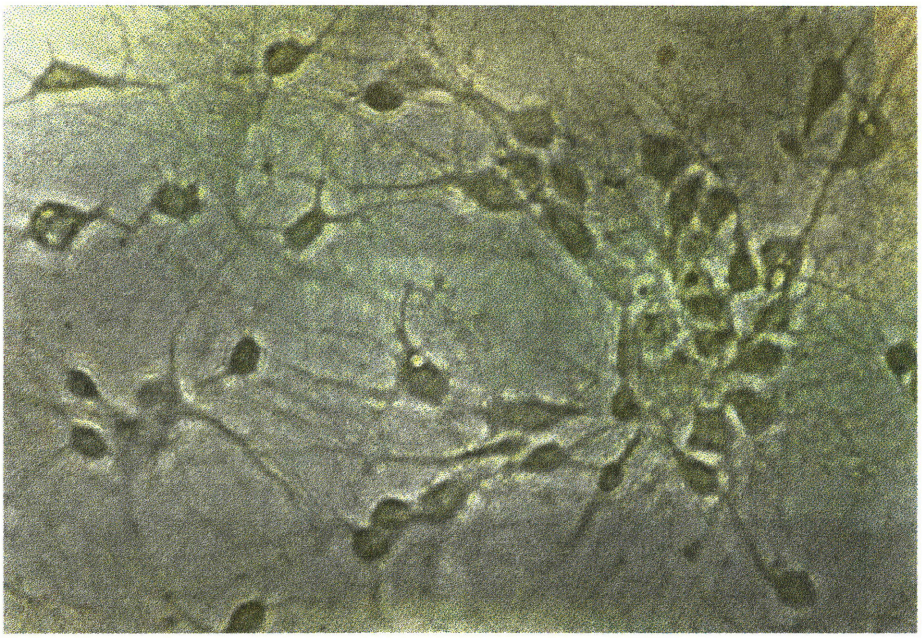


FIGURE 3. Uninfected primary cortical neurons stained with X-gal show no endogenous expression of β-galactosidase.

APP Expression

APP was detected as an approximately 100 kDa band by the mAb 22C11. Infection with HSV-APP695 and HSV-APP751 both increased the expression of APP holoprotein (Figure 4). Infection with HSV-APP695 wild-type resulted in a 9.9 fold increase in APP expression over control levels and infection with HSV-APP695 mutations resulted in APP increases that ranged from 9.3 fold to 13.4 fold over control levels. Infection with HSV-APP751 wild-type resulted in a 4.1 fold increase in APP expression over control levels and infection with HSV-APP751 mutations resulted in APP increases that ranged from 3.6 fold to 10.1 fold over control levels. For both HSV-APP751 and HSV-APP695 infections, analysis of variance (ANOVA) revealed no statistical difference ($p>0.05$) in APP expression among wild-type and all mutations; therefore, these data were pooled together to reflect mean APP expression (figure 5). Mean APP expression after infection with HSV-APP695 wild-type and mutations (10.9 fold basal) was significantly greater ($p<0.05$) than after infection with HSV-APP751 wild-type and mutations (5.3 fold basal).

Tau Expression

Total tau was detected as an approximately 55 kDa band by the mAb 5E2. Despite large increases in APP expression caused by HSV-APP695 (wild-type or mutations) infection, tau expression remained at approximately control levels (figure 6). Infection with HSV-APP695 wild-type slightly increased tau expression (1.2 fold basal) and infection with HSV-APP695 mutations resulted in tau expression that ranged from 0.9 fold to 1.3 fold over control levels. In contrast, infection with HSV-APP751 wild-type increased tau expression

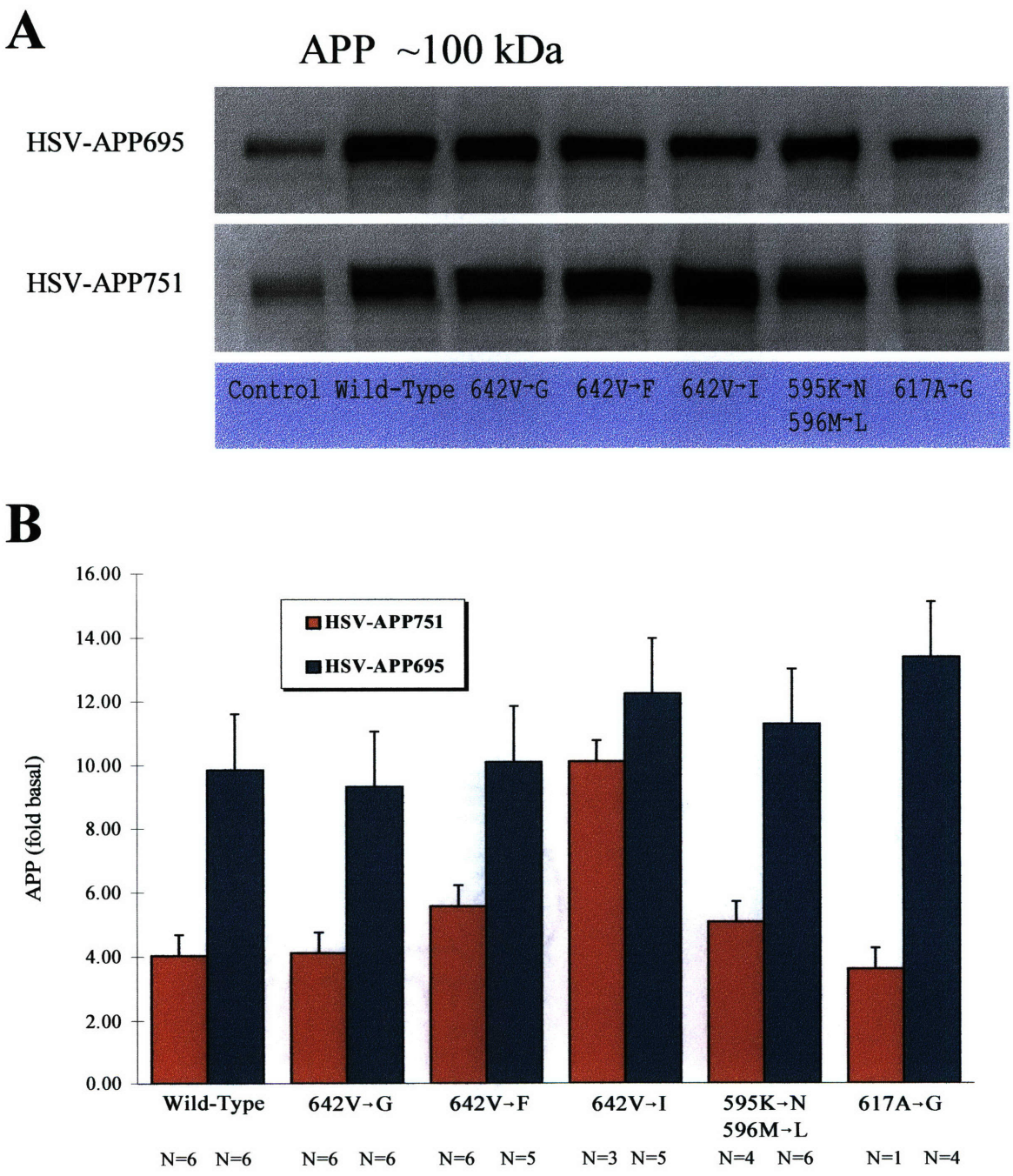


Figure 4. Effect of HSV-APP695/751 (wild-type or mutations) infection on APP protein expression. **A.** Representative Western blot showing APP expression as detected by 22C11. **B.** Summary of APP expression after infection with HSV-APP695/751 (wild-type or mutations). Both infection with HSV-APP751 and with HSV-APP695 increased APP expression above control levels. However, APP levels were significantly greater after infection with HSV-APP695 than with HSV-APP751. Generally, infections with wild-type and with mutations were not significantly different from each other for both HSV-APP isoforms.

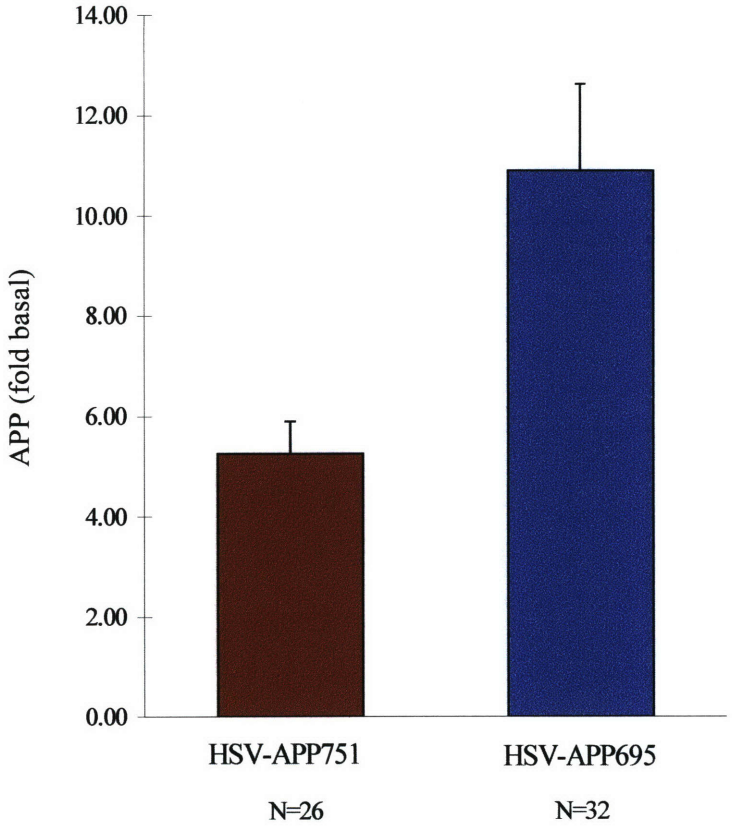
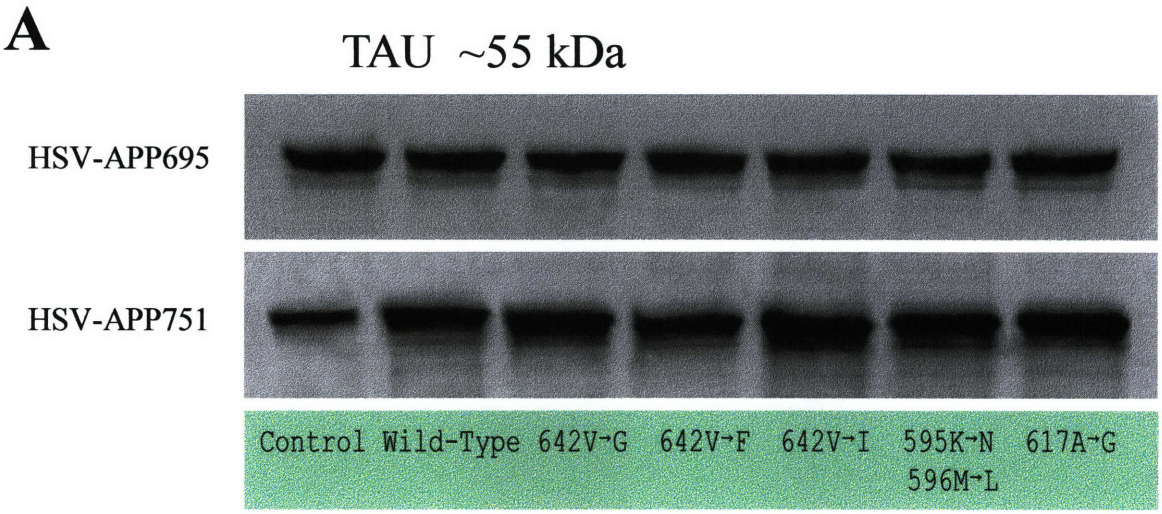


Figure 5. Mean APP protein expression after HSV-APP751 or HSV-APP695 infection. APP was significantly over-expressed compared to control levels following infection with either HSV-APP isoform. However, APP levels were significantly greater after infection with HSV-APP695 than with HSV-APP751 ($p<0.05$).



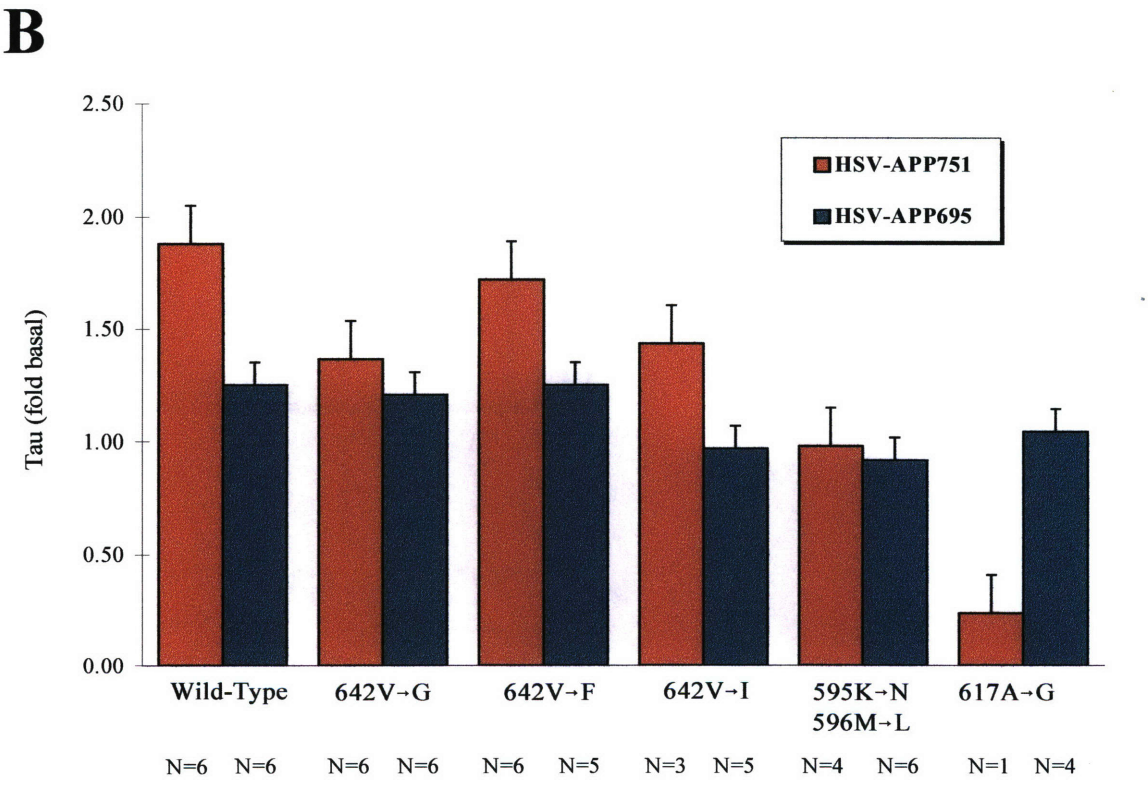


Figure 6. Effect of HSV-APP695/751 (wild-type or mutations) infection on tau protein expression. **A.** Representative Western blot showing tau expression as detected by 5E2. **B.** Summary of tau expression after infection with HSV-APP695/751 (wild-type or mutations). Tau expression was greater after infection with HSV-APP751 than with HSV-APP695. Generally, infections with wild-type and with mutations were not significantly different from each other for both HSV-APP isoforms.

1.9 fold over control levels and infection with HSV-APP751 mutations resulted in tau increases that ranged from 1.4 fold to 1.7 fold over control levels (the discrepancy of the HSV-APP751 617A→G infection is attributable to its small sample size). For both HSV-APP751 and HSV-APP695 infections, ANOVA revealed no statistical difference ($p>0.05$) in tau expression among wild-type and all mutations; therefore, these data were pooled together to reflect mean tau expression (figure 7). Mean tau expression after infection with HSV-APP751 wild-type and mutations (1.5 fold basal) was significantly greater ($p<0.05$) than after infection with HSV-APP695 wild-type and mutations (1.1 fold basal).

Tau-1 Expression

Dephosphorylated tau was detected as a 55 kDa band by the monoclonal antibody Tau-1; hence, Tau-1 immunolabeling is inversely proportional to levels of hyper-phosphorylated tau at the Ser 202 site (Kosik *et al.*, 1988). Tau-1 expression after infection with HSV-APP695/751 mutations were normalized to their wild-type counterparts (Figure 8). Infection with HSV-APP695 mutations resulted in tau-1 expression that ranged from 0.7 fold to 1.1 fold over wild-type levels. Infection with HSV-APP751 mutations resulted in tau-1 expression that ranged from 0.3 fold to 0.4 fold over wild-type levels (excepting HSV-APP751 642V→F infections). For both HSV-APP751 and HSV-APP695 infections, ANOVA revealed no statistical difference ($p>0.05$) in tau-1 expression among all mutations; therefore, these data were pooled together to reflect mean tau-1 expression (figure 9). Mean tau-1 expression after infection with HSV-APP751 mutations (0.5 fold wild-type) was significantly lower ($p<0.05$) than after infection with HSV-APP695 mutations (0.9 fold wild-

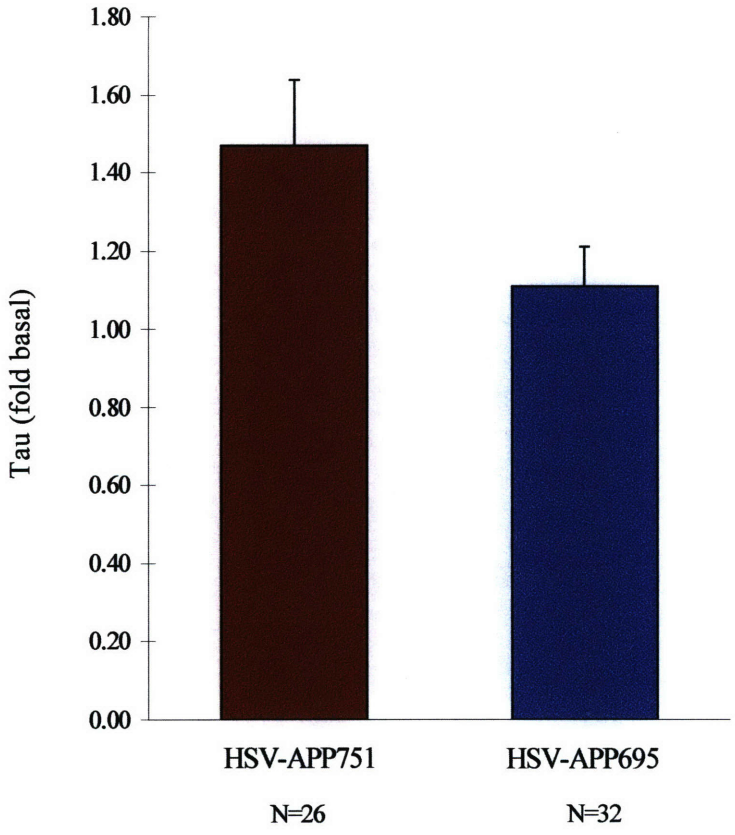


Figure 7. Mean tau protein expression after HSV-APP751 or HSV-APP695 infection. Tau levels were significantly greater after infection with HSV-APP751 than with HSV-APP695 ($p<0.05$).

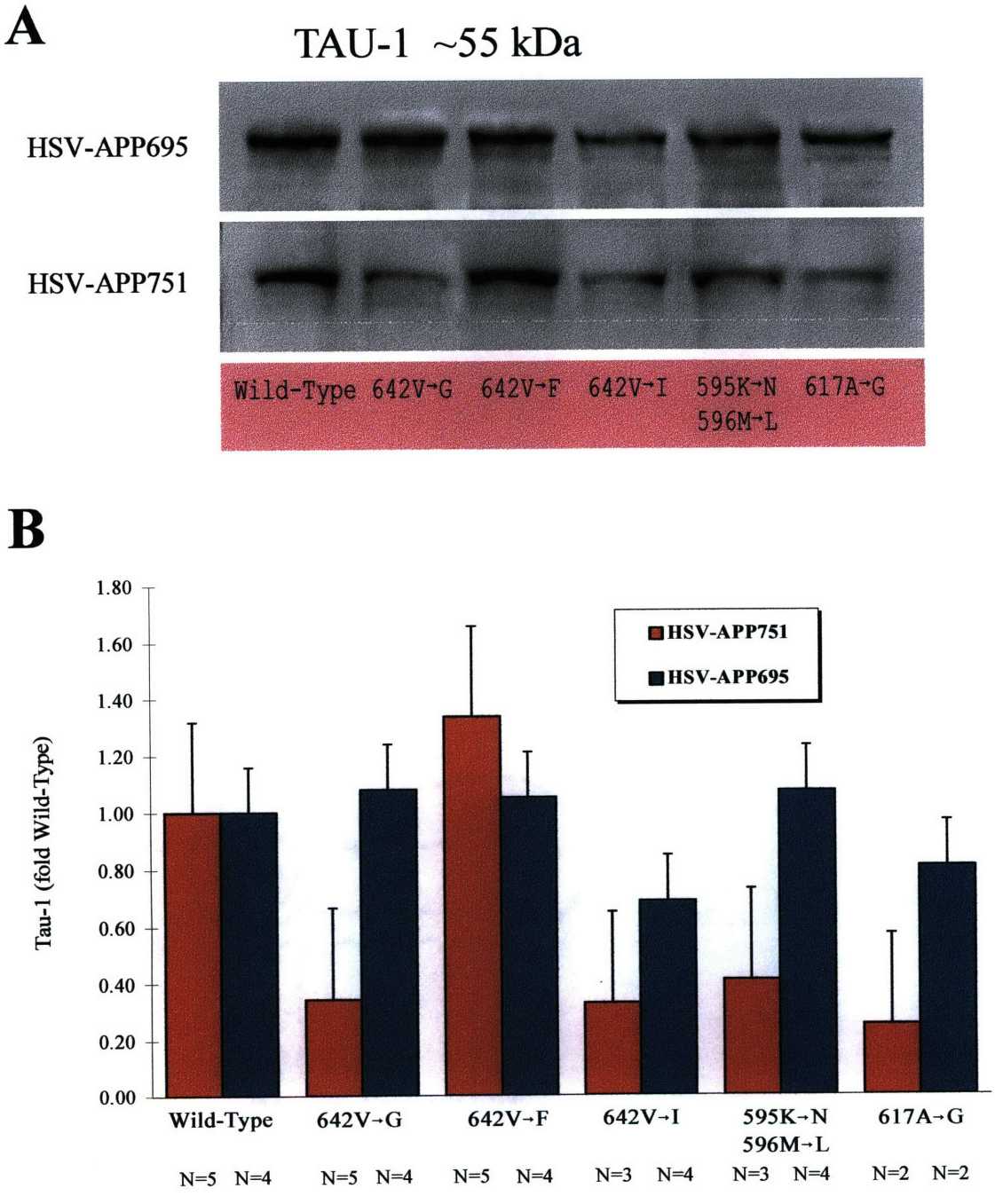


Figure 8. Effect of HSV-APP695/751 (wild-type or mutations) infection on tau-1 protein expression. **A.** Representative Western blot showing tau-1 expression as detected by Tau-1. **B.** Summary of tau-1 expression after infection with HSV-APP695/751 (wild-type or mutations). Infection with HSV-APP751 mutations, except 642V→F, resulted in decreased tau-1 expression and thus, increased amounts of hyper-phosphorylated tau relative to wild-type. Infection with HSV-APP695 mutations resulted in tau-1 expression that was not significantly different from infection with HSV-APP695 wild-type.

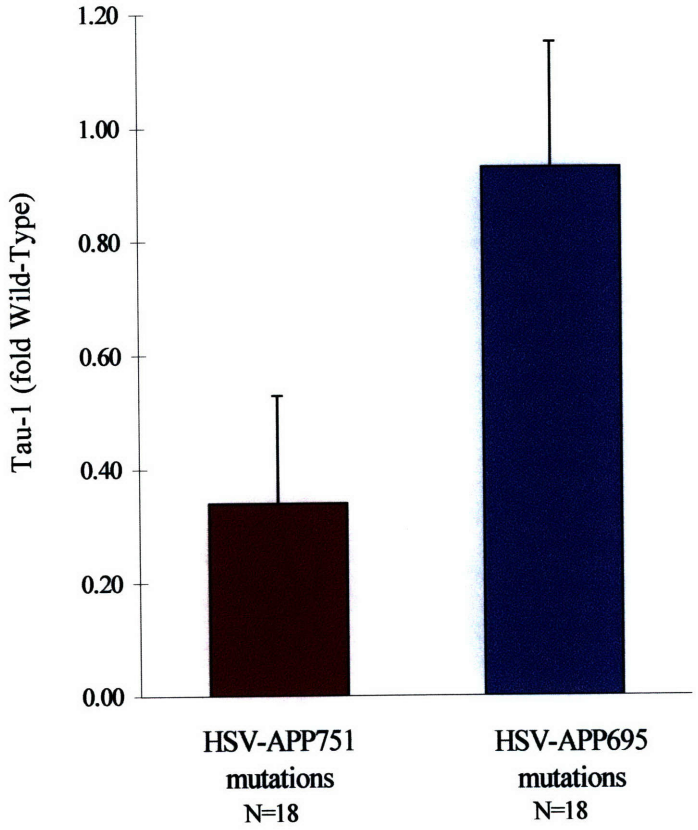


Figure 9. Mean tau-1 protein expression after infection with HSV-APP751 mutations or with HSV-APP695 mutations. Tau-1 levels were significantly greater after infection with HSV-APP695 mutations than with HSV-APP751 mutations ($p<0.05$), indicating greater amounts of hyper-phosphorylated tau after infection with HSV-APP751 mutations. (Data from HSV-APP695/751 642V→F infections were omitted in the calculation of mean tau-1 expression because of large discrepancies with data from infections with other HSV-APP751 mutations.)

type). These data indicate that tau is significantly hyper-phosphorylated at the Ser 202 site after infection with HSV-APP751 mutations relative to HSV-APP751 wild-type.

4.3 HSV-APP695 and HSV-APP751 Infections: mRNA Expression

A representative Northern blot shows the effects of infection with HSV-APP695/751 (wild-type or "London" [642V→G/F/I] mutations) or HSV-$A\beta_{1-42}$ on tau mRNA levels (figure 10) as detected by the tau probe. Neurons infected and not infected with HSV-LacZ did not differ with respect to tau mRNA levels, therefore these data were pooled together and designated as controls. Two major tau transcripts measuring 6 kb and 2 kb were detected, consistent with previous studies (Goedert *et al.*, 1988; Kosik *et al.*, 1989). Levels of the 6 kb tau mRNA transcript were significantly elevated after each of these infections than in uninfected controls. The 2 kb tau mRNA transcript was only detected after infection with HSV-APP751 642V→G and HSV-$A\beta_{1-42}$. The 8 kb and 3 kb bands that appear on all blots are produced by non-specific binding of the tau probe.

Tau mRNA Expression

For both HSV-APP751 and HSV-APP695 infections, ANOVA revealed no statistical difference ($p>0.05$) in 6 kb tau mRNA levels among the "London" mutations; these data were pooled together to reflect mean 6 kb tau mRNA levels and compared to their wild-type counterparts (figure 11). In both HSV-APP751 and HSV-APP695 infections, the "London" mutations expressed significantly higher levels of the 6 kb tau mRNA transcript than did their wild-type counterparts. Levels of 6 kb tau mRNA were not significantly different across

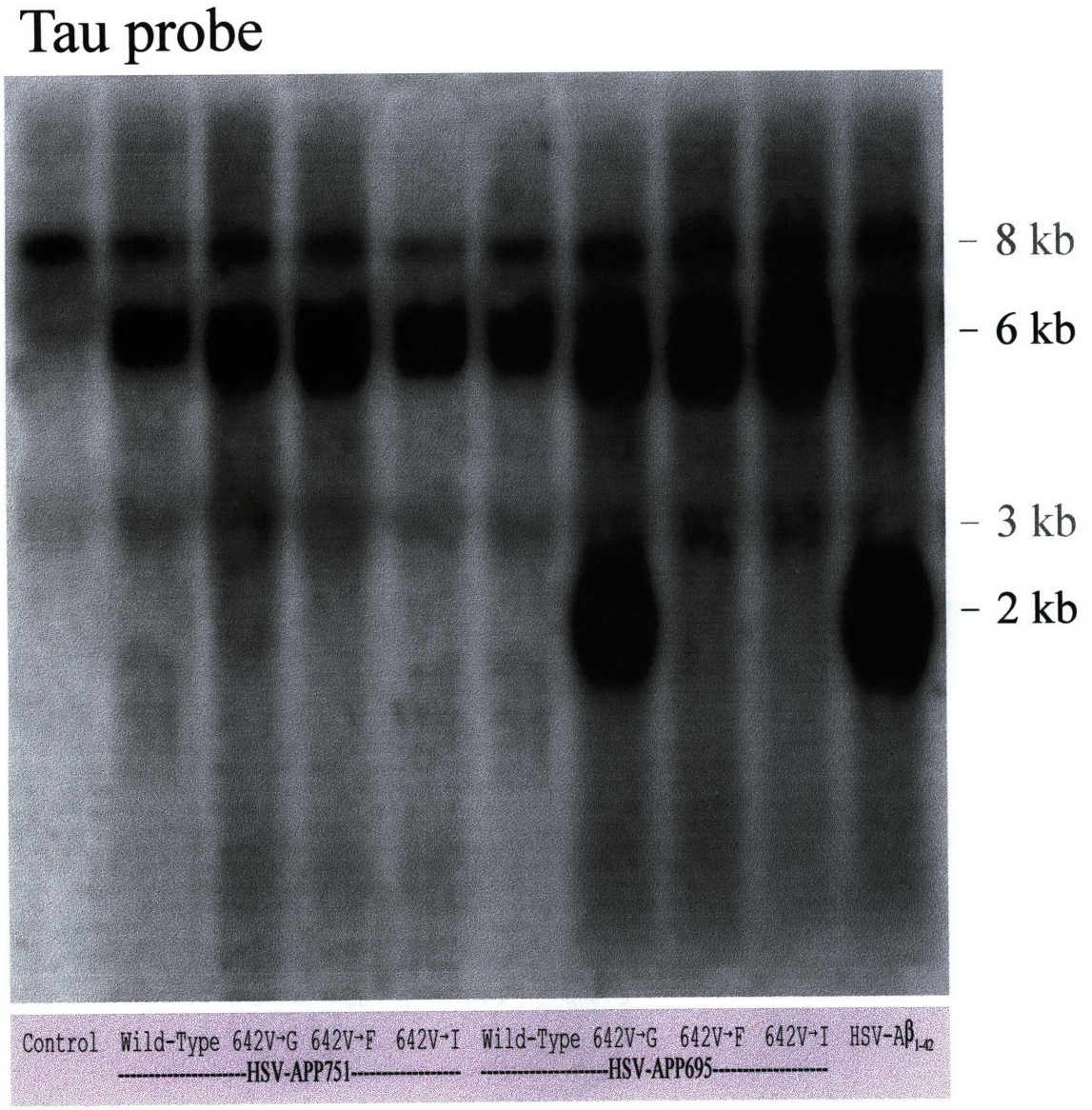


Figure 10. Representative Northern blot showing the effect of HSV-APP695/751 (wild-type or "London" mutations) or HSV-$A\beta_{1-42}$ infection on tau mRNA expression. 6 kb and 2 kb tau mRNA transcripts were detected by the tau probe. The 6 kb tau mRNA transcript was significantly increased over control levels after all infections. The 2 kb tau mRNA transcript was especially prominent after HSV-APP695 642V→G and HSV-$A\beta_{1-42}$ infections, but was absent in all other infections studied. Bands at 8 kb and 3 kb are artifacts from non-specific labeling.

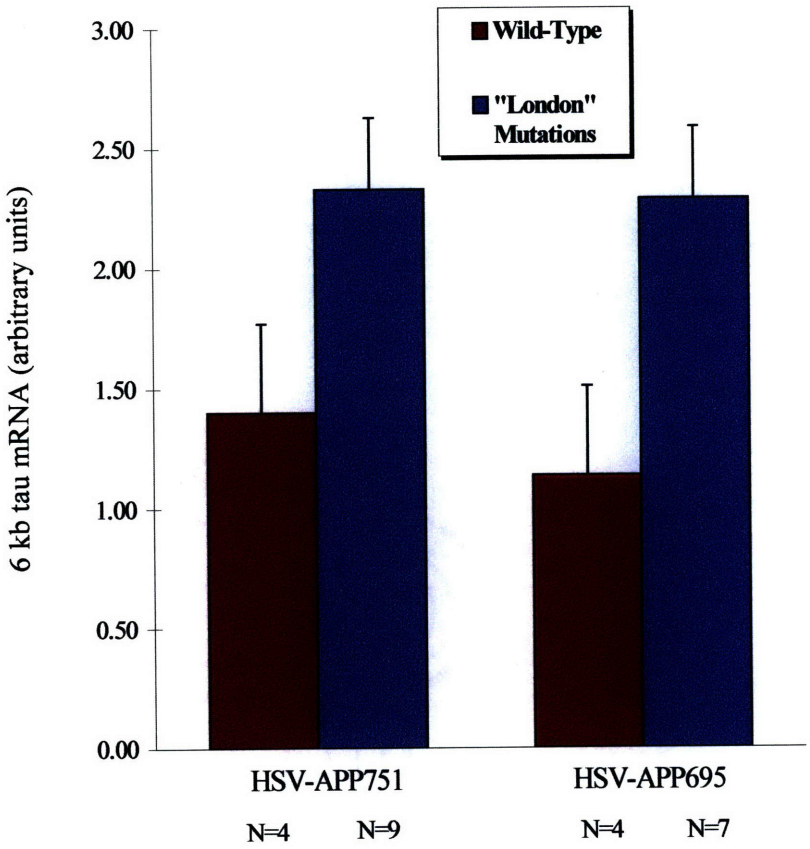


Figure 11. Effect of HSV-APP695/751 (wild-type or "London" mutations) infection on levels of 6 kb tau mRNA. After both HSV-APP751 and HSV-APP695 infections, the "London" mutations expressed significantly higher levels of 6 kb tau mRNA than wild-type ($p<0.05$). 6 kb tau mRNA levels were uniform across HSV-APP isoforms for both wild-type and "London" mutations. Due to low levels of 6 kb tau mRNA from control samples, data are graphed in arbitrary units.

APP751 or APP695 isoforms for both wild-type and mutations. Interestingly, the 2 kb tau mRNA transcript was not expressed in any of the HSV-APP695/751 infections except after infection with HSV-APP695 642V→G, where it is dramatically increased (figure 10).

4.4 HSV-Aβ_{1-42} Infection: Protein and mRNA Expression

Aβ_{1-42} mRNA expressed after HSV-Aβ_{1-42} infection was detected as an approximately 1.0 kb band and APP mRNA expressed after HSV-Aβ_{1-42} infection was detected as an approximately 3.5 kb band on Northern blots by the APP probe (figure 12). Neurons infected and not infected with HSV-LacZ did not differ with respect to Aβ_{1-42} mRNA levels, therefore these data were pooled together and designated as controls. As expected, Northern blots of control samples did not reveal a band for Aβ_{1-42} since Aβ is not a naturally occurring transcript. The effectiveness of the virus appears to be dosage dependent; the lower viral load (15λ) expressed sufficient amounts of Aβ_{1-42} mRNA and was used for all infections. Levels of APP mRNA were also dramatically elevated over control levels after HSV-Aβ_{1-42} infection in a dosage dependent manner.

C100 is the 100 amino acid C-terminus of APP that includes Aβ. HSV-C100 infection was used as a positive control experiment for HSV-Aβ_{1-42} infection. A representative Western blot shows the effect of HSV-C100 infection on APP and tau expression (figure 13). Neurons infected and not infected with HSV-LacZ did not differ with respect to APP and tau levels, therefore these data were pooled together and designated as controls. APP and tau were detected by the antibodies described in section 4.2. Both APP and tau levels were greatly reduced after HSV-C100 infection. The decreased levels of both

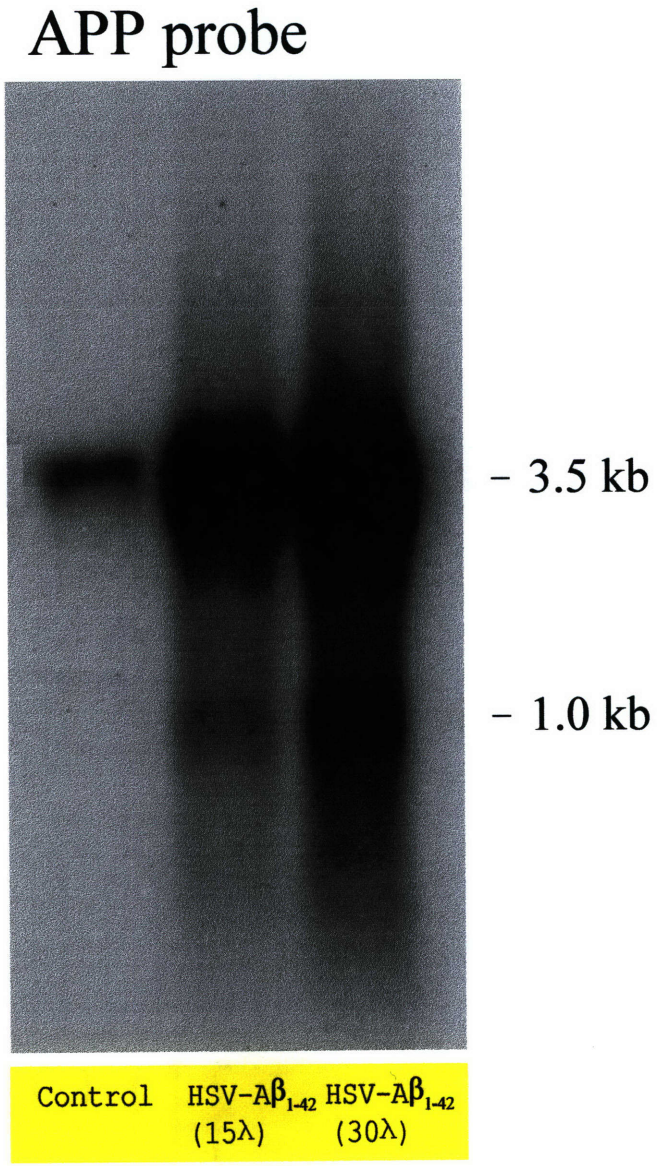


Figure 12. Representative Northern blot showing the effect of HSV-$A\beta_{1-42}$ infection on $A\beta_{1-42}$ and APP mRNA expression. $A\beta_{1-42}$ mRNA (1.0 kb) and APP mRNA (3.5 kb) were detected by the APP probe. Both $A\beta_{1-42}$ and APP mRNA levels were dramatically increased after HSV-$A\beta_{1-42}$ infection in a viral dosage dependent manner.

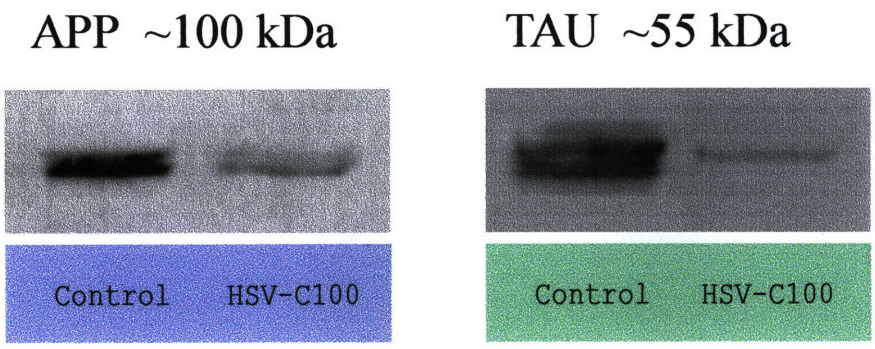


Figure 13. Representative Western blot showing the effect of HSV-C100 infection on APP and tau protein expression. Both APP (as detected by 22C11) and tau (as detected by 5E2) levels were dramatically decreased after infection with HSV-C100. These data, in addition to cell death observed under a microscope, indicate that C100 is neurotoxic.

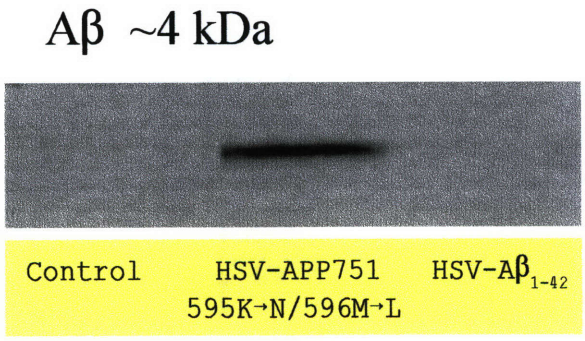


Figure 14. Effect of HSV-APP751 595K→N/596M→L and HSV-$A\beta_{1-42}$ infection on Aβ secretion. Results from immunoprecipitation of Aβ peptides from cell media show that Aβ (as detected by R1282) was secreted after HSV-APP751 595K→N/596M→L infection. However, Aβ was not secreted after HSV-$A\beta_{1-42}$ infection, suggesting that it remained intracellular.

proteins are indicative of cell death and are consistent with reports of C100 neurotoxicity (Yankner *et al.*, 1989).

Results of Aβ immunoprecipitation from conditioned cell media are shown for uninfected control, HSV-APP751 595K→N/596M→L ("Swedish" mutation) infection, and HSV-$A\beta_{1-42}$ infection (figure 14). Infection with HSV-APP751 595K→N/596M→L increased Aβ secretion into conditioned media, consistent with previous studies (Haass *et al.*, 1995). Aβ was not secreted into conditioned media in detectable amounts after HSV-LacZ infection, after HSV-$A\beta_{1-42}$ infection, or for uninfected neurons. These results indicate that Aβ is retained intracellularly following HSV-$A\beta_{1-42}$ infection.

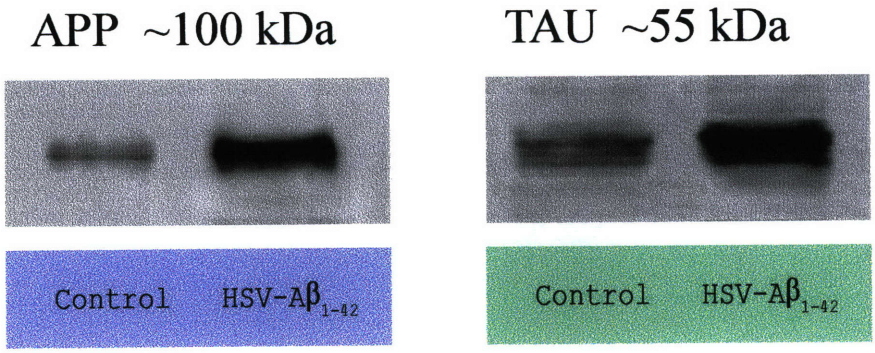
APP and Tau Expression

A representative Western blot shows the effect of HSV-$A\beta_{1-42}$ infection on APP and tau expression. HSV-$A\beta_{1-42}$ infection increased both APP and tau protein expression in cortical neurons (figure 15). Neurons infected and not infected with HSV-LacZ did not differ with respect to APP and tau levels, therefore these data were pooled together and designated as controls. Mean APP expression was 3.6 fold basal whereas mean tau expression was 1.6 fold basal after infection. These data suggest that the accumulation of intracellular $A\beta_{1-42}$ may contribute to aberrant tau and APP expression.

Tau mRNA Expression

A representative Northern blot shows significant increases in tau mRNA expression after infection with HSV-$A\beta_{1-42}$ (figure 10). Levels of the 6 kb tau mRNA transcript after

A



B

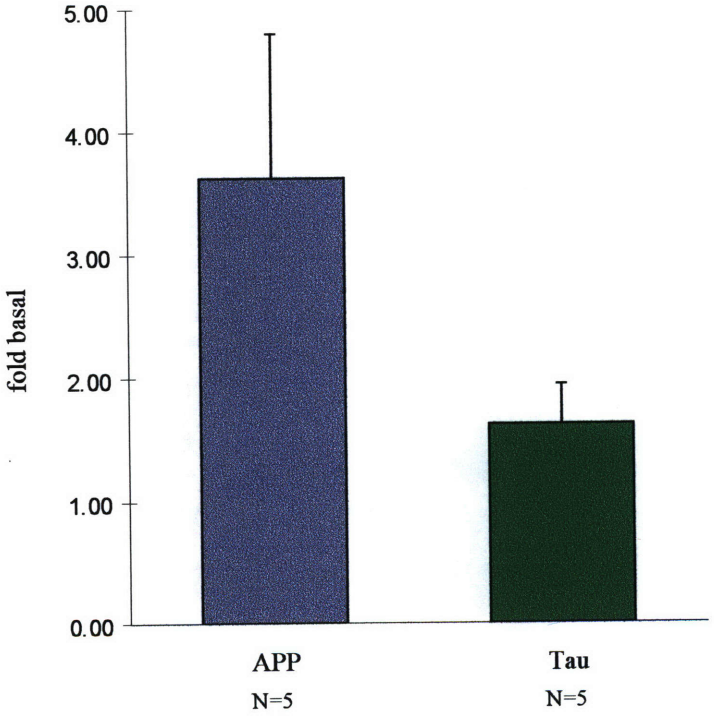


Figure 15. Effect of HSV-$A\beta_{1-42}$ infection on APP and tau protein expression. **A.** Representative Western blot showing APP expression as detected by 22C11 and tau expression as detected by 5E2. **B.** Mean APP and tau expression after HSV-$A\beta_{1-42}$ infection. Infection with HSV-$A\beta_{1-42}$ resulted in APP expression that was approximately 3.6 fold basal and tau expression that was approximately 1.6 fold basal.

HSV-$A\beta_{1-42}$ infection were significantly greater than control levels. Interestingly, as after infection with HSV-APP695 642V→G, levels of the 2 kb tau mRNA transcript after HSV-$A\beta_{1-42}$ infection were dramatically increased from undetectable levels in control samples.

CHAPTER 5

Discussion

The results from this study suggest that APP751 and its associated mutations may lead to increased tau synthesis and formation of hyper-phosphorylated tau protein. Increased APP and tau synthesis have been observed in DS, AD, and in normal aging (Johnson *et al.*, 1990; Adler *et al.*, 1991; Khatoon *et al.*, 1992; Iverfeldt *et al.*, 1993; Oyama *et al.*, 1994). We have shown that intracellular $A\beta_{1-42}$, formed by APP over-expression or mutations, may stimulate tau transcription. The increased hyper-phosphorylation of newly synthesized tau by extracellular Aβ (Busciglio *et al.*, 1995) may further accelerate the formation of PHFs and neurofibrillary tangles (Alonso *et al.*, 1996). We propose a model to account for our results (figure 16), and suggest that amyloid can initiate neurofibrillary tangle formation. Consistent with this model, amyloid plaques appear prior to neurofibrillary tangles in DS (Mann and Esiri, 1989; Murphy *et al.*, 1990). The model is also consistent with findings that neurofibrillary tangles may appear prior to amyloid plaques in AD (Braak and Braak, 1991) since intracellular Aβ need not aggregate to cause aberrant tau synthesis.

Effects of Intracellular $A\beta_{1-42}$ on APP Expression

Extracellular $A\beta_{1-40}$ increases the transcription of APP mRNA as well as other proteins (Le *et al.*, 1995; Pena *et al.*, 1995). We have shown that intracellular $A\beta_{1-42}$ dramatically up-regulates the transcription of APP, leading to significant increases in APP protein expression

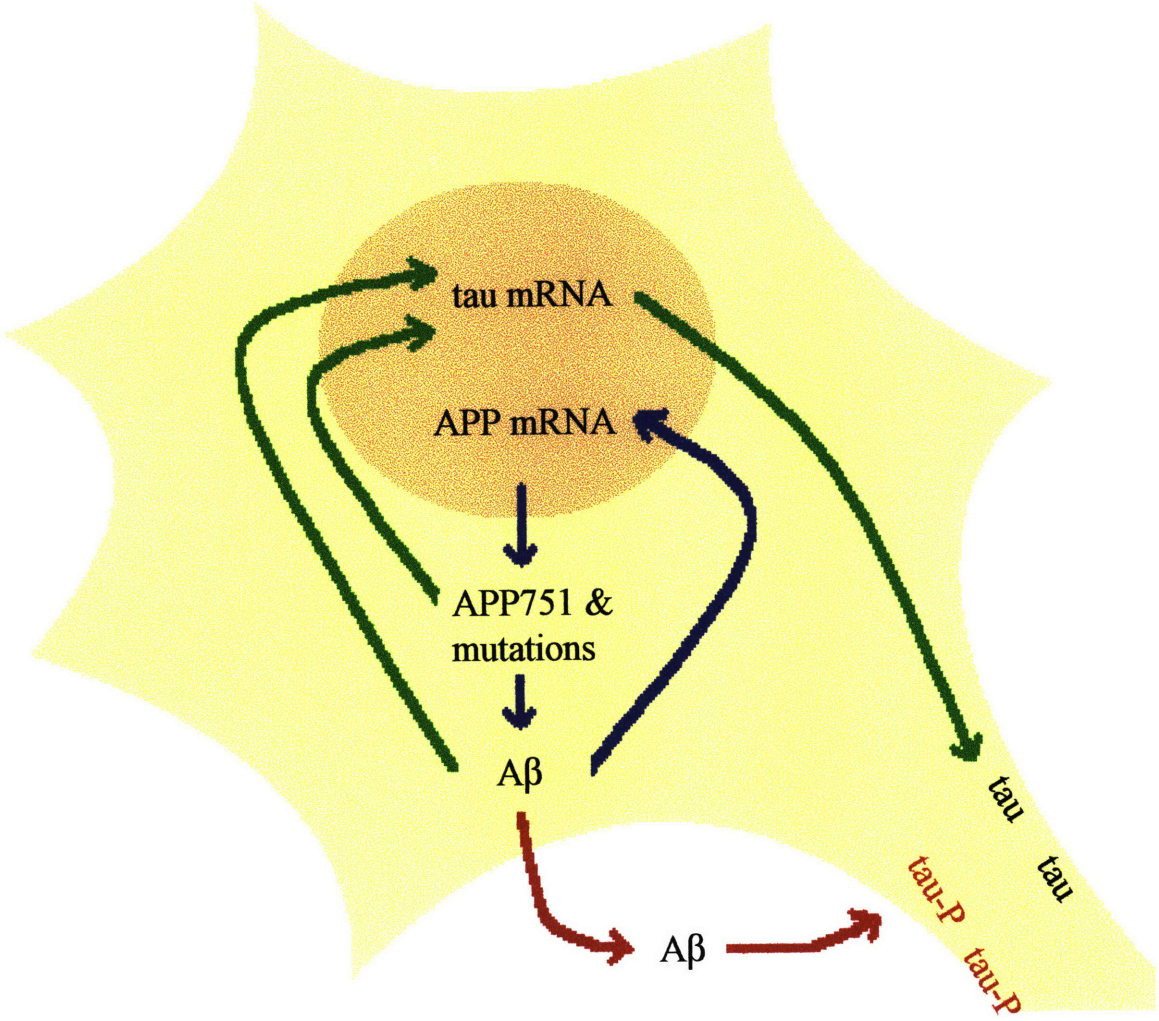


Figure 16. Regulation of tau by Aβ and APP. This study elucidated three major pathways which link APP, Aβ, and tau. The first pathway (shown in blue) is a potential positive feedback loop where aberrant processing of APP751 yields Aβ which, in turn, up-regulates the transcription of APP mRNA. The second pathway (shown in green) links APP expression to tau synthesis: APP695/751 (wild-type and mutations) and $A\beta_{1\text{-}42}$ up-regulate the transcription of tau mRNA, though the translational product is subsequently degraded in the case of APP695. The third pathway (shown in red) accounts for the generation of abnormally hyper-phosphorylated tau. When Aβ is secreted from the cell, as accelerated by APP mutations, it hyper-phosphorylates existing or newly synthesized tau protein (Busciglio *et al.*, 1995).

(3.5 fold basal). Since HSV-C100 infection resulted in cell death (Yankner *et al.*, 1989; Oster-Granite *et al.*, 1996) while equal titers of HSV-$A\beta_{1-42}$ did not, we conclude that Aβ was not neurotoxic at the levels introduced. Infection with HSV-$A\beta_{1-42}$ did not result in secretion of Aβ peptides into conditioned media, suggesting that $A\beta_{1-42}$ accumulates intracellularly. Previous studies have demonstrated that, in addition to being secreted, Aβ is generated intracellularly from proteolytic processing of APP (Martin *et al.*, 1995; Turner *et al.*, 1996). These findings suggest that Aβ may function as a transcriptional activator (Kaltschmidt *et al.*, 1997) or as a transcriptional regulator of the APP gene. Hence, there may exist a positive feedback loop whereby APP generates $A\beta_{1-42}$ by altered processing, which in turn increases the expression of APP. Although APP mRNA was found to be up-regulated after HSV-$A\beta_{1-42}$ infection, we did not ascertain which isoforms of APP were increased. If APP751 was increased, entry into this loop may be initiated by neuronal degeneration, which has been demonstrated to increase levels of KPI-containing APP (Iverfeldt *et al.*, 1993; Solá *et al.*, 1993). Since APP751 and $A\beta_{1-42}$ have both been shown to increase tau expression, this mechanism may amplify both amyloid production and tangle formation.

Effects of APP and $A\beta_{1-42}$ on Tau Expression

Several studies have reported increases of APP in AD brains (Tanaka *et al.*, 1988; Palmert *et al.*, 1988; Tanaka *et al.*, 1989; Golde *et al.*, 1990). We induced a 5 fold and an 11 fold increase in APP expression after infection with HSV-APP751 and HSV-APP695, respectively. This pattern of APP expression may arise from a higher rate of synthesis of APP695 and/or a higher rate of degradation of APP751. APP695 is normally expressed at

much higher levels in neurons (Tanaka *et al.*, 1989) and may be more viable in neuronal cultures. Despite large increases in APP levels after infection with HSV-APP695, neurons infected with HSV-APP751 over-expressed tau protein at approximately 1.5 fold over basal levels while tau levels in neurons infected with HSV-APP695 remained unchanged. These are not the results one would predict from the pattern of APP expression described above. APP751 expression appears to affect neuronal tau expression in a distinctly different way than APP695 expression.

Tau mRNA levels were greatly increased over control levels after infection with both APP isoforms. These data suggest that the differences in tau protein expression arise post-translationally. Since the KPI domain may have neurotrophic properties (Akaaboune *et al.*, 1994; Qiu *et al.*, 1995), increased tau expression may arise from neurite outgrowth promoted by APP751 (Drubin *et al.*, 1985). Alternatively, it has been shown that the secretory form of APP751 is identical to protease nexin II, a serine protease inhibitor (Van Nostrand *et al.*, 1989). The serine protease thrombin catalyzes the proteolysis of tau in vitro (Oleson, 1994; Wang *et al.*, 1996). Protease inhibition by proteins containing the KPI domain may inhibit the degradation of tau and account for elevated tau levels after APP751 infections, but not after APP695 infections. If this were the case, tau that is normally degraded may linger and become more susceptible to abnormal structural alterations, such as hyper-phosphorylation.

We have shown that intracellular $A\beta_{1-42}$ dramatically up-regulates the transcription of 2 kb and 6 kb tau mRNA, leading to significant increases in tau protein expression (1.5 fold basal). Although the 2 kb tau mRNA transcript has been observed, its up-regulation in AD brain was largely overlooked (Goedert *et al.*, 1988). This is the first study to report increases

in the 2 kb tau mRNA transcript caused by intracellular $A\beta_{1-42}$; the 2 kb transcript was absent in all other infections except HSV-APP695 642V→G. Hence, $A\beta_{1-42}$ may regulate tau as well as APP gene expression. Aβ, in addition to aggregating into insoluble amyloid plaques, may alter a neuron's normal transcriptional program and promote the formation of neurofibrillary tangles. Identification of the translational product of the 2 kb tau transcript may clarify the relationship between Aβ and aberrant tau formation.

Effects of APP Mutations on Tau Phosphorylation

Infection with HSV-APP751 mutations resulted in tau hyper-phosphorylation (at the Tau-1 site) relative to HSV-APP751 wild-type for all mutations except 642V→F. In contrast, infection with HSV-APP695 mutations did not result in significant differences in tau phosphorylation relative to HSV-APP695 wild-type infections. These data, taken together with evidence that cells infected with HSV-APP751 but not HSV-APP695 increased overall tau levels, suggest that mutations may have a particularly strong hyper-phosphorylating effect on newly formed tau as opposed to existing tau. Hyper-phosphorylation may be caused by up-regulation of kinases (Drewes *et al.*, 1992; Goedert *et al.*, 1992; Mandelkow *et al.*, 1992) or by down-regulation of phosphatases (Yamamoto *et al.*, 1988; Drewes *et al.*, 1993; Harris *et al.*, 1993). APP751 and $A\beta_{1-42}$, through mechanisms discussed above, may up-regulate tau expression. APP751 mutations may promote intracellular accumulation and secretion of Aβ (Citron *et al.*, 1992; Cai *et al.*, 1993; Suzuki *et al.*, 1994; Haass *et al.*, 1994; Martin *et al.*, 1995); extracellular Aβ may then induce phosphorylation of newly formed tau (Busciglio *et al.*, 1995). Through increased tau hyper-phosphorylation, mutations of APP751 may

accelerate the disruption of microtubule assembly and axonal transport in neurons and lead to neurofibrillary degeneration (Khatoon *et al.*, 1992; Bramblett *et al.*, 1993). Aβ is produced in normal cell metabolism and secretion increases with aging (Haass *et al.*, 1992; Turner *et al.*, 1996); this suggests a pathway for tau hyper-phosphorylation (and hence PHF formation) independent of mutations, which comprise less than 1% of all AD cases

In this study, tau hyper-phosphorylation at the Ser 202 site was measured by the mAb Tau-1. Other sites on the tau molecule have been found to be hyper-phosphorylated in AD (Iqbal *et al.*, 1989). To more fully understand the phosphorylation state of tau, other phosphorylation-dependent antibodies, such as Alz-50 (Uéda, 1990), need to be tested. It is conceivable that different mutations of APP751 may hyper-phosphorylate tau at sites other than Ser 202. In addition, other pathological aberrations of tau, such as truncation (Novák *et al.*, 1993), have to be explored to more fully evaluate the effects of APP751 mutations.

In summary, we have shown that APP751 and intracellular $A\beta_{1-42}$ can stimulate abnormal increases in tau and APP expression. Newly formed tau can be aberrantly hyper-phosphorylated by extracellular Aβ (Busciglio *et al.*, 1995), whose secretion is accelerated by APP mutations (Cai *et al.*, 1993; Suzuki *et al.*, 1994). These results suggest a potential link between amyloid plaques and neurofibrillary tangles in certain neurodegenerative diseases, including AD and DS.

References

Adler, M, Coronel, C, Shelton, E, Seegmiller, J, and Dewji, N (1991). Increased gene expression of Alzheimer disease β-amyloid precursor protein in senescent cultured fibroblasts. *Proc. Natl. Acad. Sci. USA*, **88**, 16-20.

Akaaboune, M, Ma, J, Festoff, B, Greenberg, B, and Hantai, D (1994). Neurotrophic regulation of mouse muscle β-amyloid protein precursor and α 1-antichymotrypsin as revealed by axotomy. *Journal of Neurobiology*, **25**, 503-514.

Alonso, A, Grundke-Iqbal, I, and Iqbal, K (1996). Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and dissembles microtubules. *Nature Medicine*, **2**, 783-787.

Arai, H, Terajima, M, Miura, M, Higuchi, S, Muramatsu, T, Machida, N, Seiki, H, Takase, S, Clark, C, Lee, V, Trojanowski, J, and Sasaki, H (1995). *Annals of Neurology*, **38**, 649-652.

Arriagada, P, Growdon, J, Hedley-White, E, and Hyman B (1992). Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. *Neurology*, **42**, 631-639.

Braak, H and Braak, E (1991). Neuropathological staging of Alzheimer-related changes. *Acta Neuropathology*, **82**, 239-57.

Bramblett, G, Goedert, M, Jakes, R, Merrick, S, Trojanowski, J, and Lee, V (1993). Abnormal tau phosphorylation at Ser^{396} in Alzheimer's disease recapitulates development and contributes to reduced microtubule binding. *Neuron*, **10**, 1089-1099.

Busciglio, J, Lorenzo, A, Yeh, J, and Yankner, B (1995). β-amyloid fibrils induce tau phosphorylation and loss of microtubule binding. *Neuron*, **14**, 879-888.

Butner, K, and Kirschner, M (1991). Tau protein binds to microtubules through a flexible array of distributed weak sites. *Journal of Cell Biology*, **115**, 717-730.

Cai, X, Golde, T, and Younkin, S (1993). Release of excess amyloid β protein from a mutant amyloid β protein precursor. *Science*, **259**, 514-516.

Citron, M, Oltersdorf, T, Haass, C, McConlogue, L, Hung, A, Seubert, P, Vigo-Pelfrey, C, Lieberburg, I, and Selkoe, D (1992). Mutation of the β-amyloid precursor protein in familial Alzheimer's Disease increases β-protein production. *Nature*, **360**, 672-674.

Cleveland, D, Hwo, S, and Kirschner, M (1977). Purification of tau, a microtubule-associated protein that induces assembly of microtubules from purified tubulin. *Journal of Molecular Biology*, **116**, 207-225.

De Strooper, B, Simons, M, Multhaup, G, Van Leuven, F, Beyreuther, K, and Dotti, C (1995). Production of intracellular amyloid-containing fragments in hippocampal neurons expressing human amyloid precursor protein and protection against amyloidogenesis by subtle amino acid substitutions in the rodent sequence. *The EMBO Journal*, **14**, 4932-4938.

Drewes, G, Lichtenberg-Kragg, B, Doring, F, mandelkow, E, Biernat, J, Doree, M, and Mandelkow, E (1992). Mitogen activated protein (MAP) kinase transform tau protein into an Alzheimer-like state. *The EMBO Journal*, **11**, 2131-2138.

Drewes, G, Mandelkow, E, Bauman, K, Goris, J, Merlevede, W, and Mandelkow, E (1993). Dephosphorylation of tau protein and Alzheimer paired helical filaments by calcineurin and phosphatase 2A. *FEBS Letters*, **336**, 425-432.

Drubin, D, Feinstein, S, Shooter, E, and Kirschner, M (1985). Nerve growth factor-induced neurite outgrowth in PC12 cells involves the coordinate induction of microtubule assembly and assembly-promoting factors. *Journal of Cell Biology*, **101**, 1799-1807.

Ennulat, D, Liem, R, Hashim, G, and Shelanski, M (1989). Two separate 18 amino acid domains of tau promote tau polymerization of tubulin. *Journal of Biological Chemistry*, **264**, 5327-5330.

Esch, F, Keim, P, Beattie, E, Blacher, R, Culwell, A, Oltersdorf, T, McClure, D, and Ward, P (1990). Cleavage of amyloid β-peptide during constitutive processing of its precursor. *Science*, **248**, 1122-1124.

Geller, A, During, M, and Neve, R (1994). A defective herpes simplex virus vector system for genetic intervention in the adult brain: applications to gene therapy and neuronal physiology. *Methods in Neurosciences*, **21**, 443-461.

Glenner, G and Wong, C (1984). Alzheimer's disease: Initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochemical and Biophysical Research Communications*, **120**, 885-890.

Goedert, M, Wischik, C, Crowther, R, Walker, J, and Klug, A (1988). Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer's disease: Identification as the microtubule-associated protein tau. *Proc. Natl. Acad. Sci. USA*, **85**, 4051-4055.

Goedert, M, Spillantini, M, Jakes, R, Rutherford, D, and Crowther, R (1989). Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron*, **3**, 519-526.

Goedert, M, Cohen, E, Jakes, R, and Cohen, P (1992). p42 MAP kinase phosphorylation sites in microtubule-associated protein tau are dephosphorylated by protein phosphatase 2A1. Implications for Alzheimer's disease. *FEBS Letters*, **313**, 203.

Golde, T, Estus, S, Usiak, M, Younkin, L, and Younkin, S (1990). Expression of β amyloid protein precursor mRNAs: recognition of a novel alternatively spliced form and quantitation in Alzheimer's disease using PCR. *Neuron*, **4**, 253-267.

Golde, T, Estus, S, Younkin, L, Selkoe, D, and Younkin, S (1992). Processing of the amyloid protein precursor to potentially amyloidogenic fragments. *Science*, **255**, 728-730.

Goode, L and Feinstein, S (1994). Identification of a novel microtubule binding and assembly domain in the developmentally regulated inter-repeat region of tau. *Journal of Cellular Biology*, **124**, 769-782.

Goslin, K and Banker, G (1991). In *Culturing Nerve Cells,* eds. Banker, G and Goslin, K. Cambridge, MA: MIT Press, 251-281.

Grundke-Iqbal, I, Iqbal, K, Tung, Y, Quinlan, M, Wisniewski, H, and Binder, L (1986). Abnormal phosphorylation of the microtubule associated protein τ (tau) in Alzheimer cytoskeletal pathology. *Proc. Natl. Acad. Sci. USA*, **83**, 4913-4917.

Haass, C, Schlossmacher, M, Hung, A, Vigo-Pelfrey, C, Mellon, A, Ostaszewski, B, Lieberburg, I, Koo, E, Schenk, D, Teplow, D, and Selkoe, D (1992). Amyloid β-peptide is produced by cultured cells during normal metabolism. *Nature*, **359**, 322-326.

Haass, C, Hung, A, Selkoe, D, and Teplow, D (1994). Mutations associated with a locus for familial Alzheimer's Disease result in alternative processing of Amyloid β-protein precursor. *Journal of Biological Chemistry*, **269**, 17741-17748.

Haass, C, Lemere, C, Capell, A, Citron, M, Seubert, P, Schenk, D, Lannfelt, L, and Selkoe, D (1995). The Swedish mutation causes early-onset Alzheimer's disease by β-secretase cleavage within the secretory pathway. *Nature Medicine*, **1**, 1291-1296.

Hanemaaijer, R and Ginzburg, I (1991). Involvement of mature tau isoforms in the stabilization of neurites in PC12 cells. *Journal of Neuroscience Research*, **30**, 163-171.

Harris, K, Oyler, G, Doolittle, G, Vincent, I, Lehman, R, Kincaid, R, and Billingsley, M (1993). Okadaic acid induces hyperphosphorylated forms of tau protein in human brain slices. *Annals of Neurology*, **33**, 77-87.

Hirokawa, N, Shiomura, Y, and Okabe, S (1988). Tau proteins: the molecular structure and mode of binding on microtubules. *Journal of Cell Biology*, **107**, 1449-1459.

Ihara, Y, Nukina, N, Miura, R, and Ogawara, M (1986). Phosphorylated tau protein is integrated into paired helical filaments in Alzheimer's disease. *Journal of Biochemistry*, **99**, 1807-1810.

Iqbal, K, Grundke-Iqbal, I, Zaidi, T, Merz, P, Wen, G, Saikh, S, Wisniewski, H, Alafazuff, I, and Winblad, B (1986). Defective brain microtubule assembly in Alzheimer's disease. *Lancet*, **2**, 421-426.

Iqbal, K, Grundke-Iqbal, I, Smith, A, George, L, Tung, Y, and Zaidi, T (1989). Identification and localization of a tau peptide to paired helical filaments of Alzheimer's disease. *Proc. Natl. Acad. Sci. USA*, **86**, 5646-5650.

Iverfeldt, K, Walaas, S, and Greengard, P (1993). Altered processing of Alzheimer amyloid precursor protein in response to neuronal degeneration. *Proc. Natl. Acad. Sci. USA*, **90**, 4146-4150.

Johnson, S, McNeill, T, Cordell, B, and Finch, C (1990). Relation of neuronal APP-751/APP-695 mRNA ratio and neuritic plaque density in Alzheimer's disease. *Science*, **248**, 854-857.

Kaltschmidt, B, Uherek, M, Volk, B, Baeuerle, P, Kaltschmidt, C (1997). Transcription factor NF-κB is activated in primary neurons by amyloid β peptides and in neurons surrounding early plaques from patients with Alzheimer disease. *Proc. Natl. Acad. Sci. USA*, **94**, 2642-2647.

Kang, J, Lemaire, H, Unterbeck, A, Salbaum, J, Masters, C, Grzeschik, K, Multhaup, G, Beyreuther, K, and Muller-Hill, B (1987). The precursor of Alzheimer's disease amyloid A4 protein resembles a cell surface receptor. *Nature*, **325**, 733-736.

Khatoon, S, Grundke-Iqbal, I, and Iqbal, K (1992). Brain levels of microtubule-associated protein τ are elevated in Alzheimer's disease: A radioimmuno-slot-blot assay for nanograms of the protein. *Journal of Neurochemistry*, **59**, 750-753.

Kidd, M (1963). Paired helical filaments in electron microscopy of Alzheimer's disease. *Nature*, **197**, 192-193.

Kitaguchi, N, Takahashi, Y, Tokushima, Y, Shiojiri, S, and Ito, H (1988). Novel precursor of Alzheimer's disease amyloid protein shows protease inhibitory activity. *Nature*, **331**, 530-532.

Koo, E, Park, L, and Selkoe, D (1993). Amyloid β-protein as a substrate interacts with extracellular matrix to promote neuritic outgrowth. *Proc. Natl. Acad. Sci. USA*, **90**, 4748-4752.

Kosik, K, Orecchio, L, Binder, L, Trojanowski, J, Lee, V, and Lee, G (1988). Epitopes that span the tau molecule are shared with paired helical filaments. *Neuron*, **1**, 817-825.

Kosik, K, Orecchio, L, Bakalis, S, and Neve, R (1989). Developmentally regulated expression of specific tau sequences. *Neuron*, **2**, 1389-1397.

Kosik, K (1993). Alzheimer's disease: a cell biological perspective. *Science*, **256**, 780-783.

Lamb, B, Sisodia, S, Lawler, A, Slunt, H, Kitt, C, Kearns, W, Pearson, P, Price, D, and Gearhart, J (1993). Introduction and expression of the 400 kilobase precursor amyloid protein gene in transgenic mice. *Nature Genetics*, **5**, 22-30.

Le, W, Xie, W, Okot, N, Ho, B, Smith, R, and Appel, S (1995). β-amyloid$_{1-40}$ increases expression of β-amyloid precursor protein in neuronal hybrid cells. *Journal of Neurochemistry*, **65**, 2373-2376.

Mandelkow, E, Drewes, G, Biernat, J, Gustke, N, Van Lint, J, Vandenheede, J, and Mandelkow, E (1992). Glycogen synthase kinase 3 and the Alzheimer's disease-like state of microtubule-associated protein tau. *FEBS Letters*, **314**, 315-321.

Mandelkow, E, Biernat, J, Drewes, G, Gustke, N, Trinczek, B, and Mandelkow, E (1995). Tau domains, phosphorylation, and interactions with microtubules. *Neurobiology of Aging*, **16**, 355-362.

Mann, D and Esiri, M (1989). The pattern of acquisition of plaques and tangles in the brains of patients under 50 years of age with Down's syndrome. *Journal of Neurological Science*, **89**, 169-179.

Martin, B, Schrader-Fischer, G, Busciglio, J, Duke, M, Paganetti, P, and Yankner, B (1995). Intracellular accumulation of β-amyloid in cells expressing the Swedish mutant amyloid precursor protein. *Journal of Biological Chemsitry*, **270**, 26727-26730.

Mattson, M, Culwell, A, Esch, F, Lieberburg, I, and Rydel, R (1993). Evidence for neuroprotective and intraneuronal calcium-regulating roles for secreted forms of the β-amyloid precursor protein. *Neuron*, **10**, 243-254.

Milward, E, Papadopoulos, R, Fuller, S, Moir, R, Small, D, Beyreuther, K, and Masters, C (1992). The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth. *Neuron*, **9**, 129-137.

Murphy, G, Eng, L, Ellis, W, Perry, G, Meissner, L, and Tinklenberg, J (1990). Antigenic profile of plaques and neurofibrillary tangles in the amygdala in Down's syndrome: a comparison with Alzheimer's disease. *Brain Research*, **537**, 102-108.

Neve, R, Harris, P, Kosik, K, Kurnit, D, and Donlon, T (1986). Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. *Brain Research*, **387**, 271-280.

Neve, R, Finch, E, and Dawes, L (1988). Expression of the Alzheimer amyloid precursor gene transcripts in the human brain. *Neuron*, **1**, 669-677.

Novák, M, Kabat, J, and Wischik, C (1993). Molecular characterization of the minimal protease resistant tau unit of the Alzheimer's disease paired helical filament. *The EMBO Journal*, **12**, 365-370.

Oleson, O (1994). Proteolytic degradation of microtubule associated protein tau by thrombin in vitro. *Biochemical and Biophysical Research Communications*, **201**, 716-721.

Oster-Granite, M, McPhie, D, Greenan, J, and Neve, R (1996). Age-dependent neuronal and synaptic degeneration in mice transgenic for the C terminus of the amyloid precursor protein. *Journal of Neuroscience*, **16**, 6732-6741.

Oyama, F, Shimada, H, Oyama, R, Titani, K, and Ihara, Y (1992). A novel correlation between the levels of β-amyloid protein precursor and τ transcripts in the aged human brain. *Journal of Neurochemistry*, **59**, 1117-1125.

Oyama, F, Cairns, N, Shimada, H, Oyama, R, Titani, K, and Ihara, Y (1994). Down's syndrome: Up-regulation of β-amyloid protein precursor and mRNAs and their defective coordination. *Journal of Neurochemistry*, **62**, 1062-1066.

Palmert, M, Golde, T, Cohen, M, Kovaks, D, Tanzi, R, Gusella, J, Usiak, M, Younkin, L, and Younkin, S (1988). Amyloid protein precursor messenger RNAs: differential expression in Alzheimer's disease. *Science*, **241**, 1080-1084.

Pena, L, Brecher, C, and Marshak, D (1995). β-Amyloid regulates gene expression of glial trophic substance S100 β in C6 glioma and primary astrocyte cultures. *Brain Research: Molecular Brain Research*, **34**, 118-126.

Pike, C, Walencewicz, A, Glabe, C, and Cotman, C (1991). In vitro aging of β-amyloid protein causes peptide aggregation and neurotoxicity. *Brain Research*, **563**, 311-314.

Ponte, P, Gonzalez-De Whitt, P, Schilling, J, Miller, J, Hsu, D, Greenberg, B, Davis, K, Wallace, W, Liederburg, I, Fuller, F, and Cordell, B (1988). A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. *Nature*, **331**, 525-527.

Qiu, W, Ferreira, A, Miller, C, Koo, E, and Selkoe, D (1995). Cell-surface β-amyloid precursor protein stimulates neurite outgrowth of hippocampal neurons in an isoform-dependent manner. *Journal of Neuroscience*, **15**, 2157-2167.

Saitoh, T, Sunsdmo, M, Roch, J, Kimura, N, Cole, G, Schubert, D, Oltersdorf, T, and Schenk, D (1989). Secreted form of amyloid β protein precursor is involved in the growth regulation of fibroblasts. *Cell*, **58**, 615-622.

Schubert, D, Jin, L, Saitoh, T, and Cole, G (1989). The regulation of amyloid β protein precursor secretion and its modulatory role in cell adhesion. *Neuron*, **3**, 689-694.

Seubert, P, Vigo-Pelfrey, C, Esch, F, Lee, M, Dovey, H, Davis, D, Sinha, S, Scholossmacher, M, Whaley, J, Swindlehurst, C, McCormack, R, Wolfert, R, Selkoe, D, Lieberburg, I, and Schenk, D (1992). Isolation and quantitation of soluble Alzheimer's β-peptide from biological fluids. *Nature*, **359**, 325-327.

Sisodia, S, Koo, E, Bayreuther, K, Unterbeck, A, and Price, D (1990). Evidence that β-amyloid protein in Alzheimer's disease is not derived by normal processing. *Science*, **248**, 492-495.

Solá, C, García-Ladona, F, Mengod, G, Probst, A, Frey, P, and Palacios, J (1993). Increased levels of the Kunitz-protease inhibitor-containing βAPP mRNAs in rat brain following neurotoxic damage. *Brain Research: Molecular Brain Research*, **17**, 41-52.

Suzuki, N, Cheung, T, Cai, X, Odaka, A, Otvos, L, Eckman, C, Golde, T, and Younkin, S (1994). An increased percentage of long amyloid β protein secreted by familial amyloid β protein precursor (βAPP717) mutants. *Science*, **264**, 1336-1340.

Tanaka, S, Nakamura, S, Ueda, K, Kameyana, M, Shiojiri, S, Takahashi, Y, Kitaguchi, N, and Ito, H (1988). Three types of amyloid protein precursor mRNA in human brain: their differential expression in Alzheimer's disease. *Biochemical and Biophysical Research Communications*, **157**, 472-479.

Tanaka, S, Shiojiri, S, Takahashi, Y, Kitaguchi, N, Ito, H, Kameyama, M, Kimura, J, Nakamura, S, and Ueda, K (1989). Tissue-specific expression of three types of β-protein precursor mRNA: enhancement of protease inhibitor-harboring types in Alzheimer's disease brains. *Biochemical and Biophysical Research Communications*, **165**, 1406-1414.

Turner, R, Nobuhiro, S, Abraham, S, Younkin, S, Lee, V (1996). Amyloids β_{40} and β_{42} are generated intracellularly in cultured human neurons and their secretion increases with maturation. *Journal of Biological Chemistry*, **271**, 8966-8970.

Uéda, K, Masliah, E, Saitoh, T, Bakalis, S, Scoble, H, and Kosik, K (1990). Alz-50 recognizes a phosphorylated epitope of tau protein. *Journal of Neuroscience*, **10**, 3295-3304.

Van Nostrand, W, Wagner, S, Suzuki, M, Ghoi, B, Farrow, J, Geddes, J, Cotman, C, and Cunningham, D (1989). Protease nexin-II, a potent antichymotrypsin, shows identity to amyloid beta-protein precursor. *Nature*, **341**, 546-549.

Wang, X, An, S, and Wu, J (1996). Specific processing of native and phosphorylated tau protein by proteases. *Biochemical and Biophysical Research Communications*, **219**, 591-597.

Weingarten, M, Lockwood, A, Huo, S, and Kirschner, M (1975). A protein of mammalian brain and its relation to microtubule assembly. *Proc. Natl. Acad. Sci. USA*, **72**, 1858-1862.

Wischik, C, Novák, M, Thøgersen, H, Edwards, P, Runswick, M, Jakes, R, Walker, J, Milstein, C, Roth, M, and Klug, A (1988). Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer's disease. *Proc. Natl. Acad. Sci. USA*, **85**, 4506-4510.

Yamamoto, H, Saitoh, Y, Fukunaga, K, Nishimura, H, and Miyamoto, E (1988). Dephosphorylation of microtubule proteins by brain protein phosphatases 1 and 2A, and its effect on microtubule assembly. *Journal of Neurochemistry*, **50**, 1614-1623.

Yankner, B, Dawes, L, Fisher, S, Villa-Komaroff, L, Oster-Granite, M, and Neve, R (1989). Neurotoxicity of a fragment of the amyloid precursor associated with Alzheimer's disease. *Science*, **245**, 417-420.

Yankner, B, Duffy, L, and Kirschner, D (1990). Neurotrophic and neurotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides. *Science*, **250**, 279-282.

Yoshikawa, K, Aizawa, T, and Hayashi, Y (1992). Degeneration *in vitro* of post-mitotic neurons overexpressing the Alzheimer amyloid protein precursor. *Nature*, **359**, 64-67.