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Apoptosis in Human CNS Neurons: Effect on Immediate Early Gene Expression and Key Neuron-Specific Proteins

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science
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May 1997

Abstract

The processes underlying the neurodegeneration in Alzheimer's disease (AD) are unknown. Apoptosis has been proposed as the mechanism of neuronal cell death, while altered levels of amyloid precursor protein (APP) and the amyloid β peptide, a proteolytic cleavage product of APP, are believed to contribute to the neurodegeneration in AD. Thus, defining the molecular mechanisms regulating these events are of interest. The expression of the immediate early genes (IEG), in particular *c-jun*, has been shown to be a requirement for neuronal apoptosis. Due to the presence of activator protein-1 (AP-1) consensus sequences in the promoter of the APP gene, which has been shown to be responsive to regulation by immediate early genes, we set to out determine whether expression of the immediate early genes resulted in a correlated increase in the expression of APP during neuronal apoptosis. Apoptosis was induced in highly purified human primary neuron cultures by serum deprivation and characterized by propidium iodide and TUNEL (terminal transferase mediated dUTP nick end labeling) staining to detect condensed chromatin and fragmented nuclei, distinct morphological features of apoptotic cells. Immediate early gene, *c-fos* and *c-jun*, expression was assessed at the transcriptional level by RT-PCR and immunoblotting at the protein level. We observed a transient induction of *c-fos* and *c-jun* mRNA, and c-Jun protein in neurons dying by apoptosis. There was slight increase in APP levels, however, no statistically significant changes were detected. Our results suggest the immediate early genes possibly mediate the underlying molecular mechanisms of apoptosis in human neurons *in vitro* and *in vivo*. Furthermore, we speculate the increase in Aβ peptide production previously observed in serum deprived primary human neuron cultures may not entirely result from an overexpression of APP, but from the altered metabolism of APP in primary human neuron cultures dying by apoptosis.

Alterations and proteolysis of the cytoskeletal protein tau occur during the pathogenesis of AD and result in the formation of neurofibrillary tangles (NFTs) found in the AD brain. Additionally, the expression levels of the neuron-specific proteins GAP-43 and synaptophysin are increased and decreased, respectively, and associated with neuritic alterations and loss of synapses in AD. Since apoptosis can lead to cytoskeletal

alterations and protein proteolysis, the proteolytic degradation and expression levels of tau, GAP-43 and synaptophysin were analyzed by immunoblots. We detected the appearance of degradation products and an increase in the expression of tau protein during serum deprivation-induced apoptosis. Furthermore, we observed an increase in the expression of GAP-43 and a decrease in the expression of synaptophysin. Taken together, our results further support the contribution of apoptosis to the neurodegeneration associated with AD, since human neurons in culture induced to undergo apoptosis developed features similar to those found in the AD brain. Overall, our studies substantiate the use of human primary neuron cultures as an *in vitro* model of human central nervous system (CNS) neuronal cell death.

Résumé

Les processus sous-jacents à la neurodégénération associée à la maladie d'Alzheimer (AD) sont inconnus. L'apoptose a été proposée comme mécanisme de mort des cellules neuronales, tandis qu'on croit que des niveaux anormaux des précurseurs des protéines amyloïdes (PPA) et des peptides ß-amyloïdes, qui sont des produits du clivage protéolytique des PPA contribuent à la neurodégénération associée à l'AD. Ainsi, il est intéressant de définir les mécanismes moléculaires contrôlants ces événements. Il a été démontré que l'expression des gènes précoces immédiats (GPI), en particulier *c-jun*, sont nécessaire pour l'apoptose neuronale. En raison de la présence de la séquence consensus de la protéine d'activation-1 (AP-1) dans le promoteur du gène PPA, qui est connu pour répondre à la régulation par les gènes précoces immédiats, nous voulions déterminer si l'expression des gènes précoces immédiats entraînait une augmentation correspondante de l'expression de PPA durant l'apoptose neuronale. L'apoptose a été induite sur des cultures de neurones humaines primaires hautement purifiées par privation en sérum et caractérisation par iodure de propidium et coloration TUNEL (terminal transferase mediated dUTP nick end labeling) pour détecter la chromatine condensée et les noyaux fragmentés, qui sont des caractéristiques morphologiques précises des cellules en apoptose. L'expression des gènes précoces immédiats *c-fos* et *c-jun* a été déterminée au niveau transcriptionnel par RT-PCR et au niveau des protéines par immuno-hybridation. Nous avons observé une induction transitoire de l'ARNm de *c-jun* et *c-fos* et de la protéine c-Jun dans les neurones mourant par apoptose. Il y avait une légère augmentation des niveaux de PPA, cependant, aucun changement statistiquement significatif n'a été détecté. Nos résultats suggèrent que les gènes précoces immédiats sont possiblement impliqués dans les mécanismes moléculaires sous-jacents dans l'apoptose des neurones humaines *in vitro* et *in vivo*. De plus, nous pensons que l'augmentation de la production de peptides ß-amyloïdes précédemment observée dans les cultures de neurones humaines primaires privées de sérum ne soit pas entièrement reliée à la surexpression de PPA, mais

à un métabolisme anormal de PPA dans les cultures de neurones humaines primaires mourant d'apoptose.

Il y a des modifications et protéolyse de la protéine cytosquelettique tau durant la pathogénèse de l'AD ce qui entraîne la formation d'enchevêtrements neurofibrillaires retrouvés dans les cerveaux de l'AD. En outre, les niveaux d'expression des protéines spécifiques aux neurones GAP-43 et synaptophysine augmentent et diminuent respectivement, et sont associés à des modifications neuronales et à des pertes de synapses dans l'AD. Comme l'apoptose peut provoquer des modifications du cytosquelette et la protéolyse des protéines, nous avons analysé par immuno-hybridation la dégradation protéolytique et les niveaux d'expression de tau, GAP-43 et synaptophysine. Nous avons décelé l'apparition de produits de dégradation et une augmentation de l'expression de la protéine tau durant l'apoptose induite par une privation en sérum. De plus, nous avons observé une augmentation de l'expression de GAP-43 et une diminution de l'expression de synaptophysine. Comme les neurones humaines en culture qui ont été entraînées vers l'apoptose ont développé des caractéristiques similaires à celles retrouvées dans les cerveaux d'AD, nos résultats, considérés conjointement, appuient donc la contribution de l'apoptose à la neurodégénération associée à l'AD. Finalement, nos études justifient l'usage de cultures de neurones humaines primaires en tant que modèle *in vitro* de la mort de cellules neuronales du système nerveux central (SNC).

Acknowledgments

I would very much like to thank Dr. Andrea LeBlanc for giving me the opportunity to pursue my graduate studies in her laboratory. I would also like to thank Dr. LeBlanc for the financial support and guidance for me to complete my studies. Special thanks to Dr. Cynthia Goodyer who helped us obtain the brain tissue for our studies. Many thanks to Caroline Belair and Maria Papadopoulos for their friendship and the good times over the past few years. I will definitely miss you both. I would also like to thank Maria for her assistance with the sequence analysis for the present study. I would also like to express my gratitude towards Maria Koutramanis, Helene Douillard and Eric Paradis for their technical assistance.

To my colleagues and friends at the Bloomfield Centre for Research in Aging, and at the Lady Davis Institute, thank you for answering my many questions, providing me with valuable advice and your friendship. Special thanks to Nicolas Morin for translating my abstract.

I would like to express my sincere thanks to my dear friends in Toronto who have given me much support during my studies in Montreal. To Nathalie Richard I am indebted. Thank you for your love, encouragement and patience these past few years.

To my parents and brother, thank you for all your patience, support and love through all of my studies.

Table of Contents

List of Figures

Abbreviations

A68	Paired helical filament tau
Aβ	Amyloid β protein
AD	Alzheimer's disease
Ala	Alanine
ALS	Amyotrophic lateral sclerosis
AP-1	Activator protein complex-1
APO E	Apoplipoprotein E
AP buffer	Alkaline phosphatase buffer
APP	Amyloid precursor protein
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BCS	Bovine calf serum
BDNF	Brain-derived neurotrophic factor
BH1	Bcl-2 homology 1 domain
BH2	Bcl-2 homology 2 domain
BH3	Bcl-2 homology 3 domain
bp	Base pairs
CAT	Chloramphenicol acetyl transferase
cDNA	Complementary deoxyribonucleic acid
CNTF	Ciliary neurotrophic factor
CNS	Central nervous system
dBCS	Decomplemented bovine calf serum
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
DNA	Deoxyribonucleic acid

DNase I	Deoxyribonuclease I
dNTP	Deoxyribonucleoside triphosphate
EtBr	Ethidium bromide
FAD	Familial Alzheimer's disease
FdU	Fluorodeoxyuridine
FITC	Fluorescein isothiocynate
G3PDH	Glyceraldehyde 3-phosphate dehydrogenase
GAGs	Glycosaminoglycans
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acid protein
Glu	Glutamic acid
Gly	Glycine
h	Hours
HD	Huntington's disease
ICE	Interleukin-1β-converting enzyme
IEG	Immediate early gene
Ile	Isoleucine
IRP	ICE-related protease
kDa	Kilodalton
KPI	Kunitz serine protease inhibitor
LDH	Lactate dehydrogenase
Lys	Lysine
mRNA	Messenger ribonucleic acid
MAP2	Microtubule-associated protein 2
MAPs	Microtubule-associated proteins
Met	Methionine
NBT	Nitro blue tetrazolium chloride
NF-H	High molecular weight neurofilament protein
NF-L	Low molecular weight neurofilament protein
NF-M	Medium molecular weight neurofilament protein

NGF	Nerve growth factor
nm	Nanometers
NP-40	Nonidet P-40
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PC12	Pheochromocytoma cells
PCR	Polymerase chain reaction
PHFs	Paired helical filaments
PHF-τ	Paired helical filament tau
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PNS	Peripheral nervous system
prICE	Protein resembling ICE
PS-I	Presenilin I
PS-II	Presenilin II
RNase A	Ribonuclease A
RNA	Ribonucleic acid
SCG	Superior cervical ganglion
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPs	Senile plaques
TBS-T	Tris-buffered saline with Tween-20
TUNEL	Terminal transferase-mediated dUTP nick end labeling
Tyr	Tyrosine
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
μg	Micrograms
μl	Microlitres
Val	Valine

I Introduction

In recent years apoptosis has received much attention in the areas of cellular and molecular biology because of its growing relevance in human disease. Defining the genes and the molecular events controlling the death of neurons, post-mitotic and long lived cells, has become of particular importance in the area of neurodegenerative diseases. The etiology of Alzheimer's disease (AD) is unclear, however, apoptosis is suspected to be the mode of neuronal cell death and degeneration in the development of AD. Studies have characterized some of the molecular events of neuronal apoptosis through the use of neuronal cell lines and primary rodent neuron cultures, however, they may not be entirely reflective of processes occurring in human cells. The present study utilizes a unique *in vitro* system of human primary neuron cultures to investigate the underlying molecular events of apoptosis in human neurons and to evaluate the effect of apoptosis on proteins known to be altered in the pathogenesis of AD.

1. Apoptosis

Apoptosis was first proposed by Kerr, Wyllie and Currie in 1972 to describe the phenomenon of naturally occurring cell death within a multicellular organism and is the normal physiologic process of cell deletion during the development and maintenance of an organism. In contrast, necrosis, another form of cell death, is caused by an acute metabolic or mechanical insult to the cell that is typically characterized by cell swelling, the loss of mitochondrial and plasma membrane integrity, and the release of cellular and lysosomal content that results in pathophysiological inflammatory responses (reviewed by Tomei and Cope, 1991). Apoptosis, on the other hand, is a regulated form of cell death in which the cell activates an intrinsic cell suicide program (reviewed by Tomei and Cope, 1991, 1994; reviewed by Bredesen, 1995) and is characterized by distinct changes in cellular morphology - the cells shrink, the plasma membrane blebs, the chromatin condenses, the nucleus collapses, and fragments of the cell break away to form

apoptotic bodies, while the integrity of the plasma and organelle membranes is preserved (reviewed by Tomei and Cope, 1991, 1994; reviewed by Bredesen, 1995). Neighbouring cells remove the apoptotic cells by phagocytosis in the absence of an inflammatory response (reviewed Bredesen, 1995). Biochemically, apoptosis is characterized by the internucleosomal cleavage of DNA by nucleases into large (100 to 300 kilobases) and subsequently into smaller DNA fragments that are visualized as a DNA ladder following gel electrophoresis (reviewed by Tomei and Cope, 1991, 1994; reviewed by Bredesen, 1995; reviewed by Steller, 1995). However, apoptosis has been found to occur in the absence of DNA fragmentation in some cells (reviewed by Bredesen, 1995). Numerous models have demonstrated that macromolecule synthesis is a requirement for apoptosis to occur (reviewed by Tomei and Cope, 1994), however, other models have illustrated that apoptosis can occur in the absence of a nucleus and macromolecule synthesis (reviewed by Bredesen, 1995). The consensus is that all cells contain the basic cell death machinery necessary to carry out apoptosis, which is constituitively repressed for cell survival (reviewed by Raff, 1992; reviewed by Steller, 1995). This implies that in certain cell types the synthesis of macromolecules represents a need for certain components to activate or derepress the existing cell death machinery. Thus, the means by which the apoptotic process is regulated varies from cell type to cell type and tissue to tissue.

1.1 Phases of Apoptosis

Essentially all mammalian cells proceed through a series of regulated and temporally conserved phases during apoptosis. These phases are: 1) Receipt of the apoptotic signal and initiation the apoptotic cascade; 2) Commitment to cell death; 3) Execution of the cell death program, and 4) Elimination (reviewed by LeBlanc, 1997).

Diverse signals originating from the extracellular and intracellular milieu have been shown to induce apoptosis in cells. Inducers of apoptosis include growth factor deprivation, glucocorticoids, loss of extracellular matrix and cell to cell interaction, viral

infection, ionizing radiation, free radicals, neurotransmitters, hormones and chemotherapeutic drugs (reviewed by Thompson, 1995; reviewed by Stellar, 1995; reviewed by LeBlanc, 1996). The effect of the apoptotic signal is cell-type dependent and also determined by the functional state of the cell. Mitochondrial function is also believed to regulate apoptosis. The integrity of the mitochondrion determines whether a specific signal will trigger a cell to undergo apoptosis or necrosis (Ankarcrona et al., 1995). Additionally, the magnitude of the apoptotic signal plays role in determining whether a cell undergoes apoptosis or necrosis (Bonfoco et al., 1995; reviewed by Dragunow and Preston, 1995).

The initiation phase transduces the death signal and sets into motion the events leading to cell death. In certain cell types, RNA and protein synthesis may be required to synthesize molecules that derepress or activate the existing cell death machinery, while in other types, macromolecule synthesis is not required (Jacobson et al., 1994). A number of signal transduction molecules are activated during apoptosis, most notably are the proto-oncogenes *c-myc*, *c-fos* and *c-jun*. The Fos/Jun family of proteins are inducible transcription factors that function cooperatively during signal transduction processes. Induction and sustained expression of *fos* and *jun* (reviewed by Dragunow and Preston, 1995) during the initiation phase may activate the expression of specific target genes whose products play a primary role in the apoptotic process (reviewed by Soares et al., 1994; reviewed by LeBlanc, 1997).

The commitment phase represents the time in which a cell has become irreversibly committed to cell death, even after removal of the death signal or the introduction of rescuing agents such protein and RNA synthesis inhibitors. The time of commitment is cell type specific, varies with cell age and with the apoptotic stimulus within the same cell type (Deckworth and Johnson, 1993; Edwards and Tolkovsky, 1994; reviewed by White, 1996; reviewed by LeBlanc, 1997). Execution is accomplished by

the effectors of the cell death machinery, after which the dead cells are eliminated by phagocytosis.

Each of these phases is tightly regulated and an understanding of the regulatory processes is evolving. Several of the vital regulatory and effector components of the apoptotic cascade have been identified and appear to have been conserved among species and cell types.

1.2 Regulators of Apoptosis

Numerous proteins modulate the process of apoptosis, such as c-myc, transcription factors and cell cycle control proteins, and p53 (reviewed by White, 1996), but none play a more central role than the Bcl-2 family of cell death regulators. The Bcl-2 family consists of death-suppressing homologues, Bcl-2 and Bcl-x_L (Boise et al., 1993), and death-accelerating homologues, Bax (Oltvai et al., 1993), Bcl-x_s (Boise et al., 1993), Bad (Yang et al., 1995), and Bak (Farrow et al., 1995; Chittenden et al., 1995a; Kiefer, 1995). Bcl-2 represses apoptosis through its ability to heterodimerize Bax, thereby preventing the formation of Bax homodimers and cell death (Oltvai et al., 1993). This dimerization is mediated by single amino acids within the Bcl-2 homology 1 (BH1) and Bcl-2 homology 2 (BH2) domains (Yin et al., 1994). Bcl-x_L , presumably like Bcl-2, represses apoptosis through its ability to heterodimerize with Bax via the BH1 and BH2 domains (Sedlak et al., 1995; Yang et al., 1995). The activity of Bcl-x_L to repress apoptosis is antagonized by Bcl-x_S (Boise et al., 1993). Bad interacts with Bcl-2 and more strongly with Bcl-x_L, and promotes apoptosis by displacing Bax from Bcl-2 and Bcl-x_L in vivo (Yang et al., 1995). Bak is also capable of inhibiting the ability of Bcl-2 and Bcl-x_L to block apoptosis (Farrow et al., 1995; Chittenden et al., 1995a; Kiefer et al., 1995). Recently, Han et al. (1996) have shown that a 28 amino acid region of Bax encompassing BH3 is sufficient to interact with Bcl-2 proteins in yeast. Additionally, BH3 of Bak is required for interaction with Bcl-x_L in yeast and in vitro, and is sufficient

to induce apoptosis in mammalian cells (Chittenden et al., 1995b). These results suggest that Bak and Bax may encode a death effector domain in the BH3 region, or the BH3 domain may serve as a binding site to interact with Bcl-2 and Bcl-x_L and neutralize their anti-apoptotic activity (reviewed by White, 1996). Although the specific biochemical functions of the Bcl-2 family of proteins have not been determined, it is apparent that the complexity of the cell death pathway will increase as new homologues are identified. The set point of susceptibility to apoptosis is determined by the relative ratios and a complex set of protein-protein interactions among the Bcl-2 family of apoptosis regulators.

1.3 Effectors of Apoptosis

Regardless of the activation signal, the various apoptotic pathways converge to common executioners of the cell death cascade, which act to kill the cell by dismantling the cytoskeletal and nuclear structures. Presently, the interleukin-1β-converting enzyme (ICE) family of proteases and nucleases, acting in concert, are believed to be the effectors of apoptosis (reviewed by Takahashi and Earnshaw, 1996).

1.3.1 ICE Family of Proteases

The execution of apoptosis involves numerous enzymatic pathways, all of which appear to be triggered by the activation of one or more ICE-related proteases (IRPs). The evidence that proteases may be centrally involved in the cell death process came initially from the studies by Yuan et al. (1993) in which the *Caenorhabditis elegans* cell death gene, *ced-3*, was cloned and found to exhibit 29% homology with ICE. Subsequently, apoptosis was induced in fibroblasts ectopically expressing ICE, suggesting that ICE is structurally and functionally homologous to *ced-3* (Miura et al., 1993). Presently, nine IRPs have been identified and overexpression of each of these proteases in various cell types results in apoptosis. These are: ICE, Ced-3, Nedd-2/Ich-1 (Kumar et al., 1994;

Wang et al., 1994), TX/ICErel II/Ich-2 (Kumar et al., 1994; Munday et al., 1995; Kamens et al., 1995), ICErelIII (Munday et al., 1995), CPP32/YAMA (Fernandes-Alnemri et al., 1994; Tewari et al., 1995), Mch 2 (Fernandes-Alnemri et al., 1995a), Mch 3/ICE-LAP 3 (Fernandes-Alnemri et al., 1995b; Duan et al., 1996), Mch 4 (Fernandes-Alnemri et al., 1996), and FLICE (Muzio et al., 1996). Collectively, IRPs are now classified as the caspases (Alnemri et al., 1996).

1.3.1.1 Substrates of Caspases

The caspases play a central role in the apoptotic cascade, however, the identities of the critical substrates, whose cleavage result in the dramatic morphological changes during apoptosis, remain to be elucidated. Several substrates, whose proteolytic cleavage is suspected to contribute towards the apoptotic cascade, have been identified. Poly(ADP-ribose) polymerase (PARP) was the first substrate identified (Lazebnik et al., 1994) and its cleavage inhibits most of its DNA repair activity (Kaufmann et al., 1993), which may contribute to the chromosomal degradation during apoptosis.

Some of the most characteristic changes during apoptosis affect the nucleus. Cleavage of the nuclear lamins A, B1/B2, and C; intermediate filament proteins which form the meshwork that provide the structural support of the inner nuclear membrane, by a caspase results in the disassembly of the nuclear lamina and the collapse of the chromatin into discrete blobs (Lazebnik et al., 1995).

The morphological changes in the plasma membrane during apoptosis are due to cytoskeletal rearrangements in the cell. Actin, a ubiquitous cytoskeletal protein of eukaryotic cells, is believed to be a substrate of caspases (reviewed by Bredesen, 1995). Actin is also indirectly modified by the cleavage of Gas2; a regulator of actin structure in the cell (Brancolini et al., 1995). The cleavage of Gas2 by a caspase has provided the first link to the mechanism of cytoskeletal alterations in the cell during the activation of the apoptotic cascade.

Apoptosis is recognized as a mode of cell death in numerous diseases (reviewed by Thompson, 1995). Recently, Goldberg et al. (1996) have identified the product of the Huntington's disease gene, huntingtin, as a substrate of apopain, a caspase. This finding provides insight into the mechanism of apoptosis associated with Huntington's disease. Identification of other key caspase substrates associated with a disease, in which apoptosis is suspected to be the mode of cell death, would provide a better understanding into the pathogenesis of the disease and indicate potential therapeutic targets. Presently, other constituents of the cytoskeleton or other disease-specific proteins have not been identified as substrates of caspases in apoptosis associated disorders.

1.3.2 Nucleases

It is postulated that the activity of caspases may also contribute to the demise of the cell by activating other death effectors, processing these proteins from the inactive to active state by proteolytic cleavage (reviewed by Martin and Green, 1995). Caspases may also activate the nucleases that cause the DNA fragmentation during apoptosis. Mannherz et al. (1995) propose that the cleavage of actin during the apoptotic cascade releases DNase I from its inhibition by actin, allowing DNase I to cleave the chromatin into oligosomal fragments. This mechanism, in conjunction with the observation that the activities of caspases and nucleases are both required to breakdown the chromatin and nuclear structure during apoptosis (Lazebnik et al., 1995), supports the notion that caspases and nucleases act in concert to execute the demise of the cell.

The apoptotic cascade is a complex pathway that involves many different and diverse cellular factors. Presently, the list of participating and regulatory factors of the apoptotic cascade is growing as the identity and functions of new and known proteins are discovered. Figure 1 summarizes the apoptotic cascade and the known regulatory proteins involved to date.

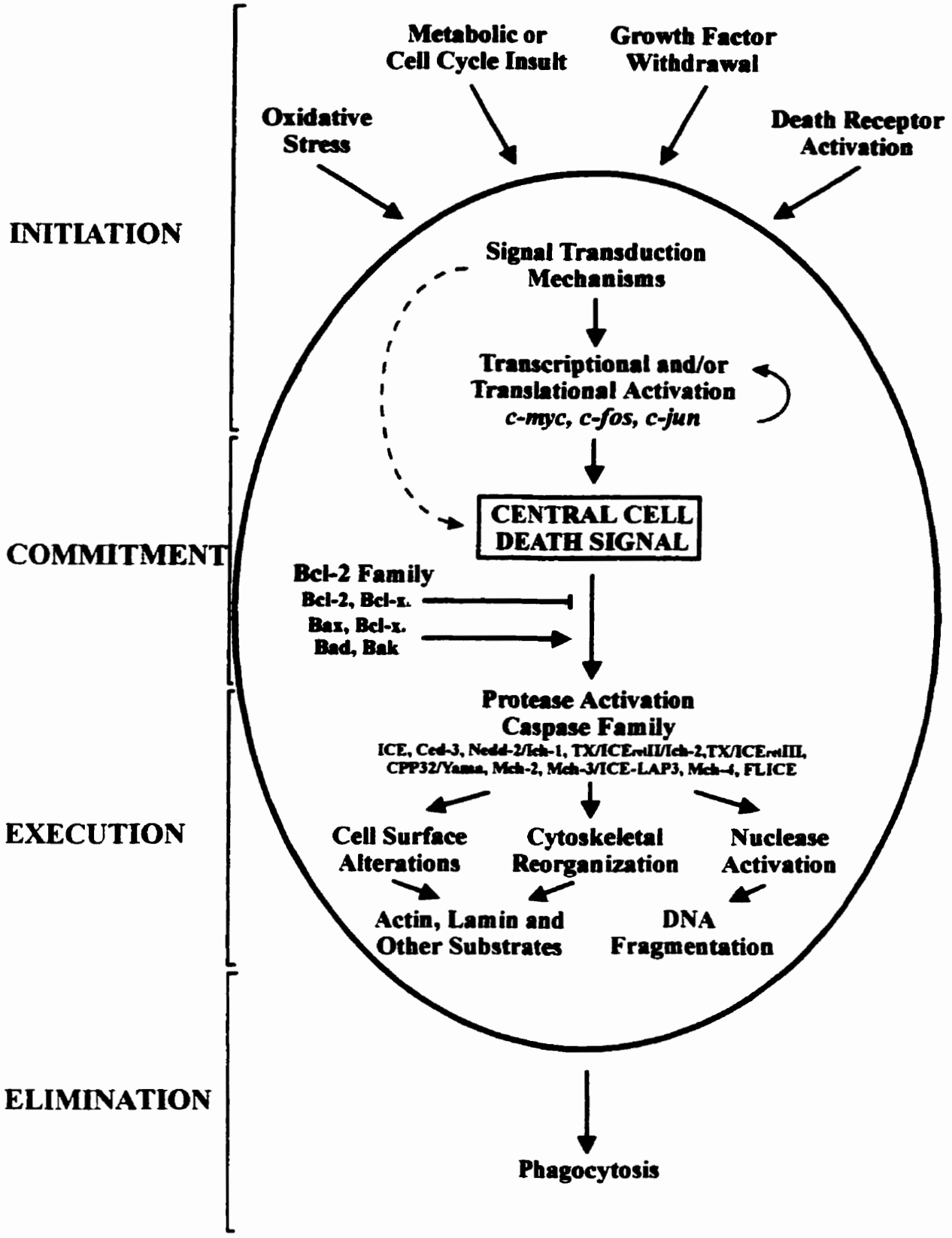


Figure 1. - Model of Apoptosis Regulation

2. Cell Death in the Nervous System

Cell death is a prominent feature in the normal development of the nervous system. During this period there is an overabundance of neurons in comparison to the number of targets that require innervation. Neuronal cell death is a normal process which prunes the initial neuronal population to match the size of the target tissues by removing supernumerary and incorrectly connected cells (Oppenheim, 1991). Approximately half of the initial population of neurons remains once the organism reaches maturity. The death of the neurons during development results from the limited availability of neurotrophic factors (Purves, 1986; Barde, 1989). Numerous studies involving nerve growth factor (Edwards et al., 1994; Deckworth et al., 1993; Silos-Santiago et al., 1995), brain-derived neurotrophic factor (Silos-Santiago et al., 1995), ciliary neurotrophic factor (Silos-Santiago et al., 1995; Johnson, 1994; Lindsay, 1995) and glial cell-derived neurotrophic factor (Henderson et al., 1994; Beck et al., 1995; Oppenheim et al., 1995; Yan et al., 1995) confirm that neuronal survival is dependent on an adequate supply of neurotrophic factors and a lack of these essential factors results in apoptotic cell death. Thus, apoptosis is the physiological mechanism of cell death in the developing nervous system.

In the mature organism programmed cell death occurs naturally to maintain the balance between cell production and loss in the proliferating tissues (Wyllie, 1980; reviewed by Thompson, 1995). The death of long-lived, terminally differentiated, post-mitotic cells, such as neurons, may result from the dysregulation of apoptosis as a homeostatic process and is believed to be associated with the pathological conditions in neurodegenerative disorders like amyotrophic lateral sclerosis (ALS), cerebral ischemia, spinal muscular atrophy, prion diseases, Huntington's disease (HD), Parkinson's disease and Alzheimer's disease (reviewed by Bredesen, 1995). Recent studies provide evidence of apoptotic cell death in models of ischemia (MacManus et al., 1993, 1994; Linnik et al., 1993), *in vitro* models of ALS (Rabizadeh et al., 1995; Greenlund et al., 1995),

Parkinson's disease (Hartley et al., 1994) and HD (Portera-Cailliau et al., 1995), and post-mortem analyses of prion (Fairbairn, et al., 1994), HD (Portera-Cailliau et al., 1995) and AD (Su et al., 1994; Smale et al., 1995; Lassman et al., 1995; Anderson et al., 1996) affected brains. The aberrant neuronal cell death seen in these diseases may be attributed to: **1)** the loss of neurotrophic support that activates an innate death program in the neurons (reviewed by Raff, 1992; reviewed by Raff et al., 1993; reviewed by Barr and Tomei, 1994); **2)** a signal that triggers the neurons to undergo "abortive mitosis"; the avoidance of reentry into the cell cycle (Heintz, 1993; Rubin et al., 1993; reviewed by Tomei and Barr, 1994) or **3)** excitatory toxicity (Choi and Rothman, 1990; reviewed by Lipton, 1994) and oxidative stress (reviewed by Bredesen, 1995). Delineating the mechanism(s) by which apoptosis occurs in the mature nervous system would provide a clearer understanding into the pathogenesis of neurodegenerative diseases.

3. Alzheimer's Disease

Alzheimer's disease (AD) is a common and devastating neurodegenerative disease that primarily affects the aging and elderly population. Clinically, AD is characterized by the progressive loss of memory and cognitive functions resulting in dementia, and presently accounts for about 70% of the observed dementias in the aged. AD is only confirmed by the post-mortem, histological identification of: **1)** a profound loss of neurons, especially the cholinergic neurons in the nucleus basalis of Meynert, hippocampus and neocortex (Davies and Maloney, 1976; Whitehouse et al., 1982; reviewed by Kandel and Schwartz, 1991) **2)** extracellular senile plaques (SPs), composed of a water insoluble core of amyloid β protein (Aβ) surrounded by a diffuse ring of dystrophic neurites, activated astrocytes and microglia (Glenner and Wong, 1984), which are found in the amygdala, hippocampus, cerebral cortex and other areas important for cognitive functions (Katzman, 1986); **3)** neurofibrillary tangles (NFTs) composed of paired helical filaments (PHFs) (Katzman, 1986) along the perforant pathway and the

CA1 region of the hippocampus; **4)** synpase loss and synaptic pathology (reviewed by Terry, 1994) and **5)** amyloid deposits in the cerebral vasculature (Glenner, 1988), in the AD brain.

Alzheimer's disease is typically classified by two criteria: **1)** the age of onset, with early onset cases occurring before the age of 65 and late onset occurring after the age of 65 and **2)** the genetic inheritance of disease termed familial AD (FAD) and sporadic cases of the disease. Only about 10% of all AD cases are classified as early or late onset FAD, while 90% of AD cases are sporadic. AD is a genetically heterogeneous disease. At present four genes have been linked to early and late onset FAD, chromosomes 1, 14, 19 and 21. Linkage of early onset FAD to chromosome 21 was established in FAD pedigrees (<3%) that possessed mutations in the amyloid precursor protein (APP) gene (St. George-Hyslop et al., 1990), which had been previously been localized to chromosome 21 (Kang et al., 1987). The ε4 allele of the apopliprotein E (ApoE) gene on chromosome 19 was linked to late onset FAD and has been deemed as a risk factor in developing AD (Corder et al., 1993; Strittmatter et al., 1993).

Recently two new genes linked with an aggressive form of early onset FAD were identified, *S182* or *presenilin-I (PS-I)* mapped on chromosome 14 (Sherrington et al., 1995) and *STM2* or *presenilin-II (PS-II)* located on chromosome 1 (Levy-Lehad et al., 1995; Rogaev et al., 1995). *PS-I* and *PS-II* encode homologous integral membrane proteins with seven transmembrane domains and a large exposed loop between the sixth and seventh transmembrane domains. To date thirty different missense mutations in *PS-I* cosegregate with early onset FAD (Cruts et al., 1996). These mutations result in amino acid substitutions that are hypothesized to disrupt the insertion of the protein into the membrane and its function. Only two missense mutations in *PS-II* have been identified in affected Volga German AD kindreds. These mutations result in amino acid substitutions Asn141Ile and Met239Val (Levy-Lehad et al., 1995b; Rogaev et al., 1995). The mutation at codon 141 occurs in an amino acid that is conserved in the human and mouse S182

homolog. Since these missense mutations occur in two highly similar genes in AD subjects, it has been proposed that mutations in both genes may be pathogenic.

3.1 The Amyloid Precursor Protein

The Aβ deposited in the senile plaques and in the cerebral vasculature ranges in length from 39-43 amino acids and is a proteolytic cleavage product of the amyloid precursor protein (APP) that is encoded on chromosome 21 (Kang et al., 1987). Alternative splicing produces five APP transcripts and results in three major isoforms of APP (reviewed by LeBlanc, 1994). The most abundant isoform of 695 amino acids (APP_{695}) is expressed highly in neurons (Koo et al., 1990) and lacks a Kunitz-protease inhibitor (KPI) domain present in the 751 and 770 amino acid isoforms (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). There are three pathways in the proteolytic processing of APP: **1) the non-amyloidogenic or secretory pathway** produces a soluble form of APP (sAPP) and a 10kDa C-terminal peptide that can be further processed to yield a 3 kDa non-amyloidogenic Aβ fragment (Haas et al., 1993); **2) the endosomal-lysosomal pathway** generates a series of C-terminal fragments of various sizes, the largest containing the entire Aβ domain which further processing may yield the 4kDa form of Aβ (Golde et al. 1992; Estus et al., 1992) and **3) the amyloidogenic pathway** produces the 4kDa Aβ (Haas et al., 1992; Shoji et al., 1992; Seubert et al., 1992). The metabolism of APP is outlined in Figure 2.

Normally the secreted 4 kDa Aβ is composed primarily (~90%) of $Aβ_{1-40}$ and a small proportion of $Aβ_{1-42/43}$ (Cai et al., 1993b). The predominant form of Aβ found in senile plaques is $Aβ_{1-42/43}$ (Roher et al., 1993). Biochemical and immunocytochemical studies show that $Aβ_{1-42/43}$ is deposited early and selectively in the AD brain (Iwatsubo et al., 1994; Gravina et al., 1995). Studies indicating that $Aβ_{1-42/43}$ nucleates more rapidly and is more fibrillogenic (Burdick et al., 1992; Jarrett et al., 1993; Jarrett and Landsbury, 1993), and neurotoxic (Lorenzo and Yankner, 1994; Busciglio et al., 1995; Howlett et al.,

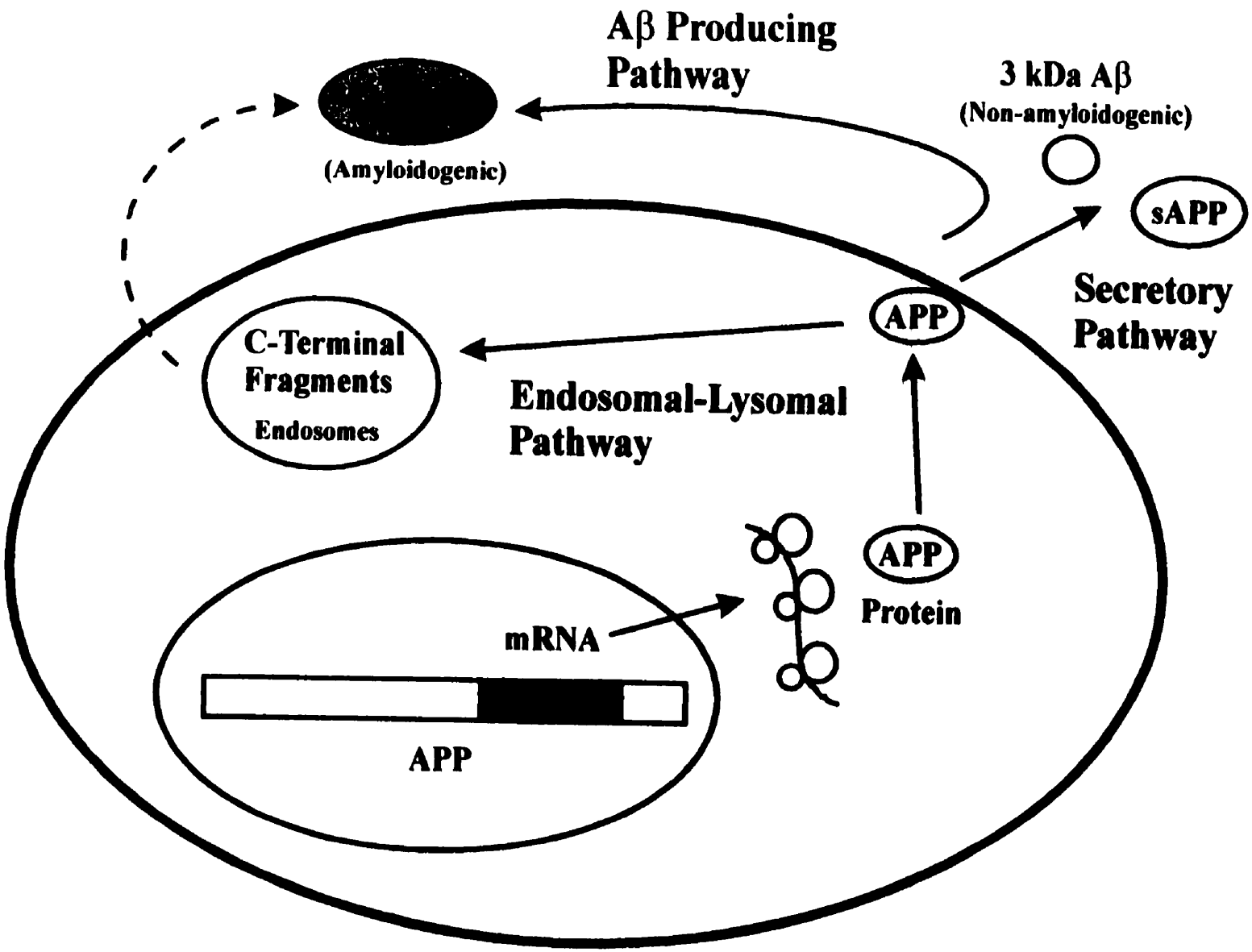


Figure 2. - APP Metabolism

1995) in comparison to $A\beta_{1-40}$ has lead to the view that $A\beta_{1-42/43}$ may play a significant role in the pathogenesis of AD.

3.2 The Role of Aβ in AD Pathogenesis

The invariant formation of the senile plaques and deposition of Aβ in the cerebral blood vessels in the pathology of AD suggests that Aβ plays a central role in the development of AD (reviewed by Haas and Selkoe, 1993). According to the **"amyloid theory"** (Hardy and Higgins, 1992), the production of Aβ that results from an overexpression and/or altered processing of APP (reviewed by LeBlanc, 1994), may be the seminal event in the disease process of AD. Evidence to support this hypothesis stems from the following observations: **1)** patients with Trisomy 21 (Down's syndrome), who carry three copies of chromosome 21, overexpress APP mRNA (Patterson et al., 1988) and invariably develop characteristic AD pathology (Wisniewski et al., 1985) with an early and selective deposition of $A\beta_{1-42/43}$ (Iwatsubo et al., 1995; Lemere et al., 1996); **2)** mutations of the APP gene are genetically linked to FAD and include: **i)** the "NL" mutation (Mullan et al., 1992) that results in the overproduction of the 4 kDa Aβ; **ii)** the APP_{717} mutation (Goate et al., 1991) which alters the metabolism of APP and increases the production of the amyloidogenic $A\beta_{1-42/43}$ (Cai et al., 1993a; Citron et al., 1992; Suzuki et al., 1994), and two additional mutations at codon 717 (Murrell et al., 1991; Chartier-Harlin, 1991) and **iii)** the $APP_{692/693}$ mutation of Hereditary Cerebral Hemorrhage with Amyloidosis of the Dutch Type (HCHWAD) (Levy et al. 1990) which generates abundant Aβ deposits in the cerebrovascular tissues; **3)** transgenic mice overexpressing the APP_{717} mutations (Games et al., 1995), $APP_{670/671}$ mutations (Hsiao et al., 1996) and the mouse $A\beta_{1-42}$ (LaFerla et al., 1996) progressively develop many of the pathogenic hallmarks and neurodegeneration characteristic of AD, and is accompanied with a significant increase in $A\beta_{1-42/43}$ (Hsiao et al., 1996); **4)** mutations in *PS-I* and *PS-II* alter the intracellular trafficking and processing of APP in fibroblasts of FAD patients,

resulting in an increase of APP mRNA (Querfurth et al., 1995) and the ratio of $A\beta_{1-42/43}/A\beta_{1-40}$ relative to fibroblasts from unaffected family members (Scheuner et al., 1996); **5)** stable, double-transfected mouse and human cells expressing wild-type APP_{695} and mutant *PS-I* or *PS-II* genes show a significant increase in the production of $A\beta_{1-42/43}$ versus PS-I and PS-II wild-type double-transfected cells (Borchelt et al., 1996; Citron et al., 1997) and **6)** mutant *PS-I* transgenic mice (Duff et al., 1996) and double-transgenic mice expressing wild-type APP_{695} and mutant *PS-I* or *PS-II* genes (Borchelt et al., 1996; Citron et al., 1997) show a significant increase in the production of $A\beta_{1-42/43}$ versus PS-I and PS-II wild-type mice. Together these studies illustrate that mutations in APP and PS-I/PS-II affect the metabolism of APP and provide strong support that the abnormal production of $A\beta_{1-42/43}$ may be a critical step in the pathogenesis of AD.

4. Aβ in Neuronal Apoptosis

There is a growing body of *in vitro* and *in vivo* evidence that suggests Aβ directly contributes to the neurodegeneration in AD through apoptosis. Yankner et al. (1990) first demonstrated that the Aβ peptide, through a region between amino acids 25-35, is neurotoxic to neurons in culture. The neurotoxicity of Aβ is dependent on its aggregation state (Pike et al., 1991, 1993). Intracerebral injections of synthetic Aβ plaque cores and Aβ aggregates into the brain of adult rats and aged primates results in neurodegeneration (Kowall et al., 1991,1992; Frautschy et al., 1991), Congo-red positive deposits of aggregated material and a loss of cholinergic neurons in the nucleus basalis (Giovannelli et al., 1995). Chronic treatment with synthetic $A\beta_{25-35}$ peptide and synthetic $A\beta_{1-42}$ peptide induces cell death in rat and mouse, primary cortical and hippocampal neuron cultures characteristic of apoptosis (Forloni et al., 1993; Loo et al., 1993; Watt et al. 1994). Recent in vivo studies show that the overexpression of Aβ induces neurodegeneration characterized by apoptosis in the hippocampal, amygdala, and neocortical regions of the brain in Aβ transgenic mice (LaFerla et al., 1995; LaFerla et

al., 1996). Indirect evidence that Aβ may effect apoptotic cell death in AD is derived from transient transfection experiments of three mutant FAD-APPs; dominantly inherited point mutations V642I/F/G in APP_{695} that increase the secretion of $A\beta_{1-42/43}$ (Suzuki et al., 1994), into COS and neuronal cells which result in apoptosis (Yamatsuji et al., 1996a; Yamatsuji et al., 1996b). These studies suggest that the induction of apoptosis is a phenotypic trait of FAD associated with the mutant APPs.

In addition to increasing the levels of $A\beta_{1-42/43}$, the presenilins have been linked to apoptosis as a mechanism of neuronal cell death in AD. A partial cDNA clone of *ALG-3*, a murine homolog of *PS-II* (*STM2*), was found to rescue a T cell hybridoma from T cell receptor and Fas induced apoptosis (Vito et al., 1996). Additionally, overexpression of a FAD PS-II mutant in PC-12 cell increased apoptosis induced by trophic factor withdrawal and Aβ (Wolozin et al., 1996). The ability of antisense PS-II to reduce the level of apoptosis induced by the FAD-APP_{PHE717} suggests that PS-II and APP share the same pathway of apoptosis (Wolozin et al., 1996). These studies indicate that PS-II may not only modulate the levels, but also mediate the apoptotic potential of Aβ, and confer susceptibiltiy of neurons to apoptosis.

4.1 Mechanisms of Aβ Toxicity

Several mechanisms have been proposed for Aβ toxicity including the production of reactive peptide free-radicals (Butterfield et al., 1994), the potentiation of excitatory amino acid neurotoxicity (Koh et al., 1990) and oxidative stress (Lockhart et al., 1994; Behl et al., 1994; reviewed by LeBlanc, 1997), the activation of the low-affinity NGF receptor (Rabizadeh et al., 1994) and the loss of Ca^{2+} homeostasis that renders neurons more susceptible to excitotoxicity (Mattson et al., 1992). Recent studies have shown that Aβ may affect Ca^{2+} homeostasis by: **1)** enhancing the binding of Ca^{2+} mobilizing second messengers inositol (1,4,5) triphosphate and inositol (1,3,4,5) tetraphosphate to their receptor sites in rat cerebral cortical membranes (Cowburn et al., 1995) and **2)**

impairing the activity of the Na^+/K^+ ATPase activity that results in the elevation of the intracellular concentration of Ca^{2+} and subsequent neuronal degeneration characteristic of apoptosis (Mark et al., 1995).

The most intriguing link of Aβ-mediated apoptosis in relation to neurodegeneration associated with AD comes from the following observations: 1) Bcl-2 is decreased in the AD brain; specifically in neurons bearing tangles and overall lower levels in neurons in comparison to astrocytes (Satou et al., 1995; O'Barr et al., 1996), which suggests certain population of neurons are more vulnerable to apoptotic mechanisms whereas astrocytes are resistant to degeneration in AD, and 2) Aβ treatment of primary human neuron cultures has been shown to modulate the levels of Bax and Bcl-2; key regulators of the apoptotic pathway, and render neurons more vulnerable to oxidative stress (LeBlanc, 1996). These neuron cultures treated with aggregated $A\beta_{1-42/43}$ show a 50% reduction in the levels of Bcl-2 and a 3-4-fold increase in the levels of Bax, and an increase in apoptotic cells following a secondary oxidative insult (LeBlanc, 1996). The study by LeBlanc (1996) illustrates that Aβ directly affects the apoptotic pathway of neuronal cell death. Taken together, the studies outlined above provide strong evidence that Aβ may mediate neurodegeneration and neuronal cell death by rendering certain populations of neurons in the brain more susceptible to apoptotsis during the development of AD.

5. The Immediate Early Genes - Fos and Jun

The proto-oncogenes *c-fos, fos-b, fra-1, fra-2, jun-b, jun-d* and *c-jun* are part of the immediate early gene (IEG) family that play an important role in the regulation of growth and differentiation (reviewed by Morgan and Curran, 1991; reviewed by Angel and Karin, 1991). These proto-oncogenes encode general transcription factors that are induced and regulated by environmental signals (reviewed by Morgan and Curran, 1991; reviewed by Angel and Karin, 1991). They function as transducers by coupling short

term extracellular signals at the cell surface, whether they be positive growth signals or negative stimuli, to long term changes in cellular phenotype by regulating the expression of selected target genes (reviewed by Soares et al., 1994). Structurally the Fos and Jun proteins contain a leucine zipper domain and a DNA binding domain which bind as Fos/Jun or Jun/Jun dimers to the AP-1 binding site or the TPA response element (reviewed by Angel and Karin, 1991). Together the Fos and Jun dimers form what is known as the AP-1 complex, and serve to regulate the transcription of target genes containing the AP-1 consensus sequence (reviewed by Morgan and Curran, 1991). C-Fos and c-Jun work in concert to activate transcription efficiently and can transform cells in culture (reviewed by Angel and Karin, 1991), while Jun-B inhibits the trans-activating and transforming activity of c-Jun (Schutte et al., 1989). Fra-1 and Fra-2 are suggested to be negative regulators of transcription (Wisdom and Verma, 1993).

5.1 Fos and Jun in the Nervous System

The IEGs have been proposed to couple transient, extracellular signals to long term changes in structure and function in the nervous system (reviewed by Morgan and Curran, 1991; Shen and Greenberg, 1990). The expression of *fos* and *jun* correlates closely with neuronal activity. IEG expression has been observed in the developing nervous system (Muller et al., 1990; Mellstrom et al., 1991) and in the hippocampus and cerebral cortex of the adult brain (Morgan et al., 1987; Sagar et al., 1988). The induction of *fos* and *jun* expression can be stimulated by growth factors and membrane depolarization (Bartel et al., 1989). Sheng et al. (1995) demonstrate that individual dorsal root ganglion neurons express several IEGs following KCl depolarization. The expression of *c-fos* is generally linked to neuronal excitation and activity (Zhang et al., 1992). Typically *c-fos* expression is relatively low at basal cellular levels in neurons, but the level of expression can be rapidly and transiently induced by numerous stimuli such as pharmacological agents (Miller, 1990) and noxious stimulation (Hunt et al., 1987). In

models of brain tissue injury (Ruppert et al., 1987; Dragunow et al., 1988), chemically and electrically induced seizure activity (reviewed by Morgan and Curran, 1991b), and excitatory amino acid induced cytotoxicity (Gorman et al., 1995) there is an induction of *c-fos* expression. Other studies show that axonal damage is associated with the expression of the IEGs (Jenkins and Hunt, 1991; Jenkins et al., 1993; Dragunow et al., 1993; reviewed by Dragunow and Preston, 1995). Thus, IEG expression is responsive to neuronal stimuli (positive or negative) and reflective of neuronal activity.

5.2 Fos and Jun - Inducible Mediators of Neuronal Cell Death

In recent years neurobiologists have exploited the inducible and transient expression pattern of the immediate early genes as markers for the induction of apoptosis. Smeyne et al. (1993) have shown that c-Fos is continuously expressed in cells preceding apoptosis. Additionally, c-Jun has been co-localized with apoptotic neurons in the developing rat brain (Messina et al., 1996). These studies implicate the IEGs in the apoptotic mechanisms of cellular deletion during nervous system development. In mature cells, there is a temporal induction in the expression of *c-fos* and *c-jun* preceding neuronal apoptosis in sympathetic neurons deprived of NGF (Estus et al., 1994). Specifically, *c-jun* must be expressed in order for apoptosis to occur (Estus et al., 1994). The level of the c-Jun protein significantly increases and expression of c-Jun itself is sufficient to induce apoptosis in NGF deprived sympathetic neurons (Ham et al., 1995). Expression of a c-Jun dominant negative mutant protects these neurons from NGF induced apoptosis, which illustrates that AP-1 activity is required for neuronal apoptosis in this model (Ham et al., 1995).

As previously discussed, Aβ induces neuronal cell death characteristic of apoptosis *in vitro*. Gillardon et al. (1996) have recently shown that $A\beta_{25-35}$ treatment of hippocampal neuron cultures results in an increase in c-Fos immunoreactivity, and that anti-sense *c-fos* can protect against Aβ toxicity. Additionally, Anderson et al. (1995)

have shown that there is a selective induction of c-Jun in hippocampal neuron cultures treated with Aβ, and a co-localization of c-Jun immunoreactivity with apoptotic cells. Collectively, these studies strongly suggest that the immediate early genes, *c-fos* and *c-jun*, may act as mediators in a pathway for activating cell death commitment genes or they may transcriptionally control the expression of target genes that act directly to elicit apoptosis in neurons (reviewed by Soares et al., 1994).

5.3 The Immediate Early Genes and Apoptosis in AD

It has previously been suggested in this discussion that the neurodegeneration and neuronal cell loss associated with AD may be the result of apoptosis, and the expression of *c-fos* and *c-jun* may be indicative of neurons activated to undergo apoptosis. Recent immunohistochemical analysis in the hippocampal and entorhinal cortices of the AD brain has revealed: **1)** DNA fragmentation by the *in situ* labeling technique TUNEL (terminal transferase-mediated dUTP-biotin nick end labeling) in apoptotic neuronal nuclei associated and unassociated with neurofibrillary tangles in the absence of apoptotic morphology in aged matched control brain samples (Su et al., 1994; Smale et al., 1995; Lassman et al., 1995); **2)** C-Fos immunoreactivity and overexpression versus aged matched control brain samples (Zhang et al., 1991), and **3)** a co-localization of c-Jun with apoptotic cells in the AD brain (Anderson et al., 1995) Since the observation in **1)** supports the hypothesis that neuronal cell death may occur via apoptotic mechanisms in AD, and the expression of c-Fos and c-Jun localized in the same regions of the AD brain that have undergone apoptosis as described in **2)** and **3)**, then it is reasonable to infer that the immediate early genes may be actively expressed in neurons undergoing apoptosis during the pathogenesis of AD.

6. Induction of the Immediate Early Genes and APP Overexpression

The preceding discussion has established that the overproduction of Aβ from the aberrant metabolism and/or overexpression of APP may be relevant to the pathogenesis of AD. Previous studies by LeBlanc (1995) have shown primary human neuron cultures induced to undergo apoptosis by serum withdrawal increase production of the 4 kDa Aβ by 3- to 4-fold. The central aim of this study is to elucidate the underlying molecular mechanisms of neuronal apoptosis and to determine the cause of the increased production of Aβ in dying primary human neuron cultures. Specifically, we set out to determine if there is a temporal induction in the expression of the immediate early genes during apoptosis in human neurons, and whether this induction is correlated with a subsequent increase the expression of APP. The basis of this study stems from the fact that the APP promoter contains three AP consensus sequences (Yoshikai et al., 1990), which regulate the transcription of APP through the AP-1 complex (Trejo et al., 1994). Furthermore, activation of a glial cell line with PMA induces the synthesis of the AP-1, increases the protein-DNA binding activity to the AP-1 sequences in the APP promoter and increases APP mRNA levels (Trejo et al., 1994). We hypothesize that serum deprivation induced apoptosis in primary human neuron cultures causes an induction in the expression of the immediate early genes, potentially resulting in the formation of AP-1 complexes that may bind to the AP-1 consensus sequences in the APP promoter, and subsequently, activate and increase the expression of APP. The increase in APP expression may lead to the increased formation of Aβ. Our hypothetical model is presented in Figure 3. Information garnished from these *in vitro* studies will provide insight into apoptosis as the mechanism of neuronal cell death and its relevance to the disease process of AD *in vivo*.

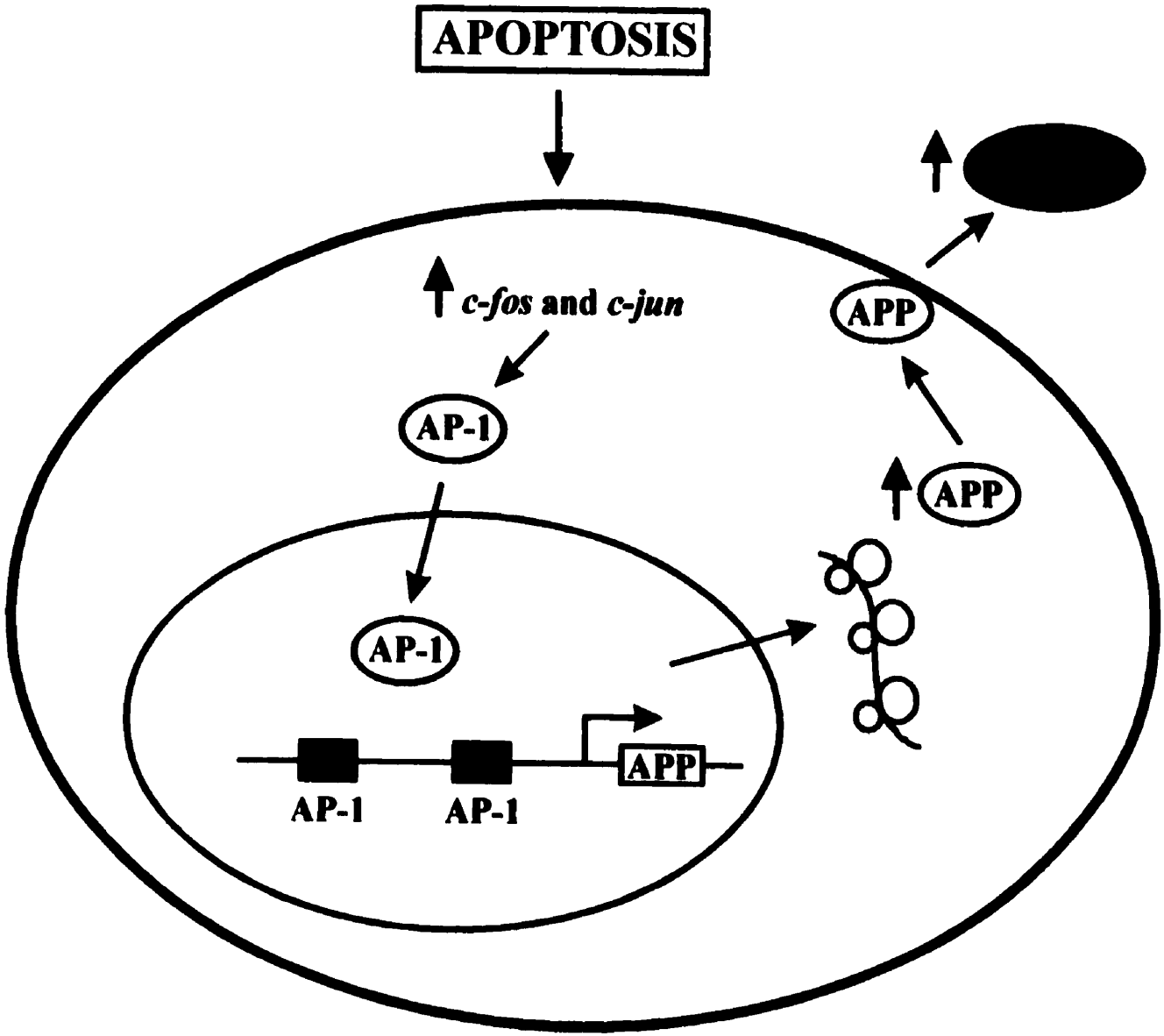


Figure 3. - Hypothetical Model of APP Expression and Metabolism During Neuronal Apoptosis

7. The Neuronal Cytoskeleton

Neurons possess extraordinarily complex and diverse shapes that determine their signaling properties, pattern of synaptic connections and their function. The dimensions of neurons are unparalleled, with some extending for meters and others with extensive axonal and dendritic branching patterns. The cytoskeleton is responsible for developing and maintaining the specialized cellular structure of neurons, and modifying the shape of neurons in response to age, experience, electrical activity, denervation and injury. The dynamics of stabilization and destabilization of various cytoskeleton polymers within neurons governs the ability the cytoskeleton to adapt and change according the functional requirements of the neurons (reviewed by Kandel and Schwartz, 1991).

The components of the neuronal cytoskeleton include the: **1) microfilaments** (8nm in diameter) formed from actin, a self-assembling 43 kDa globular protein; **2) neurofilaments** (10nm in diameter), composed three polypeptide subunits: NF-H (high, 112 kDa), NF-M (middle, 102 kDa) and NF-L (low, 68 kDa), which are only found in neurons and belong to a family of related intermediate filament proteins that includes the keratins, desmin, glial fibrillary acidic protein (GFAP), nuclear lamins and vimentin, and **3) microtubules** (25nm in diameter) formed from tubulin monomers, which are heterodimers of α and β tubulin (50 kDa each). The actin microfilaments form a cortical meshwork just under the membrane of axons and are found primarily at the periphery of axons, especially in the growth cones where they undergo rapid turnover to promote neurite extension and movement. The neurofilaments and microtubules are cross-linked to each other and other filaments of the same type, and are found throughout the cytoplasm. The neurofilaments give bulk and strength to the neuronal processes, and undergo little turnover. The microtubules are responsible for neurite extension and like the actin microfilaments, undergo rapid cycles of polymerization and depolymerization in the growth cones. Furthermore, the microtubules serve as tracks for anterograde and retrograde transport with the neurons. There are proteins known as microtubule-

associated proteins (MAPs) that play important roles in the assembly of microtubules, in transport functions and cross-linking. They include the high molecular weight MAPs (200-300 kDa) and the low molecular weight proteins that include MAP2 and tau (~60 kDa). MAPs are believed to control microtubule growth by maintaining a balance between the dynamic and stable forms of tubulin.

7.1 The Tau Protein

Tau is a MAP that is abundantly expressed in the PNS and CNS. It is primarily localized to the axons in healthy neurons. In the adult human brain, multiple isoforms of tau are produced from a single gene through alternative mRNA splicing (Goedert et al., 1989; reviewed by Goedert, 1993; reviewed by Goedert et al., 1995). Six isoforms of tau exist, which range from 352 to 441 amino acids and differ from each other by the presence or absence of three inserts. In the carboxy terminus half of tau there are three or four tandem repeats of 31 or 32 amino acids that constitute the microtubule-binding domains (Butner et al., 1991; Goode et al., 1994), while in the amino terminus some tau isoforms contain 29 or 58 amino acid inserts and is regarded as the projection domain (Refer to Figure 3) (reviewed by Goedert, 1993; reviewed by Goedert, 1995).

The expression of tau is developmentally regulated in that only the smallest isoform of the protein with three repeats, known as fetal tau, is present in the immature brain, while all six isoforms are found in the adult brain (Goedert et al., 1989). All six normal isoforms of tau are phosphoproteins (Butler et al., 1986) and the phosphorylation of tau is regulated by the balance between protein kinase and protein phosphatase activity (reviewed by Trojanowski et al., 1995). The phosphorylation of tau is developmentally regulated such that tau becomes phosphorylated at fewer sites during brain maturation, thus, tau is less phosphorylated in the adult brain in comparison to the fetal brain (reviewed by Goedert, 1993; Goedert et al., 1995). The ability of tau to bind and stabilize the microtubules is negatively regulated by phosphorylation.

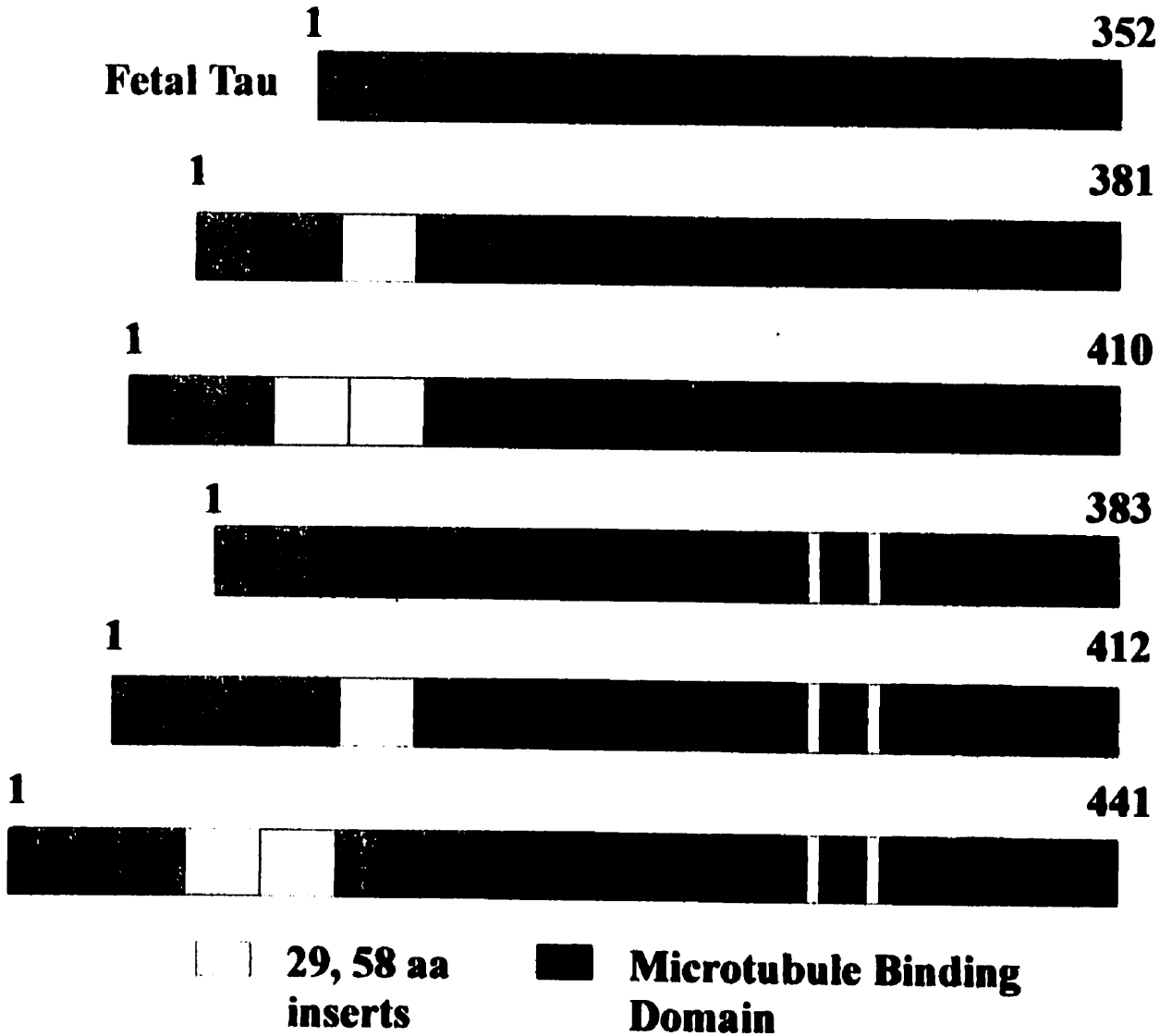


Figure 4. - Isoforms of Tau (Goedert, 1993)

7.1.1 The Neurofibrillary Tangles

NFTs are another prominent, pathological feature of AD. The number of NFTs and their regional distribution correlates with the degree of dementia in AD (reviewed by Goedert, 1993). These neurofibrillary lesions are found in neurons of the cerebral cortex and hippocampal formations that degenerate in AD. NFTs are localized in the nerve cell bodies and apical dendrites, in distal dendrites as neuropil threads and in the dystrophic neurites associated with the senile plaques (reviewed by Goedert, 1993). After the death of the neuron, NFTs appear as extracellular tangles, also known as ghost tangles, because of their insolubility. The major component of NFTs are the abnormal paired helical filaments (PHFs), while the stratight filaments (SFs) constitute a minor component (Kidd, 1963).

7.1.2 The Paired Helical Filaments

The major protein subunit of the PHFs is tau in an abnormally hyperphosphorylated form known as PHF-τ or A68 (Lee et al., 1991; reviewed by Goedert et al., 1995; reviewed by Trojanowski et al., 1995). All six isoforms of tau are components of the PHFs. The PHFs isolated from tangle fragments show cleavage of the amino and carboxy terminal of PHF-τ (Bondareff et al., 1990) and ubiquitination (Mori et al., 1987; Morishima-Kawashima et al., 1993), leaving a pronase-resistant core comprised of the three or four tandem repeats that constitute the microtubule-binding regions of tau. On the other hand, PHF-τ isolated from dispersed filaments is intact.

The ability of PHF-τ to bind microtubules is significantly reduced and is the direct result of hyperphosporylation, since dephosphoylated PHF-τ bind microtubules as well as normal tau does (Bramblett et al., 1993). It has recently been shown that hyperphosphorylated tau from the AD brain can disassemble microtubules and act as a nucleation center to assemble normal tau into filaments (Alonso et al., 1996). Since the ability of tau to bind and stabilize the microtubules is dependent on the degree of

phosphorylation, then the compromised binding of PHF-τ to the microtubules likely destabilizes the microtubule structures of neurons and leads to the degeneration of affected nerve cells in AD.

7.1.3 Formation of the Paired Helical Filaments

For years it has been hypothesized that excessive or abnormal phosphorylation of tau owing to the hyperactivation of protein kinase(s) or the hypoactivation of protein phosphatases was the pivotal event in the transformation of tau to PHF-τ and the formation of NFTs in AD (reviewed by Trojanowski et al., 1995). However, Wang et al. (1996) show that the formation of NFTs not only involves the hyperphosphorylation of tau, but also the glycosylation of PHF-τ. The authors report that abnormally phosphorylated tau from the AD brain is glycosylated and that this post-translational modification maintains the helicity of PHFs. Glycosylation of tau apparently does not have an effect on the ability of tau to promote the assembly of microtubules, thus, it is proposed that the abnormal phosphorylation of tau initially causes the polymerization of tau into straight filaments which are then crosslinked by a glycan "pin" into PHFs (Alonso et al., 1996). These findings suggest that the hyperphosphorylation and glycosylation of tau may act in tandem during the formation of NFTs.

Recently, Goedert et al. (1996) have shown that sulphated glycosaminoglycans (GAGs) may be a key factor in the development of NFTs in AD. They have shown that recombinant tau isoforms with three and four microtubule binding repeats can form PHF-like filaments and SFs , respectively, similar to those found in the AD, when incubated with GAGs such as heparin and heparin sulphate under physiological conditions in vitro. Heparin was shown to inhibit tau from binding microtubules and promote microtubule disassembly independent of the phosphorylation state of tau. Furthermore, heparin sulphate and tau immunoreactivities were found to co-localize in nerve cells of the AD brain before the formation of NFTs. The authors propose that the accumulation of

GAGs within neurons may induce the hyperphosphorylation of tau, destabilization of the microtubules and the formation of NFTs during the pathogenesis of AD (Goedert et al., 1996).

As previously described in this discussion, apoptosis results in distinct morphological changes in the cytoskeleton and the development of AD alters the neuronal cytoskeleton such that NFTs form in the degenerating regions of the brain. Since the literature presented strongly suggests that apoptosis is the mode of cell death and degeneration in AD, we set out to investigate the expression levels and intactness of tau, tubulin and vimentin in human neuron cultures during apoptosis.

8. Synaptic Pathology in AD

In addition to the hallmark neuropathological lesions, SPs and NFTs, there is also extensive synaptic loss associated with AD. It has been hypothesized that the loss of synapses is a relatively early event in the development of AD, occurring in the neocortex and hippocampal regions of the brain (Masliah et al., 1994a; Heinonen et al., 1995). The major components of small synaptic vesicles and peptides stored in large dense cored vesicles are lost in the synaptic pathology (reviewed by Lassmann et al., 1993). Synaptophysin is a 38 kDa calcium binding glycoprotein located in the membrane of presynaptic vesicles of all neocortical axonal endings that is responsible for synaptic transmission. Quantification of synaptophysin immunoreactivity by microdensitometry, confocal and electron microscopy are the current methods employed to investigate synaptic loss (reviewed by Terry, 1994; Heinonen et al., 1995). Masliah et al. (1991a) reported a 45% loss in synaptophysin immunoreactivity in the frontal and parietal cortex, and to a lesser extent in the hippocampus and entorhinal cortex in the AD brain. Another study reported a 77% reduction in synaptophysin-like immunoreactivity in the hippocampus and a 54% reduction in the temporal cortex in AD patients (Honer et al.,

1992). Heinonen et al. (1995) report up to a 53% loss of synaptophysin immunoreactivity primarily in the region of the entorhinal cortex.

The classic lesions of AD, the SPS and NFTs, do not satisfactorily account for the clinical symptoms of AD. NFTs are a better correlate of cognitive deficiency than SPs in AD. A problem arises in the fact that 20-30% of the cases of AD above the age of 70 have very rare or no tangles in the neocortex associated with dementia (Terry et al., 1987). By far the strongest correlation exists with the severity of dementia in AD is the loss of synapses (Terry et al., 1991). Taken together, the results of quantitative studies of synapse density and the correlation of dementia to the degree of synaptic loss strongly suggest that the loss of synapses is centrally involved in the pathogenesis of AD.

8.1 Aberrant Neuritic Sprouting in AD

In addition to the extensive loss of synapses in the neocortex of the AD brain, there is also the presence of abundant dystrophic neurites scattered in the neuropil and others associated with the SPs (reviewed by Masliah and Terry, 1993). Some believe they represent degenerating processes, while others suggest that at least a subpopulation of them may be aberrant sprouting neurites arising from neurons attempting a regenerative response (Geddes, et al., 1986; Masliah et al., 1991b; reviewed by Masliah et al., 1993). The latter proposal stems from the fact that GAP-43, a phosphoprotein ranging between 43-57 kDa that is highly localized in the axonal growth cones during axonogenesis and regeneration (Meiri et al., 1986; Meiri et al., 1988), has been detected in the abnormal neuritic elements in the SPs of the AD cortex (Masliah et al., 1991b; Masliah et al., 1992a; Zhan et al., 1995)) and the aged monkey (Cork et al., 1990).

Additionally, other synaptic and axon specific proteins, such as synaptophysin, synapsin, chromogranin, phosphorylated neurofilaments, tau and APP (Shoji et al., 1990; Masliah et al., 1992a; reviewed by Masliah et al., 1993; Masliah et al., 1994; Zhan et al., 1995), have been detected in abnormal neurites of SPs. Studies have revealed that APP is

transported to the presynaptic site (Koo et al., 1990), localized in the presynaptic terminal (Schubert et al., 1991; Masliah et al., 1992a; reviewed by Masliah and Terry, 1993) and found to co-localize with GAP-43 in 57% of the sprouting neurites (Masliah et al., 1992a). APP is present in the growth cone during development (Masliah et al., 1992c; Hung et al., 1992) and may be involved with synaptic plasticity (Loffler et al., 1992; Masliah et al., 1992a). In vitro studies suggest that APP and its fragments, depending on the concentration, could be neurotoxic or neurotrophic (Yankner et al., 1990). Thus, these studies suggest that altered APP processing could cause abnormal neuritic outgrowth and synaptic degeneration, and supports the hypothesis that plaque formation results from synaptic and axonal injury followed by amyloid deposition (Cork et al., 1990; Masliah et al., 1992b; Masliah et al., 1994). Cycles of aberrant sprouting and degeneration of the neurites localized in the presynaptic regions could alter the microcircuitry and cause the cognitive impairments observed in AD. Therefore, we set out to assess the protein levels of synaptophysin, GAP-43 and APP in human neurons in an in vitro paradigm of neurodegeneration to determine whether these proteins undergo similar changes in expression levels to those observed in the AD brain.

Aims of the Present Thesis

The aims of the present thesis are:

1) To induce apoptosis in primary human CNS neurons and quantify the level of cell death following different periods of serum withdrawal.
2) To define the time frame in which primary human neurons become committed to apoptosis following serum deprivation.
3) To determine the temporal expression of *c-fos* and *c-jun*, and to correlate the effect on APP expression during apoptosis.
4) To analyze the effect of apoptosis on the cytoskeletal proteins tau, tubulin and vimentin.
5) To evaluate the effect of apoptosis on GAP-43 and synaptophysin.

The experimental outline of the present thesis is shown in Figure 5.

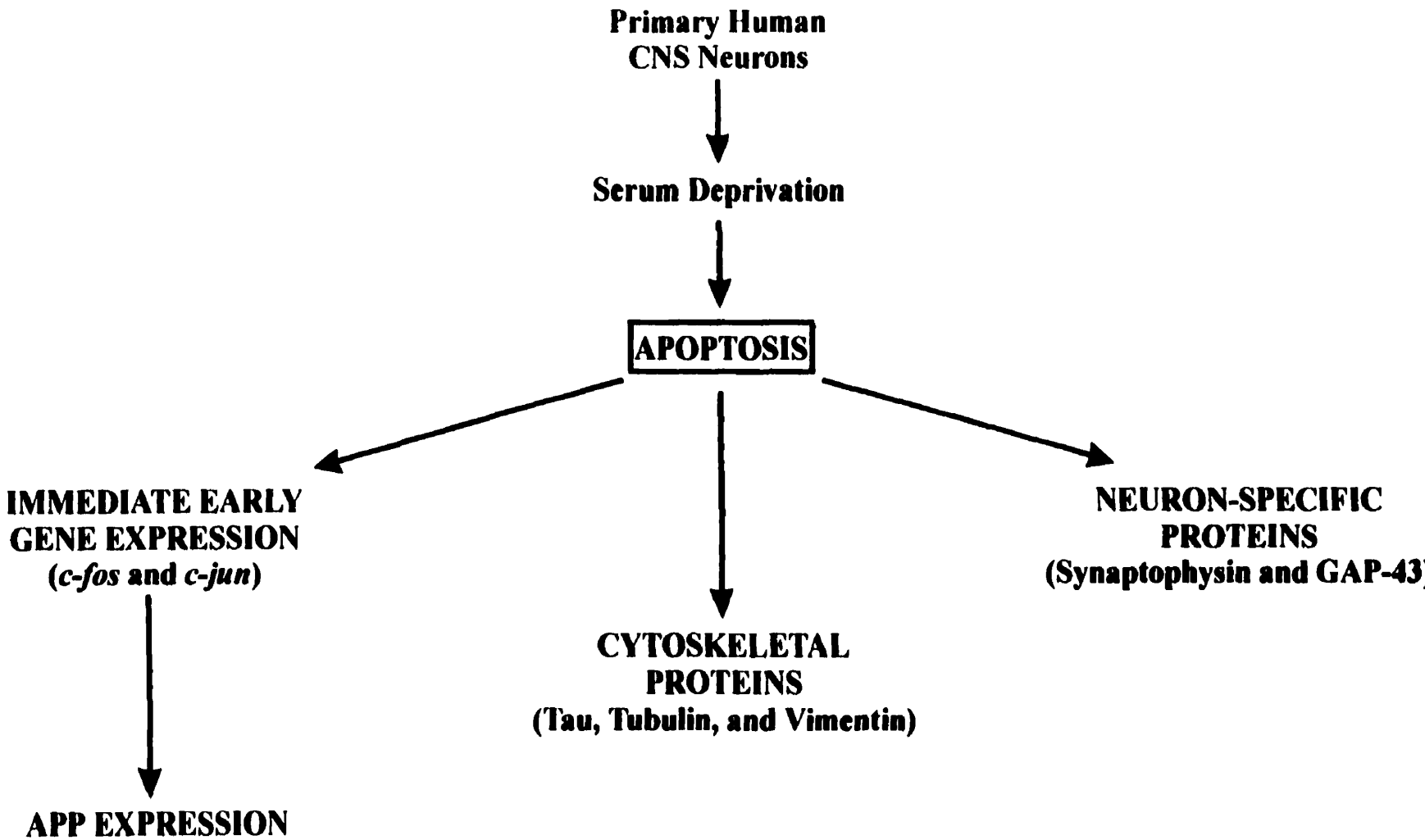


Figure 5. - Experimental Outline

II Materials and Methods

1. Primary Human Fetal Neuron Cultures

Primary human fetal neuron cultures were prepared from cerebral cortical tissue obtained from elective abortions (10-18 weeks of gestation) in accordance with guidelines established by the Medical Research Council of Canada and the National Institutes of Health, and approved by the Jewish General Hospital and McGill University Institutional Review Board ethical committees. Brain tissue was separated from the meninges and blood vessels, and minced in sterile PBS. The tissue was treated with 0.25% trypsin (Life Technologies, ON, Canada) and 1 mg/ml DNase for 15 minutes at 37°C. The action of the enzymes was neutralized with 5% decomplemented bovine calf serum (dBCS; BCS treated at 56°C for 30 minutes) (Hyclone, UT, USA). The tissue was then filtered through 132 μm and 70 μm sterile nylon mesh to separate the cells. The cells were centrifuged, washed, and resuspended in MEM media containing Earle's salts, 5% dBCS, 0.225% sodium bicarbonate, 1mM sodium pyruvate, 2mM L-glutamine, 1X penicillin/streptomycin, 0.1% glucose (all from Life Technologies, ON, Canada). Cells were plated at a density of 3×10^6 cells/ml in flasks, multiwell plates, and on ACLAR coverslips coated with 10 μg/ml of poly-L-lysine (Sigma, MO, USA). After 3 days, cultures were treated with MEM media containing 1mM fluorodeoxyuridine (FdU) for 1 week to inhibit the proliferation of contaminating astrocytes and other dividing cells. The cells were fed every third day and 11 days after initial plating the cells were ready for experimental manipulations.

1.1 Characterization of the Cultures

After 11 days, coverslips with neurons (1.5×10^4 cells) were immunostained with antibodies for tau, which normally stains the axons of neurons, and glial fibrillary acid (GFAP), which is specific for astrocytes to determine the purity of the cultures. Cells were fixed with 4% paraformaldehyde (Sigma, MO,USA) and permeablized with 0.1% Triton X-100. After washing with PBS, coverslips were incubated with 10% goat serum (Life Technologies, ON, Canada) for 1 hour at room temperature. Coverslips were incubated with polyclonal anti-bovine tau (1:200 in 1% goat serum; a kind gift from Dr.

Hemant Paudel, Lady Davis Institute, McGill University) and monoclonal anti-GFAP (1:200 in 1% goat serum; Sigma, MO, USA) antibodies overnight at 4°C. After washing with PBS, the coverslips were incubated with goat anti-rabbit IgG (H+L) linked with FITC (fluorescein isothiocynate) and goat anti-rabbit IgG linked with rhodamine secondary antibodies (1:200 in 1% goat serum; Jackson Labs, PA, USA) for 1 hour at room temperature. Following three washes with PBS, the coverslips were mounted onto glass slides with glycerol:PBS (1:1), and the cells were analyzed by phase contrast and fluorescence microscopy at low magnification (20X).

2. Apoptosis of Neuron Cultures

Apoptosis was induced in the cultures by complete serum withdrawal, removal of all essential growth factors, for various periods of time.

2.1 Analysis of Apoptosis

2.1.1 Propidium Iodide Staining and Tau Immunostaining

Neurons plated on coverslips were treated in the presence and absence of serum containing media for 0, 6, 12, 18, 24 and 48 hours. Cells were fixed with 4% paraformaldehyde, washed with PBS and permeablized with 0.1% Triton X-100. Cells were incubated with the polyclonal anti-bovine tau antibody as described above. After washing with PBS, coverslips were incubated with goat anti-rabbit-FITC in propidium iodide solution (0.1 μg/ml in PBS; Sigma, MO, USA) for 30 minutes at room temperature. The coverslips were washed 6 times with PBS and mounted on glass slides with glycerol:PBS (1:1). Cells were analyzed by phase contrast and fluorescence microscopy at low and high magnification (20X and 40X), and stained nuclei were analyzed by fluorescence microscopy at high magnification (40X and 100X).

2.1.2 DNA Ladder

Cultures were treated in the presence and absence of serum containing media for 0, 6, 12, 18, 24, 36, 48 and 72 hours. The cells (3×10^6 cells) were harvested in PBS and briefly centrifuged. The pellet was resuspended in 50 μl of proteinase K (200 μg/ml)/

0.5% sarkosyl solution (both from Sigma, MO, USA) and incubated at 50°C for 2 hours. Subsequently, 2.5 µl of RNase A (10 µg/µl) was added and the mixture was incubated for 2 hours at 50°C. Ten µl of 6X DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to the samples after which the samples were separated in parallel with a 100 bp ladder (Pharmacia, QC, Canada) on a 1.8% agarose gel at 15 volts overnight. The gels were stained with ethidium bromide (EtBr) to visualize the DNA.

2.1.3 TUNEL (terminal transferase-mediated dUTP nick end labeling) Staining

Neurons plated on coverslips were treated in the presence and absence of serum containing media for 0, 12, 24 and 48 hours. Cells were fixed with 4% paraformaldehyde, permeablized with 0.1% Triton X-100 and washed with PBS. DNA fragmentation was detected with the In Situ Cell Death Detection Kit (Boehringer-Mannheim, QC, Canada) according to the manufacturer's standard protocols. Coverslips were washed 6 times with PBS and mounted with glycerol:PBS (1:1) on glass slides. The presence of DNA fragmentation was analyzed at low magnification (20X) fluorescence microscopy.

2.1.4 Quantitating Levels of Apoptosis

After cells were stained with TUNEL as described in the previous section, approximately 500 neurons per coverslip for each time point were analyzed for positive TUNEL staining by high magnification (40X) fluorescence microscopy. The 0h time point was used as an indication of the basal level of apoptosis each culture. The ratio of apoptotic cells to the total number of cells analyzed for each time point was standardized to the ratio of apoptotic cells to the total number of cells determined at the 0h time point, and expressed as a fold increase in the number of apoptotic cells relative to the 0h time point. The data presented represents the mean and standard error of three independent cultures. A two-tailed, unpaired Student's t-test with a confidence level of 90% was used to determine the statistical significance of the changes in the levels of apoptosis between the serum deprived and untreated cultures at the corresponding time points.

2.1.5 Cell Death Commitment Experiments

Neurons cultured on coverslips were deprived of serum for 0, 6, 12, 18, 24 and 48 hours, after which serum was reintroduced in the media for another 12 hours to rescue to the cells. Subsequently, the cells were fixed with 4% paraformaldehyde, permeablized with 0.1% Triton X-100 and washed with PBS. The cells were stained with propidium iodide (0.1 μg/ml) at room temperature for 20 minutes. The coverslips were washed 6 times with PBS and mounted on glass slides with glycerol:PBS (1:1). Control cultures, neurons treated in the presence of serum for 0, 6, 12, 18, 24 and 48 hours and neurons treated in the presence of serum for 0, 6, 12, 18, 24 and 48 hours, after which fresh serum containing media was fed to the cells for another 12 hours, were also treated with the same procedures. Approximately 500 neurons per coverslip were analyzed for apoptotic morphology by high magnification (40X) fluorescence microscopy. The ratio of apoptotic cells to the total number of cells analyzed for each time point was standardized to the level of apoptosis at the 0h time point, and expressed as a fold increase in the number of apoptotic cells relative to the 0h time point. The data presented represents the mean and standard error of at least three independent cultures. A two-tailed, unpaired Student's t-test with a confidence level of 90% was used to determine the statistical significance of the changes in the levels of apoptosis between the serum deprived cultures and the control cultures at the corresponding time points.

3. Assessing *c-fos* and *c-jun* Expression

3.1 RT-PCR

3.1.1 RNA Extraction

Total RNA was extracted from cultures (~4.5 x 10^7 cells) treated in the presence and absence of serum containing media for 0, 6, 12, 18 and 24 hours by standard phenol/chloroform methods (Chomczynski et al., 1987). RNA samples were treated with 1U of RQ1 RNase-free DNase (Promega, WI, USA) in DNase buffer (40 mM Tris-HCl pH 7.9, 6 mM $MgCl_2$, 10 mM NaCl and 10 mM $CaCl_2$) for 30 minutes at 37°C to eliminate genomic DNA contamination in the samples. The samples were extracted with phenol/chlorofom and precipitated with 10 μg of tRNA to increase the efficiency of RNA

recovery. RNA concentration and purity was determined by spectrophotometry at 260nm and 280 nm.

3.1.2 cDNA Synthesis by Reverse Transcription

The RNA samples (1 μg) were reverse transcribed into cDNA with the Superscript II Reverse Transcriptase System (Life Technologies, ON, Canada) according to the manufacturer's standard protocols. One μg of RNA from the 12h serum deprived sample was subjected to the cDNA synthesis reaction in the absence of reverse transcriptase and served as negative control for genomic DNA contamination in the PCR reactions.

3.1.3 PCR Analysis

3.1.3.1 Primer Design and Specificity

Primers were designed to amplify regions of *c-fos* (2088-2721; 633 bp region), *c-jun* (2036-2445; 409 bp region) and *G3PDH* (28-598; 570 bp region) with the aid of the PC-Gene software (Intelligenetics, CA, USA). The primer sequences were: *c-fos*-forward 5'-GAGACAGACCAACTAG-3', reverse 5'-CCAGCAGCGTGGGTG-3'; *c-jun*-forward 5'-GCATGAGGAACCGC-3', reverse 5'-GCGACCAAGTCCTTC-3' and *G3PDH*-forward 5'-CGGATTTGGTCGTATTGGGC-3', reverse 5'-CCATCACGCCACAGTTCC-3'. We used *G3PDH* as an internal standard because of its constitutive expression as a housekeeping gene.

Each primer pair was tested for its ability to amplify the target sequence from plasmid containing the gene of interest, human *c-fos* plasmid, mouse *c-jun* plasmid (both kind gifts from Dr. Lorraine Chalifour, Lady Davis Institute, McGill University), and rat *G3PDH* plasmid (a kind gift from Dr. Hyman Schipper, Lady Davis Institute, McGill University). Restriction digests (5 μl of the PCR reaction digested with 1U of Xcm I (New England Biolabs, MA, USA) in 1X NEB Buffer 2 for 1 hour at 37°C, separated on a 1% agaorse gel by electrophoresis and DNA fragments visualized by EtBr staining) and direct PCR sequencing of the PCR fragments with the CircumVent Thermal Cycle Sequencing Kit according to the manufacturer's standard protocols (New England

Biolabs, MA, USA) were performed to confirm the primers correctly amplified the target sequences.

3.1.3.2 Reaction Conditions

One μl of the cDNA reaction was amplified in a 25 μl volume containing 1X PCR reaction buffer with 1 mM $MgCl_2$(Promega, WI, USA), 200 μM dNTP, 0.6 pmol of the forward and reverse primers and 0.625 U of Taq polymerase (Promega, WI, USA) in a Perkin-Elmer Thermal Cycler at 94°C for 1 minute, 50°C for 1 minutes and 72°C for 2 minutes for 20 and 30 cycles. Each cDNA sample was amplified in triplicate, and simultaneously with a positive control (human *c-fos* plasmid, mouse *c-jun* plasmid and rat *G3PDH* plasmid) and three negative controls (1 μl of cDNA mixture from the 12h serum deprived sample treated without reverse transcriptase, 1 μg of tRNA, and 1 μl of water). After amplification, the cDNAs were separated by electrophoresis on 1% agarose gels, visualized by EtBr staining and photographed.

3.2 Southern Analysis

Subsequently, the PCR products were transferred onto Nytran membrane (Schleicher and Schuell, NH, USA) and detected by hybridization with ^{32}P-dATP labeled DNA probes specific for *c-fos*, *c-jun* and *G3PDH* according to the manufacturer's standard protocols. DNA probes were obtained from PCR amplification of human *c-fos* plasmid, mouse *c-jun* plasmid and rat *G3PDH* plasmid, and labeled with ^{32}P-dATP according to the manufacturer's standard protocols supplied with the Oligolabeling Kit (Pharmacia, QC, Canada).

3.3 Quantitation of mRNA Expression

The membranes were exposed on phosphorimaging screens and the PCR products were quantitated by phosphorimaging software (Molecular Dynamics, CA, USA). The *c-fos* and *c-jun* expression levels were corrected to *G3PDH* levels at each of the corresponding time points and subsequently standardized to the expression level at the 0h time point to control for the amount of cDNA in each reaction and to establish the

baseline level of expression in each experiment. The level of *c-fos* and *c-jun* expression was expressed as a fold increase in the level of expression relative to the 0h time point, and was determined from two trials with a given experiment. Expression of *c-fos* and *c-jun* was analyzed in four independent cultures.

4. Assessing c-Fos and c-Jun Expression

4.1 Whole Cell Extract Preparation

After treatment in the absence of serum containing media for 0, 6, 12, 18, 24, 36 and 48 hours, 3 x 10^6 cells were washed in PBS (phosphate-buffered saline; 100 mM sodium phosphate, 100 mM NaCl, pH 7.5) and harvested in 100 µl of cold NP-40 lysis buffer ((50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, pH 8.0) containing 0.05% phenylmethylsulfonyl fluoride, 0.1 µg/ml pepstatin A, 1 µg/ml Nα-*p*-tosyl-L-lysine chloromethyl ketone, and 0.5 µg/ml leupeptin (all protease inhibitors from ICN, QC, Canada). The lysate was centrifuged at 14 000 rpm for 10 minutes at 4°C and the supernatant was used for analysis. Whole cell extracts were prepared from 1 x 10^6 Jurkat cells, treated with 32 nM PMA (phorbol 12-myristate 13-acetate) for 2 hours, according to the methods by Sambrook et al. (1989) and were used as a positive control.

4.2 Nuclear Extract Preparation

Nuclear extracts were prepared as described by Pinol-Roma et al. (1988). After treatment in the presence and absence of serum containing media for 0, 6, 12, 18 and 24 hours, 4.5 x 10^6 cells were washed in PBS and harvested in 5 ml of cold lysis buffer (10mM Tris-HCl, pH 7.4, 100 mM NaCl, 2.5 mM $MgCl_2$) containing 0.5% aprotinin, 2 µg/ml pepstatin A, 2µg/ml leupeptin (all protease inhibitors from ICN, QC, Canada) and 0.5% Triton X-100. The lysate was passed through a 25-gauge needle four times and centrifuged briefly at 3000g. The nuclei were resuspended in 100 µl of lysis buffer without Triton X-100 and sonicated briefly. Protein concentration was determined with BCA Protein Assay Reagents (Pierce, IL, USA).

4.3 Western Blot Analysis

Samples, containing 17 µl of whole cell extract (representative of 5.1×10^5 cells) and 3 µl of 6X SDS/Sample loading buffer (300mM Tris-HCl, pH 6.8, 600 mM DTT, 12% SDS, 0.6% bromophenol blue, 60% glycerol), were separated in parallel with Jurkat cell extracts and prestained low molecular weight markers (BioRad, ON, Canada) on 10% SDS-polyacrylamide gels by electrophoresis. Separated proteins were transferred onto Immobilon-P membranes (Millipore, MA, USA) by electroblotting. Membranes were blocked in Blotto A (5% (w/v) low fat milk in TBS-T (Tris-buffered saline; 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20)) for 1 hour and 30 minutes and incubated at 4°C overnight with primary antibodies-polyclonal anti-c-Jun (1:250 in Blotto A; sc-45, Santa Cruz Biotech, CA, USA), monoclonal anti-c-Fos (1:250 in Blotto A; sc-413, Santa Cruz Biotech, CA, USA) or polyclonal anti-c-Fos (1:200 in Blotto A; a kind gift from Dr. Eugenia Wang, Lady Davis Institute, McGill Universtiy). Blots were then washed 3 times for 10 minutes with TBS-T and incubated with the appropriate secondary antibody conjugated to alkaline phosphatase (AP)-goat anti-rabbit-AP (1:5000 in TBS-T; Jackson Labs, PA, USA) or goat anti-mouse-AP (1:1000 in TBS-T; Jackson Labs, PA, USA), for 1 hour at room temperature. Following 3 washes for 10 minutes with TBS-T and a 10 minute wash with alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$), the blots were developed with 10 ml of alkaline phosphatase buffer containing 64 µl of NBT (nitro blue tetrazolium chloride; Fisher Biotech, QC, Canada; 50 µg/ml dissolved in 70% diethylformamide) and 32 µl of BCIP (5-bromo-4-chloro-3-indoyl phosphate; Fisher Biotech, QC, Canada; 50 µg/ml dissolved in 100% diethylformamide) to detect the immunoreactive proteins.

Nuclear extracts (50 µg) were separated in parallel with 10 µg of PMA-induced HeLa nuclear extract (Santa Cruz, CA, USA), transferred to membranes and the membranes treated with the methods described above. Following incubation with the primary antibodies, the blots were washed three times with TBS-T and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (HRP)-goat anti-rabbit-HRP (1:5000 in Blotto A) or goat anti-mouse-HRP (1:10000 in Blotto A) (both a kind gift from Dr. Eugenia Wang, Lady Davis Institute, McGill University), for 1 hour at

room temperature. Immunoreactive proteins were detected with ECL Western Blot Detection Reagents (Amersham, ON, Canada) according to the manufacturer's standard protocols.

4.4 Quantitation of Protein Expression

Following detection of the proteins, the films were scanned and the protein levels quantitated by densitometric software (Molecular Dynamics, CA, USA). The 0h time point was used as the control for the basal level of expression in the culture. The expression level at each time point was standardized to the 0h time point. and expressed as a fold increase relative to the 0h time point.

4.5 Colocalization Studies

Immunocyotchemistry was performed simultaneously with PI or TUNEL staining. Neurons on coverslips, deprived of serum for 0, 6, 12, 18, 24 and 48 hours, were permeablized with 0.1% Triton-X 100, blocked with 10% goat serum and incubated with primary antibodies-polyclonal anti-c-Jun (1:100 in 1% goat serum; sc-45, Santa Cruz Biotech, CA, USA), monoclonal anti-c-Fos (1:100 in 1% goat serum; sc-413 from two different lots, Santa Cruz Biotech, CA, USA) or polyclonal anti-c-Fos (1:10000 in 1% goat serum; a kind gift from Dr. Heather Durham, Montreal Neurological Institute, McGill University) overnight at 4°C. The coverslips were washed 5 time with PBS and incubated with the appropriate secondary antibody conjugated with FITC when propidium iodide staining was performed-goat anti-rabbit-FITC (1:200 in 0.1 μg/ml of PI solution) or goat anti-mouse-FITC (1:100 in 0.1 μg/ml of PI solution). When TUNEL staining was performed, secondary antibodies conjugated to rhodamine (Rho)-goat anti-rabbit-Rho (1:200 in Converter-AP; Boerhinger-Mannheim, QC, Canada) or goat anti-mouse-Rho (1:200 in Converter-AP), were used in conjuction with the Santa Cruz antibodies. Neurons were incubated with biotinylated anti-sheep (1:100 in PBS; a kind gift from Dr. Durham) for 1 hour at room temperature when the c-Fos antibody from Dr. Durham was used. This was followed by an incubation with Texas Red-streptavidin

conjugate (1:1000 in Converter-AP). Neurons were analyzed with a fluorescence microscope at high magnification (40X).

5. Assessing APP Expression

Whole cell extracts were prepared from cultures deprived of serum for 0, 6, 12, 18, 24, 36 and 48 hours according to the method described above. Extracts were not prepared from cultures treated in the presence of serum containing media because serum did not affect *c-fos* and *c-jun*, and c-Jun expression. Twelve µl (representative of 3.6 x 10^5 cells) of each sample with 2 µl of 6X SDS/Sample loading buffer were separated by SDS-PAGE according to the methods described above. Separated proteins were transferred to Immobilon-P membrane, and the membrane treated according to the methods previously described. Separate blots were incubated with polyclonal anti-APP (anti-A1, 4) (1:100 in 1% goat serum) and monoclonal anti-$A\beta_{1\text{-}16}$ (6E10) (1:1000 in 1% goat serum) overnight at 4°C. Membranes were washed and incubated with the appropriate secondary antibody conjugated to alkaline phosphatase. The membranes were washed and developed according to the alkaline phosphatase method described above. The membranes were scanned and the protein levels quantitated with densitometric software (Molecular Dynamics, CA, USA). The 0h time point was used as the control for the basal level of expression in each culture. The level of APP expression at each time point was standardized and expressed relative to the level of expression at the 0h time point. The data shown represents the mean and standard error from three independent cultures. A two-tailed, unpaired Student's t-test with a confidence level of 90% was used to determine the statistical significance of changes in APP expression at each time point relative to the 0h time point.

6. Assessing Tau, Tubulin, Vimentin, GAP-43 and Synaptophysin Expression

Six µl (representative of 1.8 x 10^5 cells) samples; whole cell extracts that were prepared from the same cultures described in section 5, and 1 µl of 6X SDS/Sample loading buffer were separated by SDS-PAGE and stained with Coomassie Blue solution (1 g/L Coomassie Brilliant Blue R_{250}, 50% methanol, 10% glacial acetic acid) to show

equal loading and consistent protein levels over the time course of serum deprivation. Samples of whole cell extracts (6 μl) with 1 μl of 6X SDS/Sample loading buffer were separated by SDS-PAGE, transferred to Immobilon-P membrane and the membrane treated according to the methods described above. Separate blots were incubated with polyclonal anti-bovine tau (1:1000 in TBS-T; a kind gift from Dr. Hemant Paudel, Lady Davis Institute, McGill University), polyclonal anti-tubulin (1:100 in TBS-T; a kind gift from Dr. Eugenia Wang, Lady Davis Institute, McGill University), monoclonal anti-vimentin (1:1000 in TBS-T; Sigma, MO, USA), monoclonal anti-GAP-43 (1:1000 in TBS-T; Sigma, MO, USA) and monoclonal anti-synaptophysin (1:500 in TBS-T; Sigma, MO, USA) overnight at 4°C. The membranes were washed and incubated with the appropriate secondary antibody conjugated to alkaline phosphatase. The membranes were washed and developed according to the alkaline phosphatase method previously described. The blots were then scanned by a phosphoimaging/densitometric system (Molecular Dynamics, CA, USA) and the level of protein expression at each time point was quantitated. The 0h time point was used as an indication of the basal level of protein expression in each culture. The expression levels at each time point were standardized and expressed as a fold increase relative to the 0h time point. The data presented represents the mean and standard error of three independent cultures. A two-tailed, unpaired Student's t-test with a 90% confidence level was used to determine the statistical significance of changes in protein expression at each time point relative to the 0h time point.

III Results

1. Primary Human Neuron Cultures

To investigate the molecular mechanisms of apoptosis and its effect on amyloid precursor protein expression in human neurons, highly purified primary neuron cultures were established from fetal brain tissue. Cells isolated from the fetal brain tissue were cultured for 11 days before any experimental procedures were undertaken. During this period, the mixed cultures were treated with 1mM FdU to inhibit the proliferation of contaminating, mitotic cells- astrocytes, microglia, and oligodendrocytes. By the end of this period, cultures consisting primarily of neurons were established. The cultures consisted of a uniform layer of well differentiated neurons that established dense and intricate networks of neurites. This is clearly evident by phase contrast and immunofluorescence microscopy of cultures stained with anti-tau antibody (Figure 6. - a, c). As previously shown (LeBlanc, 1995), the composition of these cultures was approximately 90% neurons and 10% astrocytes. The low percentage of microglia, oligodendrocytes and fibroblasts was previously confirmed by the lack of positive LDL-DiI incorporation and immunostaining by anti-galactocerebroside and anti-fibronectin antibodies, respectively (LeBlanc, 1995). In the human brain there are approximately ten to fifty times more glial cells than neurons, of which the astrocytes are a significant proportion of the glial cells (reviewed by Kandel et al., 1991). Thus, a small population of astrocytes was beneficial to the maintenance of the cultures because neurons naturally use astrocyte factors to survive. The nominal presence of astrocytes and their close association with neurons in the cultures is shown by phase contrast and immunofluorescence microscopy of cultures stained with anti-GFAP antibody (Figure 6. - b, d).

Figure 6. - Characterization of Human Neuron Cultures

Phase contrast micrographs (a, b) and immunofluorescent pictures at low magnification (20X) of human neurons cultured for 11 days on coverslips , fixed, permeablized and stained with polyclonal anti-human tau antibody (a, c) and monoclonal anti-human GFAP antibody (b, d).

PHASE CONTRAST

GFAP

PHASE CONTRAST

Tau

2. Morphological Analysis of Apoptosis in Human Neurons

Serum deprivation was used to induce apoptotic cell death in the primary human neuron cultures. In the normal human brain there are numerous neurotrophic factors, for example, NGF, BDNF, CNTF and GDNF , that support the survival of the brain cells (reviewed by Lindsay, 1995; reviewed by Silo-Santiago, 1995). The withdrawal of a specific neurotrophic factor may not have been enough to induce apoptosis in these cultures because various neuron types could have utilized other neurotrophic factors as a compensatory response for survival. Thus, completely removing the serum from the cultures ensured that the neurons were deprived of all neurotrophic factors. To confirm that serum deprivation induced apoptosis in these cultures, neurons cultured on coverslips for 11 days in the presence of serum were deprived of serum containing media for 48 hours, fixed and stained with propidium iodide. Typically, apoptotic cells possess fragmented nuclei with condensed chromatin (reviewed by Bredesen, 1995). The cells highlighted by arrowheads in Figure 7A. clearly show the morphological characteristics of apoptotic cells. These cells have obvious pyknotic and fragmented nuclei with condensed chromatin that stain intensely. In contrast, the normal cells (Figure 7A., arrows) have intact and diffusely stained nuclei that appear larger than the apoptotic cells.

Changes in cell morphology were analyzed in neuron cultures deprived of serum for different periods of time. By propidium iodide staining it is evident that neurons deprived of serum for 48h underwent apoptotic cell death (Figure 7B. - c), whereas untreated (cells immediately fixed after 11 days of culture, shown as 0h in Figure 7B. - a) and neurons treated in the presence of serum for 48h (Figure 7B. - e) do not exhibit apoptotic morphology. This was confirmed by the *in situ* labeling technique TUNEL (terminal transferase-mediated dUTP-biotin nick end labeling) (Gavrieli et al., 1992). TUNEL specifically detects apoptotic cells by utilizing terminal transferase to incorporate biotinylated nucleotides on the 3'-OH groups of fragmented DNA, which can then be visualized by a reaction with alkaline phosphatase and a suitable substrate. Numerous apoptotic cells were detected in cultures deprived of serum containing media for 48h (Figure 7B. - d), whereas untreated (Figure 7B. - b) and cultures treated with serum containing media for 48h (Figure 7B. - f) did not exhibit positive TUNEL staining.

Typically, changes in the cellular organization accompany the characteristic changes associated with apoptosis. Apoptotic cells undergo membrane blebbing and in addition, apoptotic neurons undergo degeneration of the neurites and retract (LeBlanc, 1995). Neurons deprived of serum for 48h following 11 days of culture in the presence of serum show marked changes in their cellular organization. By phase contrast and immunofluorescence microscopy of tau immunostaining, it is evident the neurons retracted, which decreased the density of the neurite networks. Furthermore, these dystrophic neurites show beading and degeneration (Figure 7C. - c, arrows), and the cell bodies and neurites have diffuse, non-uniform staining of tau (Figure 7C. - d, arrows). In contrast, untreated and neurons treated in the presence of serum for 48h maintained a consistent density and an intricate network of healthy neurites as shown by phase contrast microscopy (Figure 7C. - a, e). Additionally, immunofluorescence analysis of tau immunostaining shows cell bodies and neurites have a uniform, polar staining pattern characteristic of healthy cells (Figure 7C. - b, f, arrows). In addition, propidium iodide and tau double staining showed that only neurons and not astrocytes undergo apoptosis following serum withdrawal. Previous studies have shown that serum deprivation does not induce human neurons to die by necrosis (LeBlanc, 1995). Clearly, serum deprivation induced morphological changes in primary human neuron cultures characteristic of apoptosis.

To quantify the level of apoptosis associated with the time of serum deprivation, neurons on coverslips were treated in the absence of serum containing media for various periods of time (0h, 12h, 24h and 48h) following 11 days of culture in the presence of serum and subsequently the number of apoptotic cells was scored by positive TUNEL staining. To demonstrate that the length of time in culture did not induce apoptosis, neurons on coverslips were treated in the presence of serum for 0h, 12h, 24h, and 48h, and also scored for positive TUNEL staining. The number of apoptotic cells was expressed as a percent of apoptotic cells to the total number of cells scored, and then expressed as a fold increase relative to the percent of apoptotic cells at 0h. Standardization to the control (0h) was used to establish a baseline level of apoptosis in each independent culture.. The results from three independent cultures show that there

was a statistically significant increase in the number of apoptotic cells by 12 hours of serum deprivation, a 5-fold increase in the number of apoptotic cells by 24 hours and a 15-fold increase in the number of apoptotic cells by 48 hours of serum deprivation (Figure 7D.; $p < 0.1$). Few cells were found floating in the media. Neurons treated in the presence of serum did not show an increase in the number of apoptotic cells, thus the length of time in culture did not have an effect on the level of apoptosis.

3. Cell Death Commitment Assays

In order to understand the temporal events associated with apoptosis in serum deprived human neuron cultures, the time in which these cultures became irreversibly committed to cell death was determined. Neurons cultured on coverslips for 11 days in the presence of serum were treated in the absence of serum containing media for 0h, 6h, 12h, 18h, 24h and 48h, after which serum containing media was reintroduced into the cultures for another 12 hours. The cells were stained by propidium iodide and the coverslips were scored for apoptotic cells following the criteria established previously. To show that the length of time in culture did not affect the level of cell death, coverslips treated in the presence of serum containing media for 0h, 6h, 12h, 18h, 24h, and 48h were also stained and scored in this manner. To control for the effect of the addition of fresh serum and the disturbance of feeding the cultures, neurons cultured on coverslips for 11 days in the presence of serum were treated in the presence of serum containing media for 0h, 6h, 12h, 18h, 24h and 48h, after which serum was reintroduced to the cultures for another 12 hours. These coverslips were stained and scored in the same manner as described. Based on data from at least three independent cultures (Figure 8.), it is evident that the length of time in culture and the process of reintroducing serum back to the neurons had no effect on the level of apoptosis, while neurons deprived of serum for greater than 6 hours could not be rescued from undergoing apoptotic cell death with the return of serum to the media. Statistically, the difference between the serum deprived culture and the control cultures became significant after 18 hours of serum deprivation. Thus, the time in which human neurons in culture become irreversibly committed to apoptosis following serum withdrawal is 18 hours.

Figure 7. - Characterization of Human Neurons in Serum Deprived Cultures

A) Morphological characterization of apoptotic cells in human neuron cultures by propidium iodide staining after 48 hours of serum deprivation. Shown at high magnification (100X, of a small field in 2B. - c), normal cells have diffusely stained, intact nuclei illustrated by the arrows, while apoptotic cells possess brightly stained, pyknotic and fragmented nuclei with condensed chromatin highlighted by the arrowheads.

B) Immunofluorescence analysis showing apoptosis in serum deprived human neuron cultures by propidium iodide and TUNEL staining. Cultured neurons on coverslips at 0 hour (0h) (a, b), deprived of serum containing media for 48 hours (48h) (c, d) and not deprived of serum containing media for 48h (e, f) were fixed and stained by propidium iodide (a, c, e - high magnification, 40X) or TUNEL (b, d, f - low magnification, 20X). Apoptotic cells with fragmented nuclei and condensed chromatin are highlighted by the arrowheads. Notice that there is only positive TUNEL staining present in (d). Coverslips in a, c, e are not the same as those shown in b, d, f.

C) Morphological characterization of serum deprived human neuron cultures by phase contrast and immunofluorescence analysis of tau staining. Cultured neurons on coverslips at 0h (a, b), deprived of serum containing media for 48h (c, d) and treated with serum containing media for 48h (e, f) were fixed, stained with rabbit polyclonal anti-tau antibody and analyzed by phase contrast (a, c, e) and immunofluorescence (b, d, e) microscopy. Contrast the uniform, polarized staining of the cell body and neurites in the neurons shown in (b, f - arrows) to the non-uniform, diffuse staining of the cell body and beading of the dystrophic neurites in the neurons shown in (d - arrows). The fields outlined by the dashed boxes in (a, c, e) correspond to (b, d, f) at high magnification (40X), respectively.

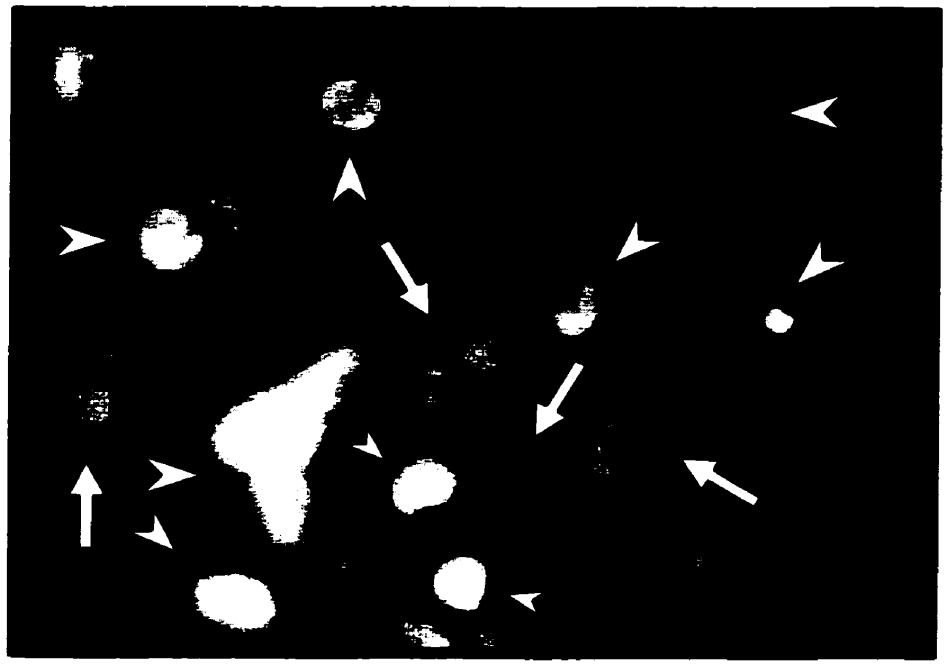
D) Quantitative analysis of apoptosis in serum deprived human neuron cultures by TUNEL. Neurons cultured on coverslips were deprived and not deprived of serum containing media for 0, 12, 24 and 48h, fixed, labeled by TUNEL and scored for apoptotic cells by high magnification (40X) fluorescence microscopy. The data

represents the fold increase in the number of apoptotic cells at 12h, 24h and 48h relative to the control (0h). Time points in which the fold increase in apoptotic cells in the serum deprived cultures (closed diamonds, - **Serum**) is statistically significant from the corresponding cultures treated in the presence of serum (open circles, + **Serum**) are marked by asterisks ($p < 0.10$). The data shown is the mean and standard error of three independent cultures.

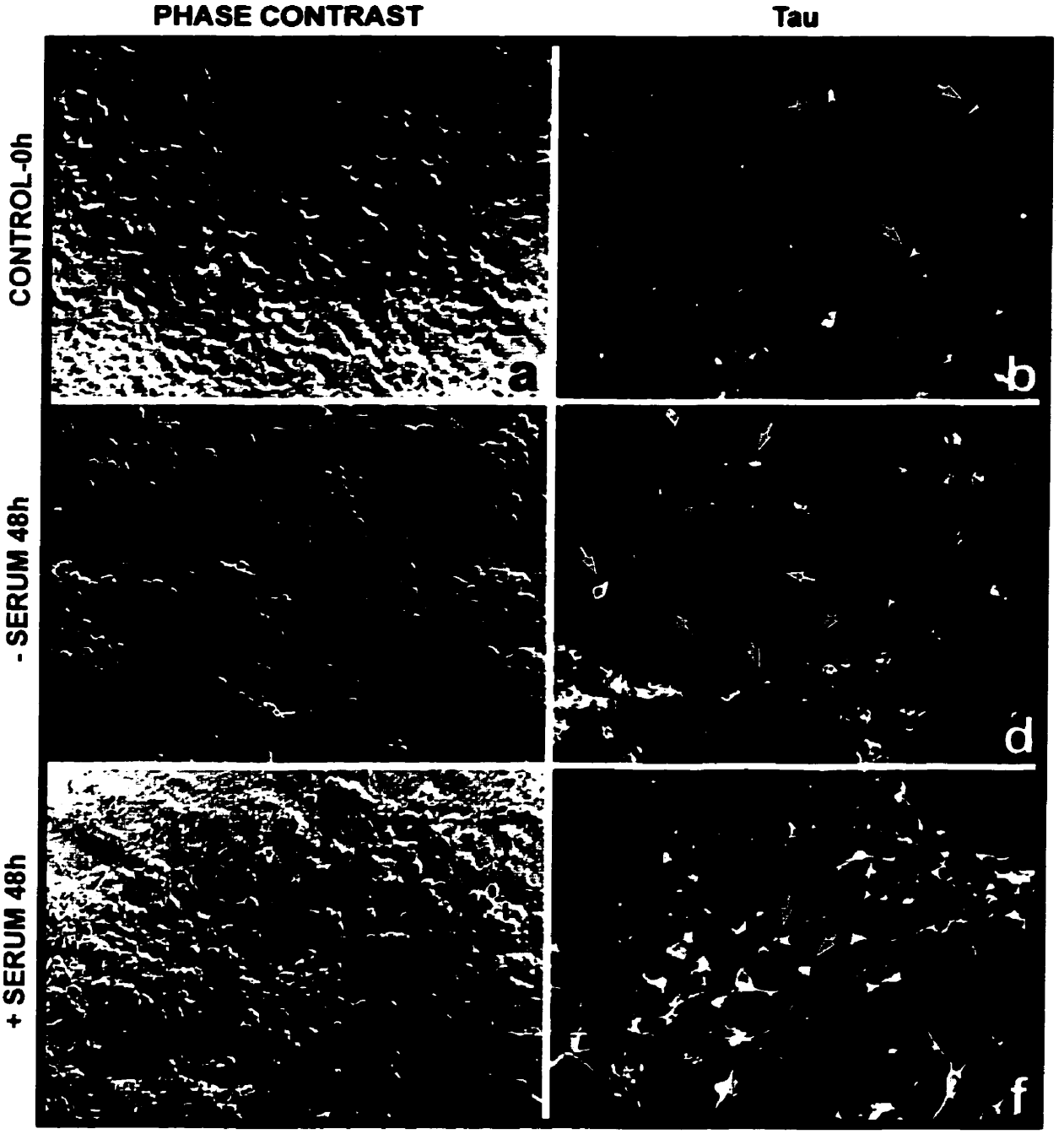
A

PROPIDIUM IODIDE

- SERUM 48h



C



D

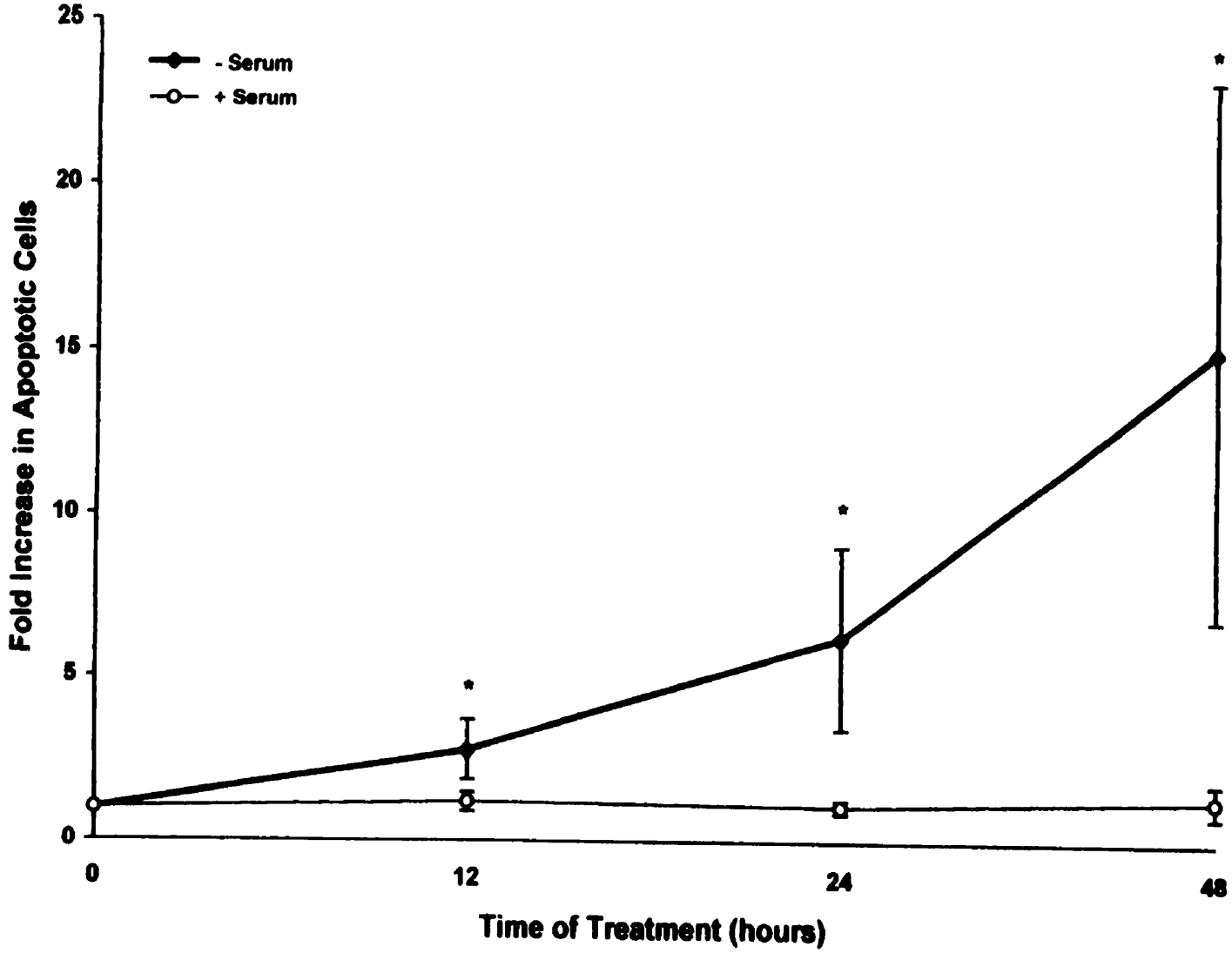
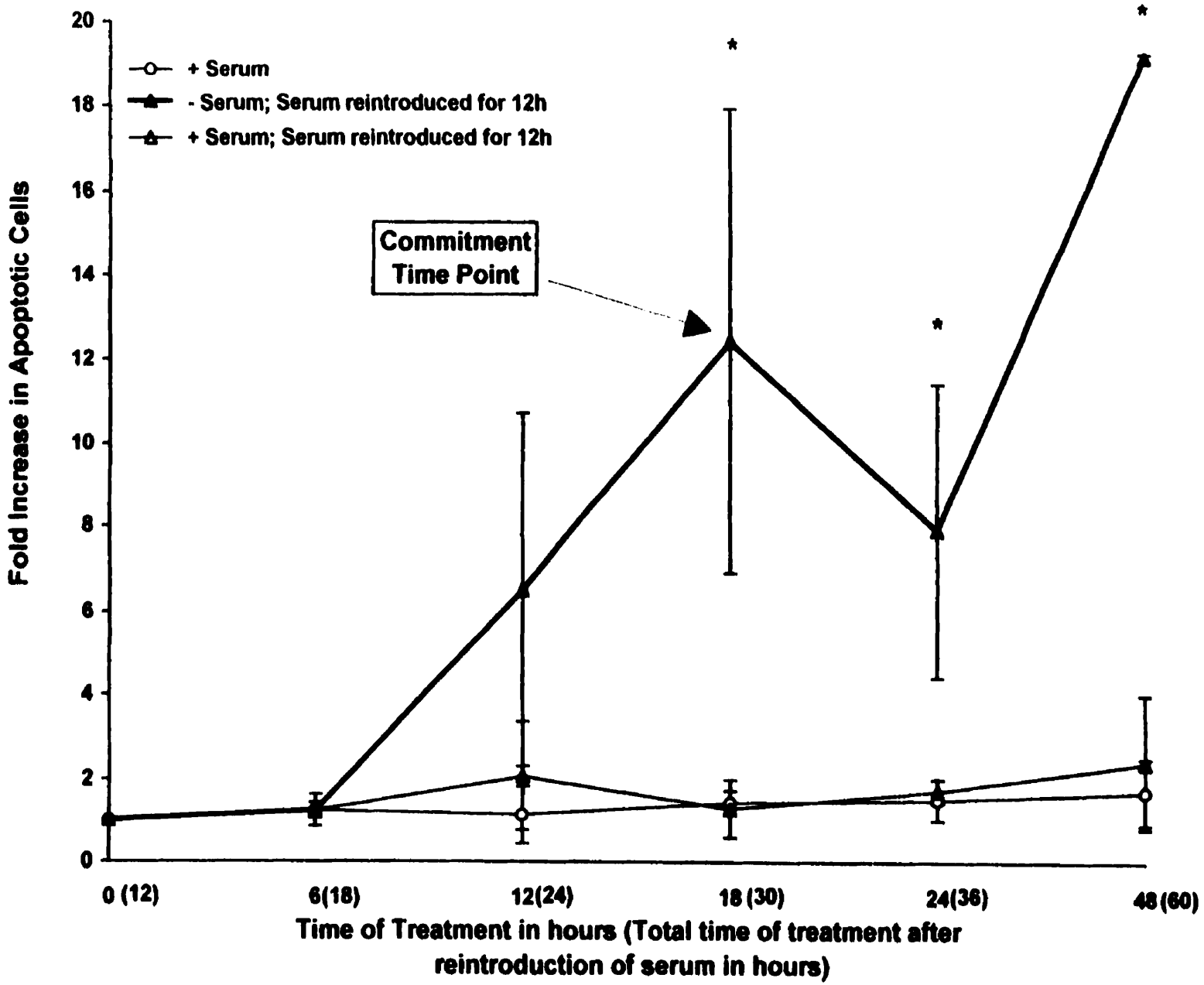

25
20
15
10
5
0
- Serum
+ Serum
Fold Increase in Apoptotic Cells
*
*
*
0
12
24
48
Time of Treatment (hours)

Figure 8. - Quantitative Analysis of the Time of Commitment to Apoptosis in Serum Deprived Human Neuron Cultures

Neurons cultured on coverslips were deprived of serum containing media for 0h, 6h, 12h, 18h, 24h and 48, after which serum containing media was reintroduced for an additional 12h (represented as **closed triangles, - Serum; Serum reintroduced for 12h).** Sister cultures were treated in the presence of serum containing media for 0h, 6h, 12h, 18h, 24h and 48h, after which serum containing media was reintroduced for an additional 12h (represented as **open triangles, + Serum; Serum reintroduced for 12h**). The cells were fixed after this 12h period (the total time of treatment is shown in brackets on the bottom of the graph), stained with propidium iodide and scored for the number of apoptotic cells by high magnification (40X) fluorescence microscopy. Additionally, neurons on coverslips were treated with serum containing media at 0h, 6h, 12h, 18h, 24h and 48h, fixed, stained and scored in the same manner as described (shown as **open circles, + Serum**). The data shown represents the fold increase in the number of apoptotic cells at each time point relative to 0h. The commitment time point represents the time of serum deprivation after which the replacement of serum cannot prevent the cells from undergoing apoptosis. The commitment time point is shown to be 6h in human neuron cultures deprived of serum containing media, after which there is an increase in the number of apoptotic cells in comparison to the cultures treated with serum containing media. Each point represents the mean and standard error of at least three independent cultures. Asterisks mark the time points in which the fold increase in apoptotic cells in the serum deprived coverslips is statistically significant in comparison to the coverslips cultured in serum containing media ($p < 0.1$).


+ Serum
- Serum; Serum reintroduced for 12h
+ Serum; Serum reintroduced for 12h
Commitment Time Point
Fold Increase in Apoptotic Cells
0
2
4
6
8
10
12
14
16
18
20
0 (12)
6(18)
12(24)
18 (30)
24(36)
48 (60)
Time of Treatment in hours (Total time of treatment after reintroduction of serum in hours)

4. Immediate Early Gene Expression During Neuronal Cell Death

The expression of the immediate early genes, *fos* and *jun*, has been reported as a necessary event for apoptosis to occur in rodent neuron cultures (Estus et al., 1994; Ham et al., 1995). To determine immediate early gene expression in human neuron cultures, immediate early gene expression was analyzed in serum deprived neuron cultures by RT-PCR. Total RNA was extracted from cultures treated in the absence of serum containing media for 0h, 6h, 12h, 18h and 24h. One μg of total RNA from each sample was reverse transcribed into cDNA and 1μl of the resultant cDNA mixture was amplified by PCR with primers specific for *c-fos*, *c-jun* and *G3PDH* for 20 and 30 cycles in parallel with a positive control (human c-fos plasmid, mouse c-jun plasmid and rat G3PDH plasmid) and three negative controls (1 μl of cDNA mixture from the 12h serum deprived sample treated in the absence of reverse transcriptase, 1μg of tRNA, and 1 μl of water). To demonstrate that the effect of the addition of fresh serum and the disturbance associated with feeding did not induce the expression of *c-fos*, *c-jun* and *G3PDH*, total RNA was also extracted from sister neuron cultures whose serum containing media was changed at the same time as the serum deprived cultures. Following separation by electrophoresis on 1% agarose gels, the PCR products indicating the levels of expression of *c-fos*, *c-jun* and *G3PDH* were visualized by EtBr staining (Figure 9A.). PCR products were only detected by ethidium bromide staining after 30 cycles of amplification and verification these PCR products were amplified from the genes of interest was obtained by restriction enzyme mapping (Figure 9B.) and sequence analysis (data not shown).

The identity of the *c-fos*, *c-jun* and *G3PDH* products was confirmed by Southern blotting. After transfer to nylon membranes, *c-fos*, *c-jun* and *G3PDH* specific products were detected by hybridization with ^{32}P-dATP labeled DNA probes specific for each PCR product, while non-specific amplification products were not. Thus, the use of nested primers was not required to enhance the sensitivity or specificity of the PCR reaction. Blots were exposed on phosporimaging screens and the PCR products were quantitated by phosphorimaging software. The level of expression of *c-fos* and *c-jun* in the absence and presence of serum was expressed as a ratio relative to *G3PDH* because the expression level of this gene is constant, typical of a 'housekeeping' gene and serves as a

control for the amount of cDNA obtained from each sample. The expression levels of *c-fos* and *c-jun* for the various times of treatment was also standardized to the expression levels at 0h to establish a baseline level of expression for each culture. The level of *c-fos* and *c-jun* expression from four independent experiments are presented as individual graphs in Figure 9C and Figure 9D because the time of induction and maximal level of expression varied in each experiment, which did not permit an overall average of the of the data and a generalization for the time of induction of *c-fos* and *c-jun* in human neuron cultures following serum deprivation. The data presented in the graphs represents the mean of two trials within a given experiment.

In each of the four experiments there was an induction of *c-fos* expression following serum deprivation (Figure 9C.). The maximal increase of *c-fos* expression occurred after 6 hours of serum deprivation in experiments 1 and 3. Despite the coincident time of induction of *c-fos* expression in experiments 1 and 3, there was a 10-fold increase of *c-fos* in experiment 1 and a 2.5-fold increase in experiment 3. In experiment 2, the peak level of *c-fos* expression, a 4.5-fold increase, occurred after 12 hours of serum deprivation, while in experiment 4 a 10-fold increase occurred after 18 hours of serum deprivation.

In each of the four experiments there was also an induction of *c-jun* expression (Figure 9D.). In experiment 1 the level of *c-jun* expression appeared cyclic. The peak level of *c-jun* expression occurred after 6 hours of serum deprivation, which was followed by a decline at the 12h time point and a subsequent increase in expression at the 24h time point. The maximal level of *c-jun* expression was an 8-fold increase in experiment 1. Experiment 3 showed a 5-fold maximal increase in *c-jun* after 6 hours of serum deprivation, while experiment 2 showed a 12-fold increase after 12 hours of serum deprivation. In experiment 4 there was a steady increase in the level of *c-jun* expression following 6 hours of serum deprivation that peaked to a 5-fold increase by 24 hours of serum deprivation.

Within each experiment there was a coincident peak in the level of expression of *c-fos* and *c-jun*, but the maximal level of expression differed. In experiment 1 the peak level of *c-fos* and *c-jun* expression occurred after 6 hours of serum deprivation with a 10-

fold increase for *c-fos* and a 8-fold increase for *c-jun*. Additionally, the expression pattern for *c-fos* was not cyclic. After 12 hours of serum deprivation, the levels of *c-fos* and *c-jun* peaked in experiment 2, with a 4.5-fold increase in *c-fos* and a12-fold increase in *c-jun*. In experiment 3 the level of expression for *c-fos* and *c-jun* peaked after 6 hours of serum deprivation, with a 2.5-fold increase in *c-fos* and a 5-fold increase in *c-jun*. The expression *c-fos* and *c-jun* in experiment 4 did not peak at the same time following serum withdrawal, however, the expression patterns showed a similar trend, with *c-fos* reaching a maximum level (10-fold increase) by 18 hours and *c-jun* reaching a 5-fold increase by 24 hours after serum deprivation.

Based on the data, there appears to be no trend as to whether neurons in culture are induced to express higher amounts of *c-fos* or *c-jun* transcripts following serum deprivation, given that in two of four experiments (Experiments 1 and 4) the maximum level of *c-fos* expression is greater than the maximum level of *c-jun* expression, while in the other two experiments (Experiments 2 and 3) the level of *c-jun* expression is greater than the level of *c-fos*. Furthermore, there is no consistent time of induction of the immediate early genes in neuron cultures, given that in two of four experiments the peak levels of expression occurred after 6 hours, while the other experiments the peak levels occurred at 12 and 18 hours after serum deprivation. The differences in the time and the level of expression of *c-fos* and *c-jun* can be attributed to the genetic differences between the cultures for each experiment, possibly the age of the fetal brain when it was cultured or differences in the cellular components of the neuronal subtypes in the cultures. However, it is clear that the expression of *c-fos* and *c-jun* was induced in neuron cultures deprived of serum, and it is important to note that neither the time in culture, the addition of fresh serum or the disturbance of feeding induced the expression of *c-fos* or *c-jun* in each of the four experiments (Figure 9C and 9D).

Figure 9. - RT-PCR Analysis of Immediate Early Gene Expression in Serum Deprived Human Neuron Cultures

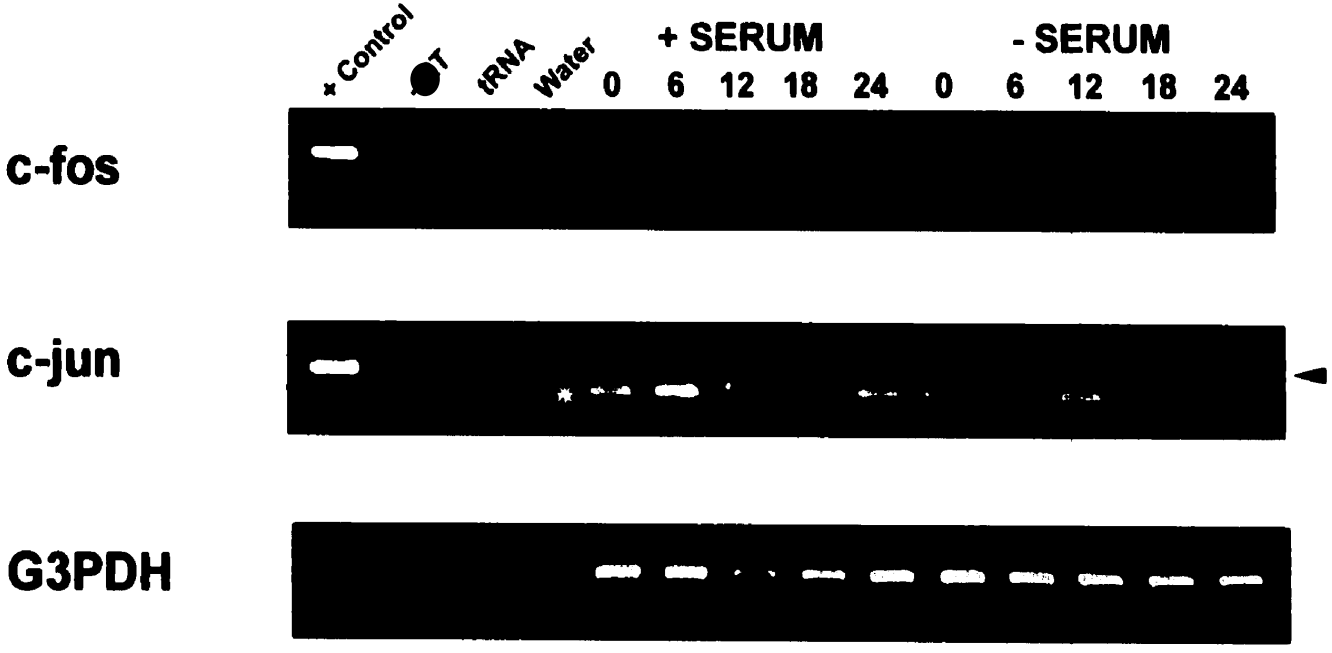
A) Expression of *c-fos*, *c-jun* and *G3PDH* in human neuron cultures. Total RNA was extracted from neuron cultures deprived of serum containing media (**- Serum**) and cultures treated with serum containing media (**+ Serum**) for 0h, 6h, 12h, 18h and 24h. Following reverse transcription into cDNA, 1µl of cDNA from each sample was amplified by *c-fos*, *c-jun* and *G3PDH* specific primers by PCR for 20 and 30 cycles. The PCR products were separated by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining. The pattern of expression after 30 cycles of amplification is shown.. The arrowhead highlights the *c-jun* PCR product, while the asterisk marks a non-specific amplification product. No amplification was detectable after 20 cycles with EtBr.

B) Restriction enzyme mapping to verify the identity of the PCR products. **Lane 1**- 633 bp PCR product of *c-fos;* **Lane 2**- 382 bp and 251 bp Xcm I restriction digest products of the 633 bp *c-fos* PCR product shown in lane 1; **Lane 3**- 409 bp PCR product of *c-jun*; **Lane 4**- 236 bp and 173 bp Xcm I restriction digest products of the 409 bp *c-jun* PCR product shown in lane 3; **Lane 5**- 570 bp PCR product of *G3PDH*; **Lane 6**- 366 bp, 106 bp and 98 bp Hind III restriction digest products of the *G3PDH* PCR product shown in lane 5. The arrowheads highlight the restriction digest products and the asterisk marks a non-specific PCR product.

C) Quantitative analysis of c-fos expression in serum deprived human neuron cultures from four independent experiments. PCR products were transferred to nylon membrane and probed with c-fos and G3PDH specific ^{32}P-labeled DNA probes. Blots were exposed on phosphorimaging screens and the PCR products quantitated. A ratio of the level of c-fos expressed to the level of G3PDH expressed was calculated for each sample (**- Serum** and **+ Serum**) at the corresponding time points to determine the level of c-fos expression relative to the level of G3PDH expression. The graphs show the ratio of the level of c-fos expression to the level of G3PDH expression normalized to the ratio of c-fos/G3PDH level of expression at the 0h time point and is expressed as a fold increase, and represents the mean of two trials in a given experiment.

D) Quantitative analysis of c-jun expression in serum deprived human neuron cultures from four independent experiments. PCR products were treated as described above. The graphs show the ratio of the level of c-jun expression to the level of G3PDH expression normalized to the ratio of c-jun/G3PDH level of expression at the 0h time point and is expressed as a fold increase, and represents the mean of two trials in a given experiment.

A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Water
+ SERUM
- SERUM
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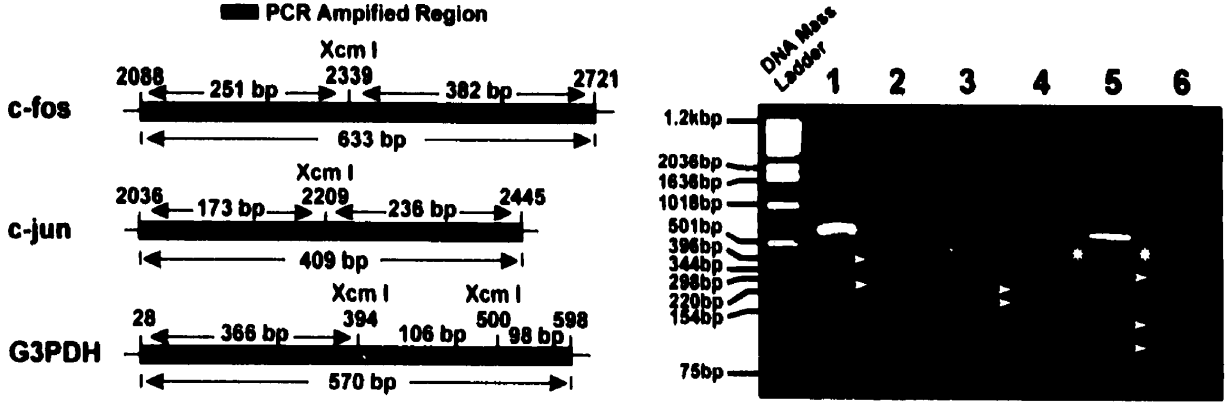
c-fos
c-jun
G3PDH

B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2339
382 bp
2721
633 bp
c-fos
Xcm I
2036
173 bp
2209
236 bp
2445
409 bp
c-jun
Xcm I
Xcm I
28
366 bp
394
106 bp
500
98 bp
598
570 bp
G3PDH
DNA Mass Ladder
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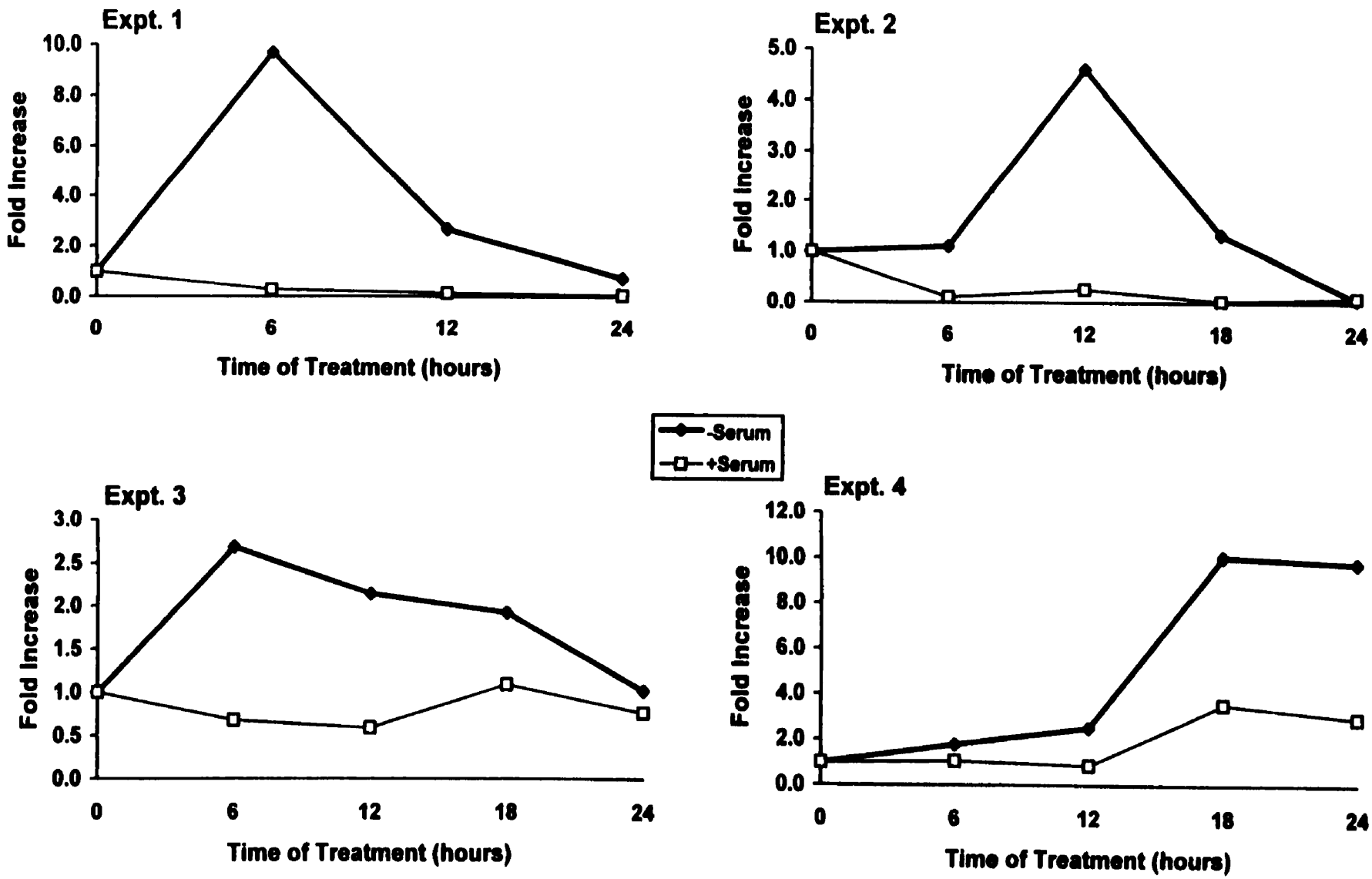
220bp
154bp
75bp

C


Expt. 1
Fold Increase
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8.0
6.0
4.0
2.0
0.0
0
6
12
24
Time of Treatment (hours)
Expt. 2
Fold Increase
5.0
4.0
3.0
2.0
1.0
0.0
0
6
12
18
24
Time of Treatment (hours)
-Serum
+Serum
Expt. 3
Fold Increase
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
6
12
18
24
Time of Treatment (hours)
Expt. 4
Fold Increase
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6.0
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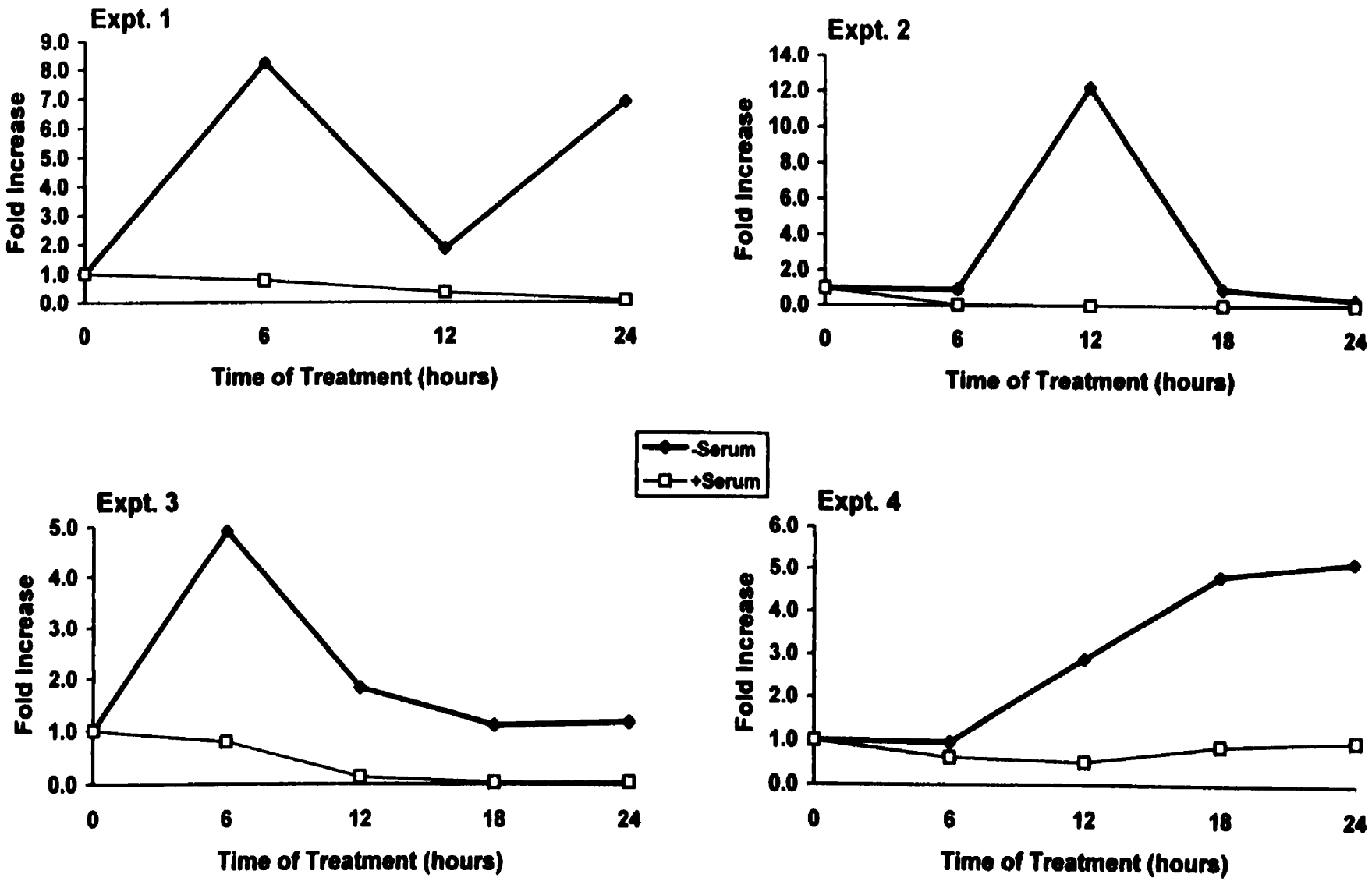
2.0
0.0
0
6
12
18
24
Time of Treatment (hours)

D


Expt. 1
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3.0
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1.0
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0
6
12
24
Time of Treatment (hours)
Expt. 2
Fold Increase
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0
6
12
18
24
Time of Treatment (hours)
-Serum
+Serum
Expt. 3
Fold Increase
5.0
4.0
3.0
2.0
1.0
0.0
0
6
12
18
24
Time of Treatment (hours)
Expt. 4
Fold Increase
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
6
12
18
24
Time of Treatment (hours)

5. Induction of c-Jun Protein During Neuronal Cell Death

To investigate the expression pattern of c-Fos and c-Jun during apoptosis in human neurons, nuclear extracts were prepared from cultures treated in the absence of serum containing media for various periods of time (0h, 6h, 12h, 18h, 24h) following 11 days of culture in the presence of serum. Nuclear extracts were also prepared from neurons cultured for 11 days and treated in the presence of serum for 0h, 6h, 12h, 18h, and 24h. The concentration of protein in each of the samples was quantitated by a BCA colourmetric assay and spectrophotometry. Fifty μg of protein extract from each sample was separated in parallel with 10 μg of nuclear extract prepared from phorbol ester induced HeLa cells by SDS-PAGE. Following transfer, blots were probed with polyclonal anti-c-Jun antibodies and monoclonal anti-c -Fos antibodies.

C-Jun was clearly detected as a 39 kDa protein in the phorbol ester induced HeLa nuclear extract, but not in normal neuron cultures (Figure 10A.). The level of c-Jun increased significantly in neuron cultures deprived of serum (Figure 10A., - SERUM) compared to those treated in the presence of serum (Figure 10A., + SERUM) to a maximum 3.5-fold after 18 hours of serum deprivation, and decreasing to normal levels by 24 hours. The increase in c-Jun began 6h following serum withdrawal and peaked at 18h, decreasing thereafter to initial levels by 24h (Figure 10C.). This increase was accompanied with an apparent change in the mobility of c-Jun (Figure 10A., - SERUM, arrow) as previously observed for activated and phosphorylated c-Jun (Ham et al., 1995). There was another strong lower molecular weight cross-reacting species detected by the c-Jun antibody in each of the neuronal samples, but not in the HeLa nuclear extracts. The identity of this band is not clear at this time (Figure 10A., - SERUM, asterisk). Induction of c-Jun and a change in its mobility did not occur in neurons treated in the presence of serum (Figure 10B., + SERUM).

The expected 62 kDa protein for c-Fos was not detected in any of the samples treated in the absence or presence of serum, while only a weak signal was detected in the phorbol ester induced HeLa nuclear extract (Figure 10B.). Two additional antibodies specific for c-Fos, a monoclonal and a polyclonal, were utilized and failed to show c-Fos induction following serum deprivation.

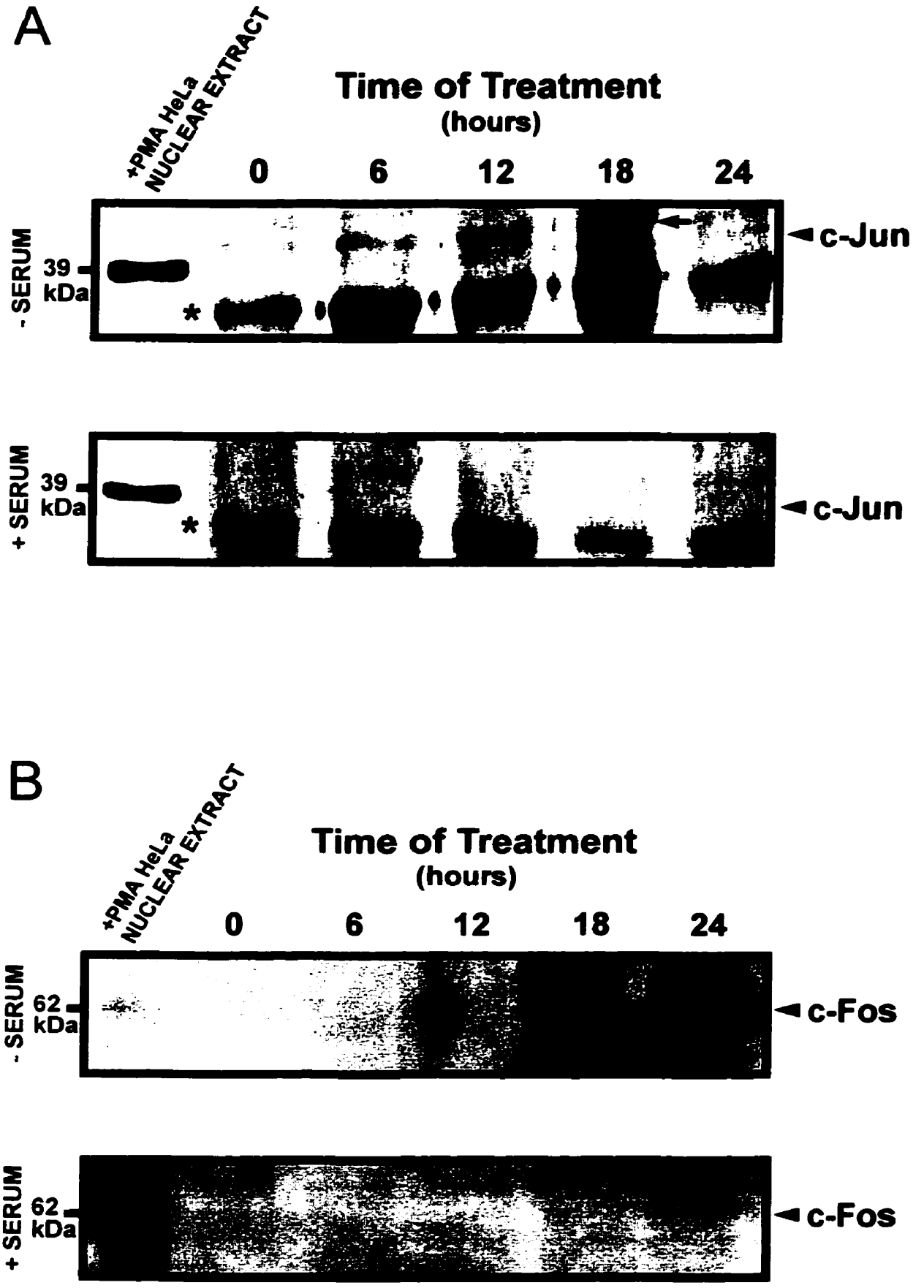
Figure 10. - Western Blot Analysis Showing the Induction of c-Jun Protein in Serum Deprived Human Neuron Cultures

Nuclear extracts were prepared from neuron cultures deprived of serum and control (serum fed) cultures for 0h, 6h, 12h, 18h and 24h. Fifty μg of nuclear extract was separated by SDS-PAGE in parallel with 10 μg of nuclear extract prepared from phorbol ester induced HeLa cells. After transfer, the blots were probed with a polyclonal anti-c-Jun antibody and a monoclonal anti-c-Fos antibody.

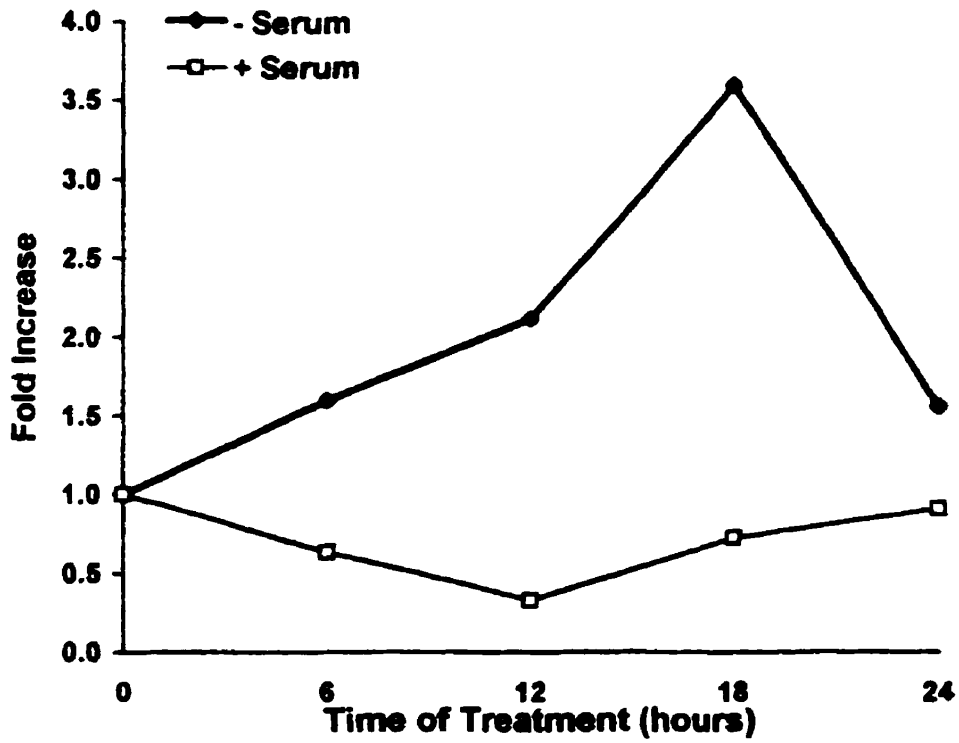
A) A 39 kDa band corresponding to c-Jun was detected in the phorbol ester induced HeLa nuclear extract lane. Notice the appearance of a band corresponding to c-Jun by 6h of serum deprivation, followed by an increase in intensity and shift in mobility of the band by 18h of serum deprivation. No appearance of c-Jun is apparent in the control (serum fed) cultures. The asterisk marks a nonspecific band detected by the anti-c-Jun antibody.

B) A 62 kDa band corresponding to c-Fos was faintly detected in the phorbol ester induced HeLa nuclear extract lane. The presence of c-Fos protein was not detected in the serum deprived and serum fed cultures.

C) Quantitative analysis of c-Jun expression in serum deprived neuron cultures. The bands corresponding to c-Jun and phospho-c-Jun (arrow) were quantitated as a whole by densitometer. The data shows the fold increase in the level of c-Jun expression at the respective times of serum deprivation relative to 0h in a single experiment.


A
+PMA HeLa NUCLEAR EXTRACT
Time of Treatment
(hours)
0
6
12
18
24
- SERUM
39 kDa
c-Jun
+ SERUM
39 kDa
c-Jun
B
+PMA HeLa NUCLEAR EXTRACT
Time of Treatment
(hours)
0
6
12
18
24
- SERUM
62 kDa
c-Fos
+ SERUM
62 kDa
c-Fos

C


- Serum
+ Serum
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Fold Increase
0
6
12
18
24
Time of Treatment (hours)

6. Expression of Amyloid Precursor Protein During Neuronal Cell Death

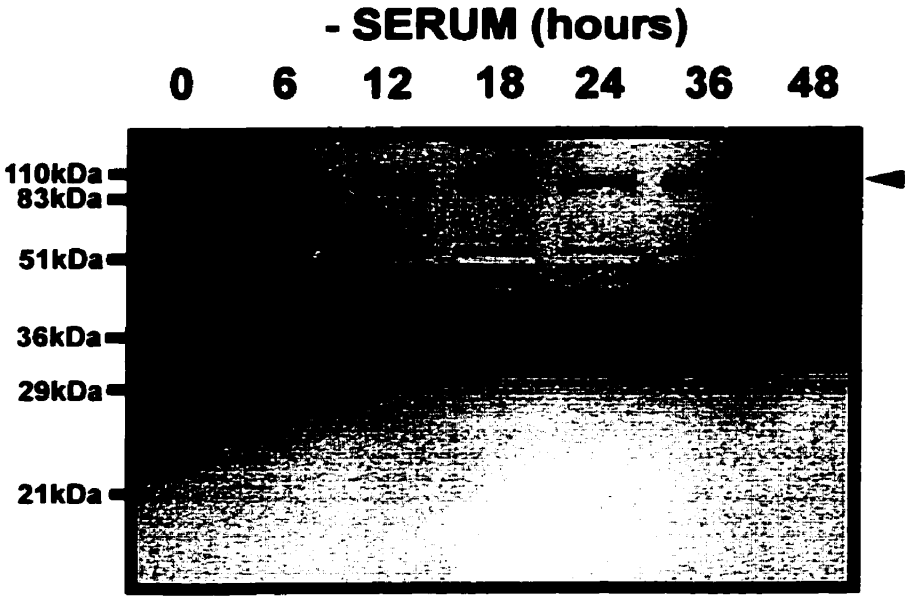
The APP promoter contains AP-1 binding sites which are under the control of c-Fos and c-Jun (Trejo et al., 1994). To determine if the induction of the *c-fos* and *c-jun* influenced the expression of APP, whole cell extracts were prepared from neurons cultured for 11 days in the presence of serum and treated in the absence of serum for 0h, 6h, 12h, 18h, 24h, 36h and 48h. Extracts were not prepared from cultures treated in the presence of serum because the addition of fresh serum and the disturbance of changing the media did not have an effect on the expression of *c-fos* and *c-jun* as previously shown. Equal amounts of protein were not separated because sample sizes were small and the extract was harvested in a small volume such that conventional protein assays could not accurately determine the protein content in the samples. Instead, equal volumes of each extract was separated by SDS-PAGE since cells are prevented from lifting and floating away by the network of neurites and neuronal loss in cultures is not significant until 72 hours of serum deprivation (LeBlanc, 1995). After the proteins were transferred to nylon membranes, separate blots were probed with polyclonal anti-APP (anti-A1,4) and monoclonal anti-$A\beta_{1\text{-}16}$ (6E10) antibody for APP. APP appears as a condensed group of bands at 97 kDa representing the APP_{695} isoform (Figure 11A.). The level of APP expression at each time point for all the APP isoforms was quantitated by densitometer and expressed as a fold increase relative to the level of expression at 0h (Figure 11B.). Based on three independent cultures tested with the 6E10 and anti-A1,4 antibodies, there was a slight 1.5-fold increase in APP levels after 18 hours of serum deprivation that decreased slightly by 48 hours. However, no statistically significant changes in the level of APP expression were determined.

Figure 11. - Western Blot Analysis of APP Expression in Serum Deprived Human Neuron Cultures

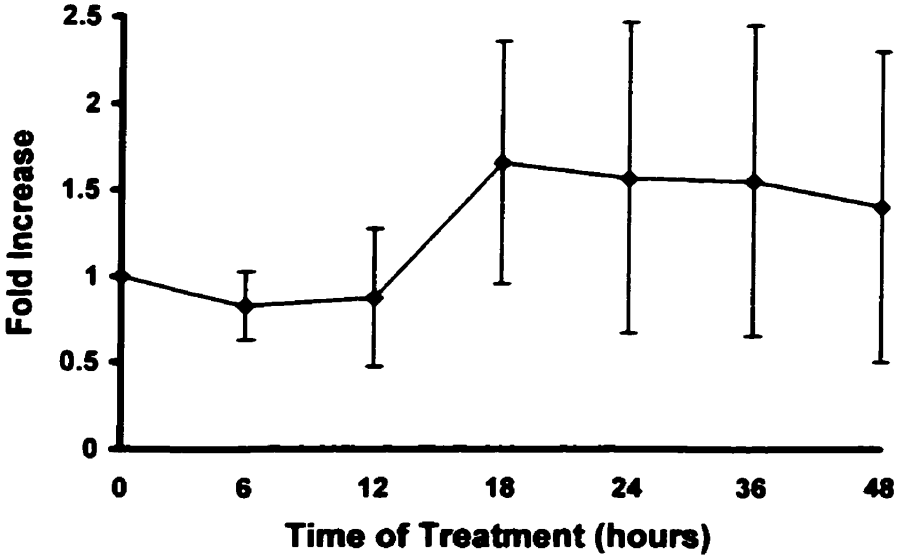
A) Whole cell extracts were prepared from neuron cultures deprived of serum for 0h, 6h, 12h, 18h, 24h, 36h and 48h and equal volumes (12 μl, representative of 3.6×10^5 cells) of the extracts were separated by SDS-PAGE. After transfer, blots from three independent cultures were separately probed for APP with polyclonal anti-APP (anti-A1,4) antibody and monoclonal anti-$A\beta_{1-16}$ (6E10) antibody. APP appears as dense group of bands, representing the different isoforms of APP, at 97 kDa (arrowhead).

B) Quantitative analysis of APP expression in serum deprived neuron cultures. The entire group of bands at 97 kDa on the blots were quantitated by densitometer. The data shows the fold increase in the level of APP expression at the respective times of serum deprivation relative to 0h. The data represents the mean and standard error of three independent cultures. No statistically significant changes in the level of APP expression were detected.

A


- SERUM (hours)
0 6 12 18 24 36 48
110kDa
83kDa
51kDa
36kDa
29kDa
21kDa

B


Fold Increase
2.5
2
1.5
1
0.5
0
0 6 12 18 24 36 48
Time of Treatment (hours)

7. Cytoskeletal Protein Degradation During Neuronal Cell Death

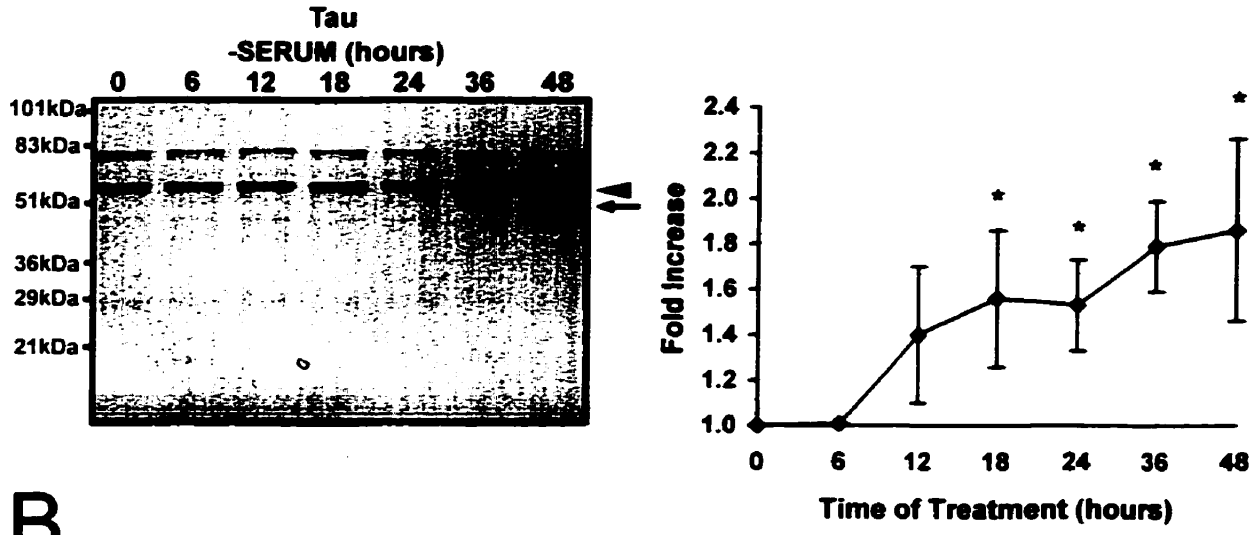
Serum deprivation induces apoptosis in human neurons and results in the formation of dystrophic neurites and alterations in the neuronal cell body, which is evident from tau immunostaining shown in Figure 7C. To determine if neuronal cytoskeletal proteins undergo changes in expression levels and/or proteolytic degradation during apoptosis, the level of expression and the presence of degradation products of tau, tubulin and vimentin was assessed by Western blots. Whole cell extracts were prepared from neurons cultured for 11 days in the presence of serum and subsequently deprived of serum containing media for 0h, 6h, 12h, 18h, 24h, 36h and 48h. Extracts were not prepared from cultures treated in the presence of serum because as previously shown, cultures treated in the presence of serum for 48h do not undergo apoptosis. Equal volumes of protein extracts were separated by SDS-PAGE and transferred to nylon membranes. Blots were probed with polyclonal anti-tau antibody, polyclonal anti-tubulin antibody and monoclonal anti-vimentin antibody. The level of expression of each protein at each time point was quantitated by densitometer and expressed as a fold increase relative to the level of expression at 0h. As expected, fetal tau is detected as one band at 54 kDa (Figure 12A., arrowhead). The additional band at approximately 80 kDa may represent the known cross-reactivity of this polyclonal anti-tau antibody to MAP2 (Dr. H. Paudel, personal communication). We observed additional bands of 48-50 kDa at 36 and 48 hours of serum deprivation, possibly the result of proteolytic degradation of tau (Figure 12A., arrow). In three of four independent cultures, degradation products of tau were detected, however, the time following serum withdrawal in which these degradation products appeared varied, some appearing as early as 12h in two experiments. In contrast, no degradation products of tubulin (56 kDa) and vimentin (58 kDa) were detected (Figure 7B. and 7C.).

In addition, the expression level of tau increased following serum deprivation. By 18h of serum deprivation there was a statistically significant increase in the level of tau, which continued to a maximal two-fold increase by 48h (Figure 7A.; $p < 0.1$). There was a slight 1.5-fold, but not a statistically significant increase in the levels of tubulin and vimentin expression (Figure 12B. and 12C.).

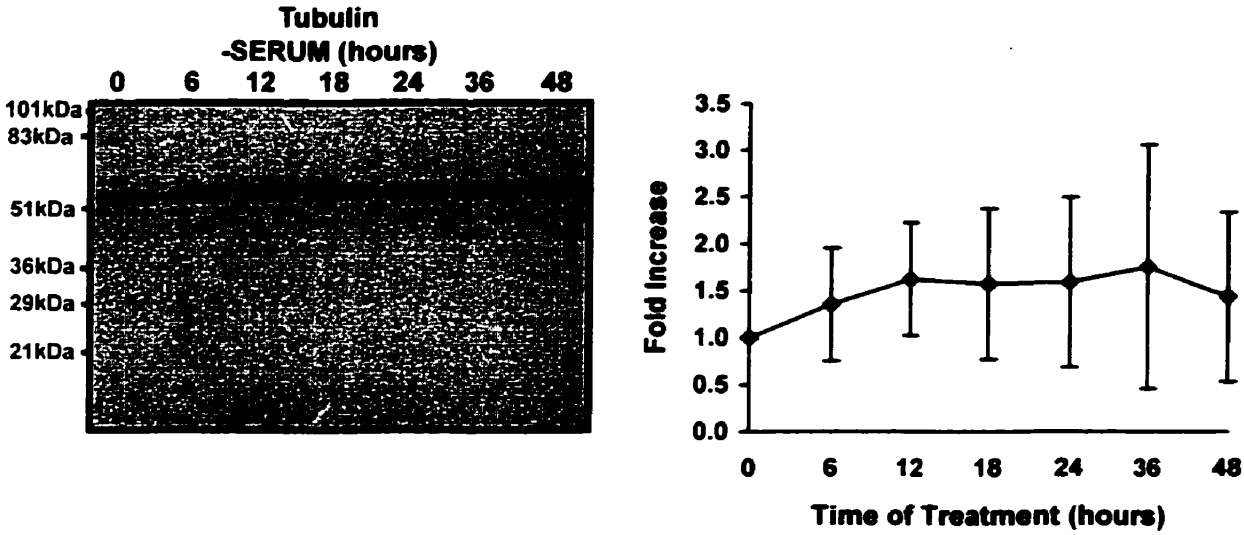
Figure 12. - Western Blot Analysis of Cytoskeletal Proteins in Serum Deprived Human Neuron Cultures

Whole cell extracts were prepared from neuron cultures deprived of serum for 0h, 6h, 12h, 18h, 24h, 36h and 48h and equal volumes (6 µl, representative of 1.8×10^5 cells) of the extracts were separated by SDS-PAGE. After transfer, blots were probed with polyclonal anti-tau antibody, polyclonal anti-Tubulin antibody and monoclonal anti-vimentin antibody. **A)** Fetal tau is detected as a 54 kDa band (arrowhead). The arrow marks the appearance of degradation products. **B)** Tubulin is detected as a 53 kDa band, while vimentin **(C)** is detected as a 55kDa band. The graphs represent the level of protein expression at the respective times of serum deprivation relative to the protein level at 0h in **(A, B, C)** as determined by densitometer. The data represents the mean and standard error of four independent cultures. Statistically significant changes in the level of protein expression are marked by asterisks ($p < 0.1$).

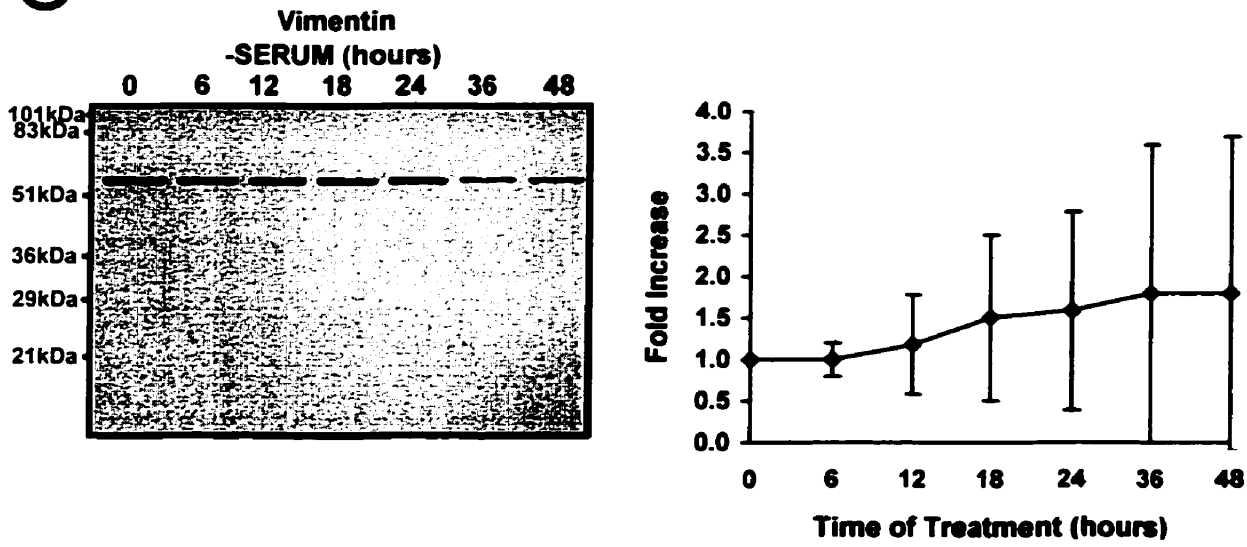
A


Tau
-SERUM (hours)
0 6 12 18 24 36 48
101kDa
83kDa
51kDa
36kDa
29kDa
21kDa
Fold Increase
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0 6 12 18 24 36 48
Time of Treatment (hours)

B


Tubulin
-SERUM (hours)
0 6 12 18 24 36 48
101kDa
83kDa
51kDa
36kDa
29kDa
21kDa
Fold Increase
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 6 12 18 24 36 48
Time of Treatment (hours)

C


Vimentin
-SERUM (hours)
0 6 12 18 24 36 48
101kDa
83kDa
51kDa
36kDa
29kDa
21kDa
Fold Increase
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 6 12 18 24 36 48
Time of Treatment (hours)

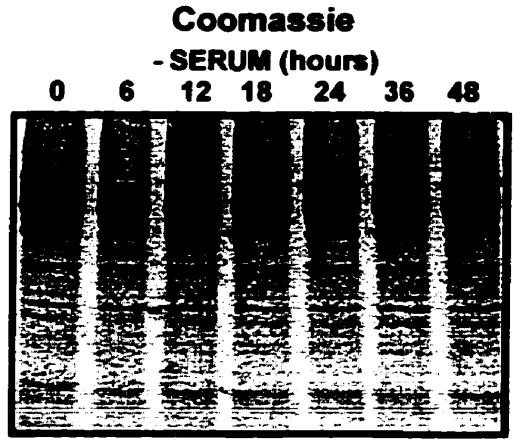
8. Expression of Neuron-Specific Proteins During Neuronal Cell Death

The neuron-specific proteins Gap-43 and synaptophysin undergo changes in expression during the pathogenesis of AD (Masliah and Terry, 1993), in which apoptosis is suspected to be the mode cell death (reviewed by Cotman, 1994; reviewed by Johnson, 1994). The expression levels of Gap-43 and synaptophysin neuron cultures during apoptosis were analyzed by Western blots. Whole cell extracts were prepared as described in the section assessing cytoskeletal proteins. Equal volumes of the extracts were separated by SDS-PAGE and gels were stained by Coomassie blue to show equal loading and consistency in the protein content in the cultures over the time course of treatment. Following transfer, blots were probed with monoclonal anti-Gap-43 antibody and monoclonal anti-synaptophysin protein. As shown in Figure 13A., there was equal loading of the amount of protein for each time point and the total protein content remained consistent throughout the time course of serum deprivation. There was a significant decrease in the expression level of Gap-43 by 6h of serum deprivation which was followed by a continuous and statistically significant increase in the expression level that reached a maximal 3-fold increase by 36h (Figure 13B., $p < 0.1$). The expression of synaptophysin showed a statistically significant decrease by 12h of serum deprivation, and continued to decline to 90% of starting levels by 48h of serum deprivation (Figure 13C., $p < 0.1$).

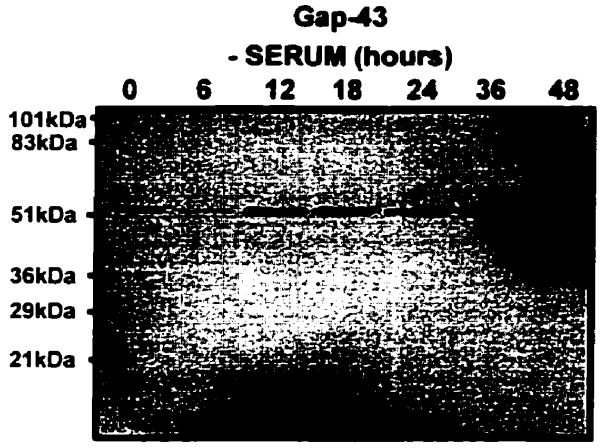
Figure 13. - Western Blot Analysis of Neuron-Specific Proteins in Serum Deprived Human Neuron Cultures

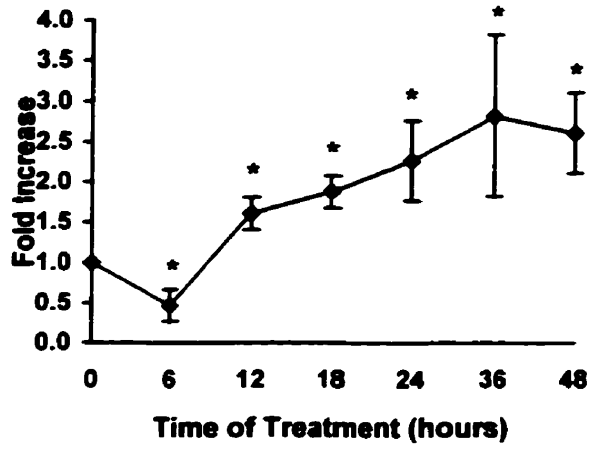
Whole cell extracts were prepared from neuron cultures deprived of serum for 0h, 6h, 12h, 18h, 24h, 36h and 48h and equal volumes (6µl, representative of 1.8×10^5 cells) of the extracts were separated by SDS-PAGE. Total protein content is shown by Coomassie blue staining **(A)**. After transfer, blots were probed with monoclonal anti-Gap-43 antibody to detect a 53kDa protein **(B)** and monoclonal anti-synaptophysin antibody to detect a 38kDa protein **(C, arrowhead)**. Graphs represent the protein levels at each time of serum deprivation as quantitated by densitometer, and is expressed relative to the protein level at 0h. Statistically significant changes in protein levels are marked by asterisks. The data represents the mean and standard error of four independent cultures.

A

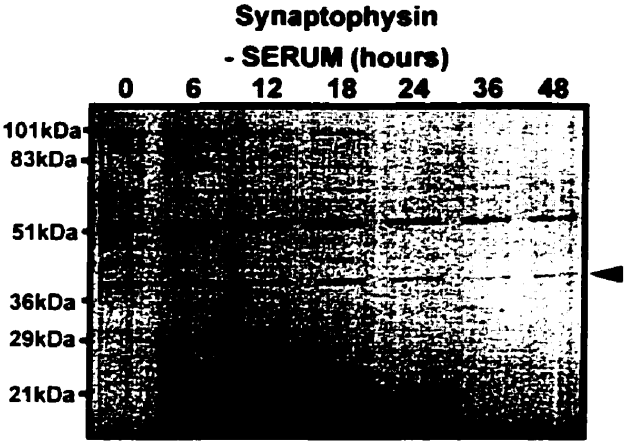

Coomassie
- SERUM (hours)
0 6 12 18 24 36 48

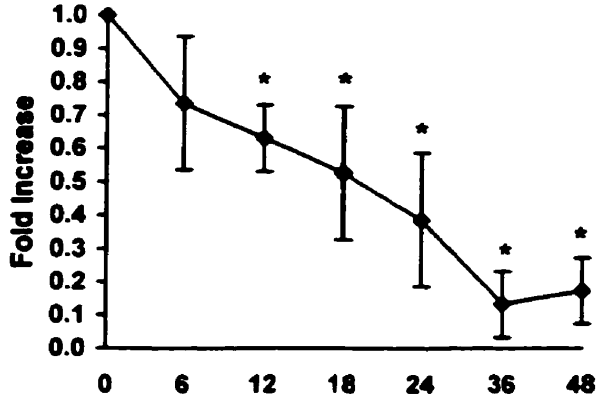
B


Gap-43
- SERUM (hours)
0 6 12 18 24 36 48
101kDa
83kDa
51kDa
36kDa
29kDa
21kDa


Fold Increase
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 6 12 18 24 36 48
Time of Treatment (hours)

C


Synaptophysin
- SERUM (hours)
0 6 12 18 24 36 48
101kDa
83kDa
51kDa
36kDa
29kDa
21kDa


Fold Increase
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 6 12 18 24 36 48

IV Discussion

1. Primary Human Neurons as a Model of Neuronal Apoptosis

Presently, the majority of systems used to define the molecular mechanisms of neuronal apoptosis utilize neuronal cell lines or primary rodent neuron cultures. While these systems offer the advantages of unlimited cell numbers, synchronicity and a uniform genetic background, the information gained from these models may not be entirely relevant to the pathogenesis of human neurodegenerative diseases. However, it appears that key players of the apoptotic pathway have been conserved, thus, these models serve as a basis of comparison and provide a good starting point to identify the proteins involved in human models of apoptosis. The following study, based on a unique system of primary human neuron cultures, provides novel information regarding the molecular processes of apoptotic cell death in human neurons.

1.1 Apoptosis in Primary Human CNS Neurons

For the purposes of the present study it was necessary for us to establish primary human neuron cultures and successfully induce apoptosis by serum withdrawal. Phase contrast and immunofluorescence analysis show we successfully established healthy and viable primary human neuron cultures from fetal brain tissue, consisting primarily of neurons (~90%) and a small population of astrocytes (~10%). Propidium iodide staining of nuclei and fluorescence microscopy allowed us to clearly identify and differentiate between apoptotic and normal neurons following serum deprivation based on morphological changes, such as chromatin condensation and nuclear fragmentation, that are typical of apoptotic cells (reviewed by Bredesen, 1995). Furthermore, we identified, by phase contrast analysis of the neurons and immunofluorescence of tau immunostaining, that changes in the neurites and the cytoskeleton occurred in the dying neurons. Clearly, the neurites became dystrophic, showed beading and degeneration, and the distribution of tau became irregular and non-uniform in the cell bodies and neurites of the neurons. Although the gross identification of DNA fragmentation, determined by the visualization of a DNA ladder, was negative in the present study (data not shown), a previous study by LeBlanc (1995) has shown that primary human neuron cultures exhibit

DNA laddering following 12 hours of serum withdrawal. The difference in the observations between the two studies may have been due to the techniques used. Following DNA extraction, the DNA was separated by agarose gel electrophoresis and stained with ethidium bromide in the present study, whereas the study by LeBlanc (1995) employed a more sensitive method of end-labeling the fragmented DNA with ^{32}P-dCTP to visualize the DNA ladder by autoradiography. However, TUNEL staining revealed DNA fragmentation at the single cell level in the present study. Therefore, the two methods employed in the present study, PI and TUNEL staining, confirmed that neurons underwent apoptotic cell death following serum deprivation.

We quantitated the level of apoptosis in our cultures following various periods of serum withdrawal. In conjunction with TUNEL staining, we observed a statistically significant increase in the number of apoptotic cells by 12 hours of serum deprivation, a 5-fold increase by 24 hours and a 15-fold increase in the number of apoptotic cells by 48 hours of serum deprivation, whereas serum-fed, sister neuron cultures did not exhibit an increase in the number of apoptotic cells. These results are in agreement with other studies in which increasing periods of NGF deprivation results in an increase in the level of apoptosis in rodent sympathetic cervical ganglion (SCG) neuron cultures (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994; LeBlanc, 1995).

After determining that increasing periods of serum withdrawal induced quantifiable levels of apoptosis in our cultures, we proceeded to determine the time frame in which our neurons lost the ability to respond to the readdition of serum with long term survival and became committed to die by apoptosis. Completing this aspect of our study allowed us to correlate gene and protein expression associated with the time course of apoptosis in our system, and will be discussed in the following sections. We observed that, beyond 6 hours of serum deprivation, neurons could not be rescued from undergoing apoptosis by the reintroduction of serum, since the quantitative levels of apoptotic cells in the serum starved cultures began to diverge from the sister, serum fed control cultures. However, the difference was not statistically significant. The difference was appreciable by 12 hours, but did not reach statistically significant levels until 18

hours of serum deprivation (Figure 3.). Based on these results, we believe that primary human neuron cultures become committed to apoptosis by 18 hours of serum deprivation.

The commitment time point to apoptosis in primary human neuron cultures is similar to the commitment time point determined in other neuronal systems. Studies of SCG neurons has revealed that these neurons become committed to apoptotic cell death by 17-18 hours of NGF withdrawal (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). Neuron-like PC12 cells commit to apoptotic cell death following 14 hours of NGF withdrawal. Neurons in these systems display the classic morphological features of apoptotic neurons, condensed chromatin, fragmented nuclei and degenerating neuritic processes, obtained with continued NGF deprivation beyond the commitment time point (Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994; Pittman et al., 1993). The similarities in the commitment time points among the different studies suggests that human neurons and other mammalian neurons may share a common pathway of apoptosis activation, and that the underlying molecular mechanisms acting in the apoptotic pathway may have been conserved among different species.

In the commitment experiment, we observed an average decline in the number of apoptotic cells at the 24h (36h total time of treatment) in comparison to the 18h (30h total time of treatment) time point of serum withdrawal. On average, we expected the level of apoptosis to increase with longer periods of serum withdrawal once the neurons had become committed to die, such that the curve (- Serum; Serum reintroduced for 12h) shown in Figure 3 would be linear. It is unlikely the lower average level of apoptosis at the 24h (36h total time of treatment) was due to an under estimation of the number of apoptotic cells; a result of an absolute decrease in the number of cells on the coverslips in different experiments, since dying cells are prevented from lifting and floating away by the network of neurites and neuronal loss does not become significant until 72 hours of serum deprivation (LeBlanc, 1995). Also, the quantitative analysis of apoptosis by TUNEL (Figure 2D) showed that the level of apoptosis did linearly increase with longer periods of serum deprivation. Most importantly, the difference in the levels of apoptosis between the 24h (36h total time of treatment) and the 18h (30h total time of treatment) was not statistically significant. Therefore, our results are still valid.

1.2 Immediate Early Gene Expression During Apoptosis

We have used RT-PCR to study the pattern of transcriptional expression of *c-jun* and *c-fos* in human neurons undergoing apoptosis. In four independent experiments, we have clearly shown there was an induction in the expression of *c-fos* and *c-jun* during apoptosis. Although there was largely a coincident time for the peak level of induction of *c-fos* and *c-jun* within a given experiment following serum withdrawal, the time of induction between different experiments varied. The peak levels of *c-fos* and *c-jun* induction occurred after 6h in experiments 1 and 3, 12h in experiment 2, and 18h for *c-fos* and 12h for *c-jun* in experiment 4. Also, the pattern of *c-jun* expression in experiment 1 appeared cyclic. Additionally, the magnitude (fold-increase in mRNA levels) of *c-fos* and *c-jun* induction varied within and between different experiments. These variations did not allow us to average the data and provide an overall summation for the time of *c-fos* and *c-jun* induction in primary human neurons undergoing apoptosis. Variations were unlikely to have arisen from differences in cell numbers in each culture, the RT-PCR reaction or the quantitative analysis since the number of cells harvested, the amount of input RNA into the cDNA synthesis reaction, the amount of input cDNA into the PCR reaction were carefully controlled and fixed for each experiment, while the quantitation of *c-fos* and *c-jun* expression was corrected to an internal control (*G3PDH*). However, a major limitation in the use of primary human neurons is the lack of experimental control over the genetic differences between each culture. Control of the genetic differences cannot be achieved because every individual fetal brain, from which the cells were cultured, had a different genetic background. Thus, the variability in our data has to be considered as a reflection of the inherent genetic heterogeneity between cultures. Nonetheless, our findings are significant and we may compare them to those of Estus et al. (1994), who examined IEG expression in SCG neurons undergoing NGF deprivation-induced apoptosis.

Similar to our study, Estus et al. (1994) reported an induction of *c-fos* and *c-jun* in SCG neurons undergoing apoptosis. However, with the exception of experiment 4, *c-fos* expression was induced and reached peak levels earlier (by as much as 9h in

experiments 1 and 3) in our primary human neuron cultures in comparison to SCG neurons undergoing apoptosis. Estus et al. (1994) observed an induction of *c-fos* beginning after 10h, reaching a maximal level of expression by 15h, and subsequently returning to normal levels by 25h of NGF withdrawal. In comparison to the pattern of *c-fos* expression in SCG neurons, the induction and peak level of *c-fos* expression occurred later and did not decline in experiment 4 of our studies. The pattern of *c-jun* expression in each of the four experiments of our study was distinctly different to the pattern of expression for *c-jun* in SCG neurons. Estus et al. (1994) observed *c-jun* induction beginning after 5h, reaching the same maximal level as *c-fos* by 15h, and decreasing to above normal levels by 25h of NGF deprivation. In experiments 1 and 3 of our studies, *c-jun* induction and the maximal level of expression occurred much earlier in comparison to *c-jun* expression in SCG neurons. In experiment 2, *c-jun* expression was induced later, but reached a peak level of expression earlier, while the pattern of *c-jun* expression in experiment 4 was entirely different in comparison to SCG neurons. It is difficult to ascertain whether the differences in the patterns of *c-fos* and *c-jun* expression in primary human neurons in comparison to SCG neurons undergoing apoptosis are truly different when one considers that the data presented by Estus et al. (1994) is based only on a single preparation of neuronal cultures. Even though the researchers state each gene induction was confirmed in a least two experiments, this does not exclude the possibility that these researchers observed the same variations in *c-fos* and *c-jun* expression as we did in our studies, since their graphical representation of *c-fos* and *c-jun* expression is not based on the mean from several independent experiments (Estus et al., 1994). Even so, Estus et al. (1994) have shown that the induction of *c-fos* and *c-jun* preceded the commitment time point, and through the injection of c-Jun neutralizing antibodies, demonstrated that the expression of *c-jun* was required for apoptosis to occur in SCG neuron cultures deprived of NGF. Since *c-fos* and *c-jun* expression was activated and preceded the commitment time point in our primary human neuron cultures similar to SCG neurons, then it is reasonable to infer that the expression of *c-fos* and *c-jun* may play a role in the apoptotic pathway of human neurons.

We proceeded to determine if the increase in transcription of *c-fos* and *c-jun* resulted in an increase in c-Fos and c-Jun protein levels. Through immunoblotting we were anticipating to find a correlated increase in the protein levels of c-Fos and c-Jun following the transcription of the mRNA. Using an immunocytochemical approach we hoped to identify a colocalization of c-Fos and c-Jun with apoptotic cells. Positive results from these experiments would support the involvement of the IEG in apoptosis in human neurons.

We did not detect an induction of c-Fos protein levels or a colocalization of c-Fos immunoreactivity with apoptotic cells. Numerous immunoblotting experiments carried out with whole cell extracts from four neuron cultures and nuclear extracts from three neuron cultures, utilizing two different lots of the same monoclonal antibody and one polyclonal antibody specific for c-Fos, failed to yield a c-Fos specific signal, and only weak signals in two different positive controls (phorbol ester induced Jurkat cell extracts and HeLa nuclear extracts). There was also an absence of c-Fos immunoreactivity from immunocytochemistry experiments. Although an increase in *c-fos* mRNA was detected in SCG neurons undergoing apoptosis (Estus et al., 1994), it was not translated into protein, since Ham et al. (1995) did not detect c-Fos by immunoblotting in dying SCG neurons. Furthermore, Ham et al. (1995) observed c-Fos immunoreactivity only in a subpopulation (<1%) of dying cells. Similarly, Messina et al. (1996) reported only a minor proportion of sympathetic neurons undergoing developmental apoptosis were positive for c-Fos immunoreactivity. Based on these studies and our findings, we believe that poor antibody quality contributed to the complete lack of c-Fos detection and the increase in *c-fos* mRNA may not have been translated into c-Fos protein in dying human neurons.

We were unable to detect a colocalization of c-Jun immunoreactivity with apoptotic cells in several attempts with samples from three neuron cultures. However, were able to detect a c-Jun specific signal in nuclear extract preparations from one neuron culture. Protein levels of c-Jun began to increase by 6h and reached maximal levels by 18h of serum deprivation, coincident with the commitment time point. Also, an apparent change in the mobility of c-Jun occurred, beginning at about 6h of serum

deprivation. This change in mobility has previously been correlated with phosphorylation of the c-Jun transactivation domain by Jun kinases (Pulverer et al., 1991; Kyriakis et al., 1994) that are activated by various stimuli including cellular stress (Davis, 1994). Since phosphorylated c-Jun autoregulates its own expression by activating transcription of the *c-jun* gene itself (Angel et al., 1988;Pulverer et al., 1991; Smeal et al., 1991; Karin et al., 1994), it is plausible that serum deprivation, a form of cellular stress, activated Jun kinases that phosphorylated c-Jun, which increased the transcription of *c-jun* and c-Jun protein levels in our human neuron cultures during apoptosis. Our findings are consistent with those of Ham et al. (1995), who confirmed and extended the results of Estus et al. (1994) in SCG neurons. Ham et al. (1995) observed changes in the protein level and phosphorylation state of c-Jun that preceded the commitment time point, and showed that changes in the mRNA level of *c-jun* observed by Estus et al. (1994) actually resulted in changes in c-Jun protein levels in SCG neurons undergoing apoptosis. Furthermore, Ham et al. (1995) demonstrated by the overexpression of c-Jun and through a transactivation-defective c-Jun dominant negative mutant that c-Jun was essential for apoptosis to occur in SCG neurons. Due to the similarities between our studies and to those of Ham et al. (1995), and the possibility that mechanisms of neuronal apoptosis may have been conserved among mammals, we suggest that c-Jun likely plays a role in the activation of apoptosis in human neurons.

The findings of our study only correlate the involvement of c-Jun in the apoptotic process of human neurons. Several studies are required to confirm a direct relationship between c-Jun and apoptosis in human neurons, but were unfortunately beyond the scope of the present study: **1)** performing peptide competition assays to confirm the specificity of the c-Jun antibody and continue studies to identify a colocalization of c-Jun and apoptotic cells; **2)** metabolically labeling neurons with ^{35}S-methionine and immunoprecipitating with c-Jun antibodies to show that c-Jun is phosphorylated during apoptosis induction; **3)** injecting neutralizing antibodies specific for c-Jun or transfecting anti-sense oligonucleotides to *c-jun* mRNA or injecting a dominant negative c-Jun mutant and determining whether these methods prevents serum deprivation-induced apoptosis in primary human neuron cultures.

The formation and activity of the AP-1 complex is not inhibited by the absence of c-Fos expression since the AP-1 complex can consist of Fos/Jun heterodimers or Jun/Jun homodimers (reviewed by Angel and Karin, 1991). Thus, there several possible functions of c-Jun in dying human neurons. For example, we postulate the expression of c-Jun could possibly: **1)** activate the transcription of target genes whose protein products are triggers or effectors of the cell death program, such as the Bcl-2 or ICE family of proteins; **2)** activate genes involved with cell proliferation, like cyclin D1, and cause post-mitotic neurons to undergo 'abortive mitosis' (Freeman et al., 1994); **3)** activate a regenerative mechanism or **4)** activate the transcription of a target gene whose protein product could potentiate neuronal cell death and degeneration.

1.3 The Effect of AP-1 on APP Expression During Apoptosis

Previous studies by LeBlanc (1995) showed that serum deprived primary human neuron cultures increased production of Aβ. We set out to determine whether APP would be a target gene for AP-1 during apoptosis. We observed an increase in the level of APP beginning after 12h and reaching a maximum 1.5-fold increase by 18h of serum deprivation. The increase in APP occurred shortly after the increase in c-Jun and expression of both proteins reached maximal levels by 18h of serum deprivation. Thus, it appears that the expression of APP was regulated by the expression of c-Jun. However, the increase in APP was not statistically significant due to variations between different cultures, and therefore, we cannot conclusively state that the increase in c-Jun resulted in a correlated increase in APP. Several studies could determine if c-Jun has a causal effect on the expression of APP. For example, gel-shift assays, utilizing a nucleotide construct that contains the APP promoter region and nuclear extracts of apoptotic neurons from various times of serum deprivation, would verify if AP-1 from human neurons binds to the AP-1 consensus sequences in the APP promoter. Secondly, functional assays, such as chloramphenicol acetyl transferase (CAT) or luciferase assays, would determine if the nuclear extracts from apoptotic neurons could activate the transcription of a reporter gene under the control of the APP promoter.

The findings from the present study extend those of LeBlanc (1995), who observed a two-fold decrease in the metabolism of APP through the non-amyloidogenic pathway. The study by LeBlanc (1995) was only conducted after 12 hours of serum deprivation and did not assess the possibility of AP-1 action on the expression of APP and consequent effect on the increase in Aβ production. The present study has shown over a time course of serum deprivation-induced apoptosis that the level of APP does not decrease below initial levels or significantly increases in dying neurons. Thus, we speculate that an increase in Aβ production would not result from the normal metabolism of a larger pool of APP, but from a decrease in the metabolism of consistent levels of APP through the non-amyloidogenic pathway in neurons during apoptosis. Future studies analyzing the metabolism of APP through the different pathways over time course of serum deprivation will further define the mechanism(s) responsible for increased Aβ production during apoptosis in human neurons.

In summary, we have been able to induce and characterize apoptosis in primary human neuron cultures. Contrary to our hypothesis, it does not appear that the expression of c-Jun induces the expression of APP in dying neurons, but further studies are required to confirm this finding. Nonetheless, we have determined that the expression of *c-fos* and *c-jun*, and c-Jun occurs in primary human neurons preceding the commitment to apoptosis. Because of the similarities in the molecular events during the induction of apoptosis between rodent neuron cultures, in which the expression c-Jun has been shown to be essential apoptosis to occur, and our human neuron cultures, we believe the expression of c-Jun is likely involved in the activation of apoptosis in human neurons. Based on the present study we have established that primary human neurons cultures are a good model of neuronal apoptosis. Moreover, because they involve human cells, primary human neurons are more relevant to address the role of apoptosis in AD than other mammalian neuron culture models.

2. Primary Human Neurons as an *In Vitro* Model of AD

2.1 Expression of c-Fos and c-Jun *In Vivo*

The expression of c-Fos and c-Jun has been observed to be associated with apoptotic cell death *in vivo* (Smeyne et al., 1993; reviewed by Dragunow and Preston, 1995; Messina et al., 1996). The localization of c-Fos and c-Jun immunoreactivity (Zhang et al., 1991; Anderson et al., 1994) to the same regions of the AD brain where DNA fragmentation has been detected (Su et al., 1994; Smale et al., 1995; Lassman et al., 1995) has led to speculation of a relationship between IEG expression and neuronal cell death in AD. However, *in vitro* (Estus et al., 1994; Ham et al., 1995) and *in vivo* evidence; c-Jun is colocalized with apoptotic cells in the AD brain (Anderson et al., 1996), suggest it is c-Jun that mediates neuronal cell death during the pathogenesis of AD. Therefore, it is postulated that the expression of c-Fos *in vivo* may have no causal relationship to neuronal apoptosis, but merely a reflection of altered signal transduction mechanisms or a coincidental response to cellular stress (Smeyne et al., 1993). The lack of c-Fos expression and the induction of c-Jun in our neuron cultures dying by apoptosis is consistent with this hypothesis. Altered signal transduction mechanisms during the induction of apoptosis and the possible hyperactivation of signal transduction systems in AD relating to protein kinase C (PKC) (Masliah et al., 1991c), known to regulate the activity of c-Jun (reviewed by Angel and Karin, 1991), are important points of consideration in the following sections.

2.2 The Effect of Apoptosis on Tau

Since apoptosis causes alterations in the cytoskeleton and alterations in the neuronal cytoskeleton result in the neurofibrillary lesions in AD, we proceeded to assess the expression levels and intactness of tau, tubulin and vimentin in our neuron cultures undergoing apoptosis. By phase contrast and immunofluorescence microscopy of tau immunostaining we found that untreated neurons maintained their neurite integrity and a strong localization of tau in the axons, similar to the localization of tau in the normal brain. In contrast, the neurites of the serum deprived neurons became dystrophic, displayed beading, and tau became diffusely localized in the cell bodies of the

degenerating neurons. Similarly, tau is found in the cell bodies and the dendrites of affected neurons in the AD brain (Kosik et al., 1989). Thus, it appears that primary human neurons dying by apoptosis undergo changes in the localization of tau like the neurons in AD.

During the time course of serum deprivation-induced apoptosis the expression levels of tau increased almost two-fold by 48h. A recent study by Sadot et al. (1996) has isolated tau promoter sequences and characterized the regulation of tau expression in NGF stimulated PC12 cells. NGF stimulation has been shown to activate the IEGs in PC12 cells (Bartel et al., 1989). Sadot et al. (1996) propose the IEGs may have an effect on the tau promoter activity. With this in mind, we propose that the increase in tau protein levels was the result of transcriptional activation of the tau gene by c-Jun in our cultures. A hypothesized consequence of the elevated expression of tau is the accumulation of tau inside the axons, possibly resulting in the abnormal phosphorylation of tau and PHF formation (Gotz et al., 1995) that would destabilize the microtubule structures and cause the degeneration of the nerve cell. Similar to overexpression of tau in our cultures is the observation that the level of abnormally phosphorylated tau is elevated by eight-fold in the AD brain (Khatoon et al., 1992). Thus, the overexpression of tau in our degenerating neurons parallel the changes in the neurons of the AD brain. Identifying an increase in the phosphorylation state of the overexpressed tau in serum deprived neuron cultures would lend further support to the notion that tau undergoes changes during apoptosis which are similar to those during AD pathogenesis.

In addition, we observed the appearance of degradation products of tau over the time course of apoptosis. Although the time of appearance varied, degradation products were detected in three of four independent cultures undergoing apoptosis. Recently, Bossy-Wetzel et al. (1997) have shown that the expression and activation of c-Jun induces apoptosis and activates a caspase that cleaves of α-fodrin, a substrate of the caspase family. Additionally, Goldberg et al. (1996) identified the protein huntingtin as a substrate of apopain, a caspase, and provided further support to the role apoptosis plays in the development of Huntington's disease. Moreover, based on the amino acid sequence of human tau (Goedert et al., 1989), we have identified two potential caspase

cleavage sites in the N-terminal region of tau. A possible CPP32/apopain cleavage site is located between amino acids 22-25, Asp-Arg-Lys-Asp; the consensus cleavage site for apopain is Asp-X-X-Asp (Nicholson et al., 1995), and a possible cleavage site for a caspase similar to ICE is located between amino acids 146-148, Gly-Ala-Asp-Gly, and is very similar to one of the two ICE cleavage sites (Glu-Val-Asp-Gly) in pro-interleukin-1-β (Lazebnik et al., 1994). Cleavage at either or both of these sites would produce a tau protein between 45-50 kDa, similar in size to the degradation products we observed in our studies. In light of these findings, we speculate that an unidentified caspase is activated through c-Jun mediated pathways and cleaves tau during apoptosis in human neurons. Because cleavage products have been identified as a component of NFTs (Bondareff et al., 1990), we propose that a similar mechanism occurs during the development of neurofibrillary lesions in AD. It is known some cell death processes are cell type specific, thus, activation of the proposed mechanism of NFT development could occur in the hippocampal neurons of the CA1 region of the brain, where neurofibrillary pathology commonly occurs. Based on the similar changes in tau that we observed in primary human neurons dying by apoptosis to changes in tau observed in the AD brain, we believe that apoptosis is likely a mechanism of neurodegeneration, which alters the cellular organization of tau and causes the development of the neurofibrillary pathology, in AD. Our model of neurofibrillary tangle development is summarized in Figure 14.

Several studies could be pursued to test our model. Immunoblot analysis of nuclear extracts for the cleavage of PARP, a death signature molecule which undergoes specific cleavage during apoptosis (Nicholson et al., 1995), could confirm the activation of a caspase during serum deprivation induced apoptosis in human neurons and determine whether this activation is coincident with the proteolysis of tau. We could also evaluate the effect of a cell permeable caspase inhibitor, like z-Val-Ala-Asp-fluoromethylketone for example, on serum deprived neuron cultures by determining whether the morphological changes characteristic of apoptosis, and the proteolysis of tau and PARP are inhibited. If activation of a caspase is confirmed, then cleavage inhibition studies utilizing extracts of chicken hepatoma cells committed to apoptosis (S/M extracts) (Lazebnik et al., 1994), *in vitro* translated [^{35}S] labelled human tau and

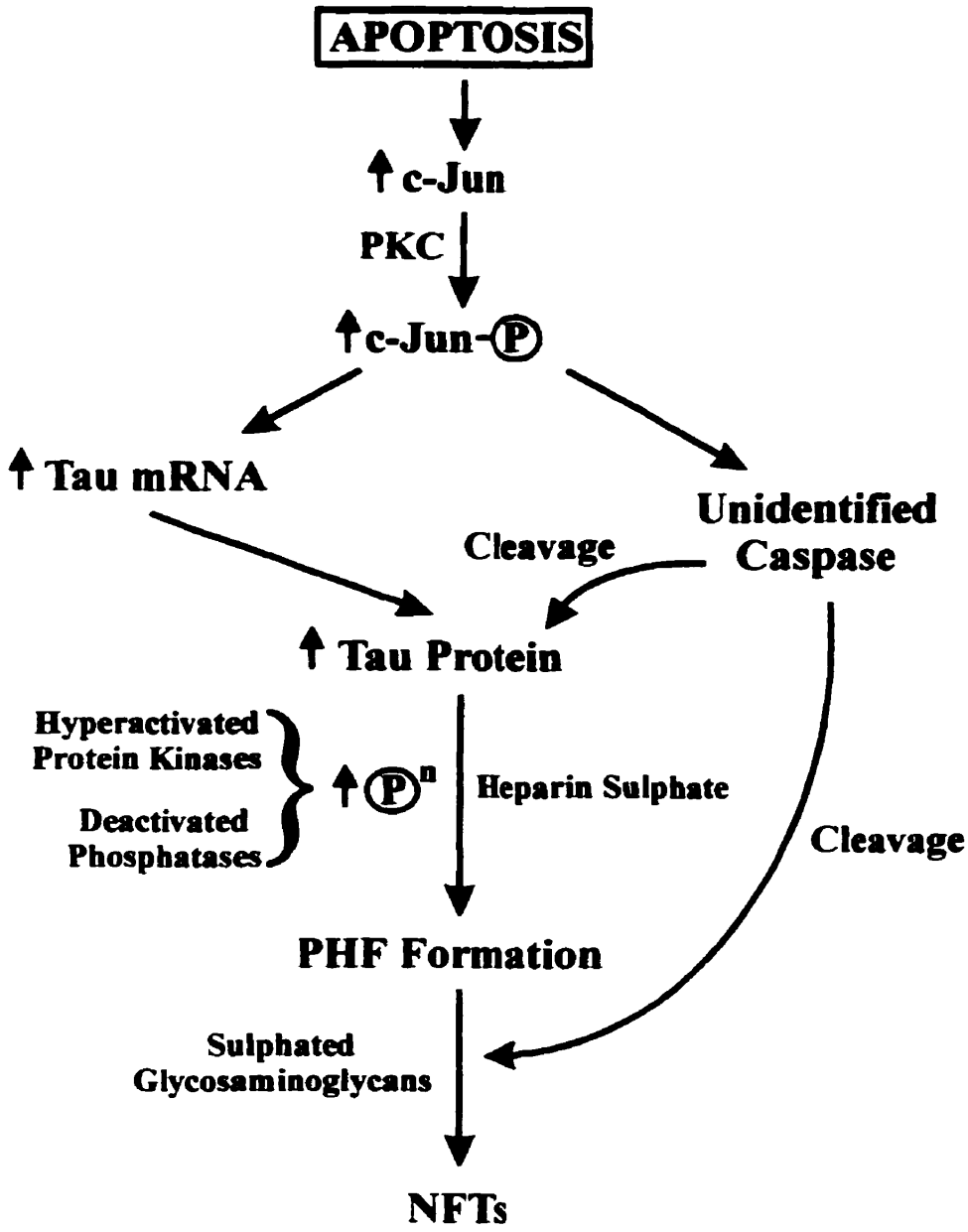


Figure 14. - Proposed Model of Neurofibrillary Tangle Development

tetrapeptide aldehyde inhibitors, Ac-Asp-Glu-Val-Asp-CHO (a potent inhibitor of apopain) and Ac-Tyr-Val-Ala-Asp-CHO or CrmA (both potent inhibitors of ICE), would determine whether tau cleavage during serum deprivation induced apoptosis was due to a caspase with apopain-like or ICE-like activity in human neurons.

2.3 The Effect of Apoptosis on GAP-43 and Synaptophysin

We analyzed the expression levels of GAP-43 and synaptophysin in our dying neuron cultures because the expression of these two proteins is dramatically altered in the AD. We found the expression of GAP-43 increased three-fold in our cultures, indicating that not only were the neurons dying by apoptosis, but also attempting a regenerative response. Although the expression of the IEGs (reviewed by Dragunow and Preston, 1995) has been previously suggested to be associated with nerve regeneration, it does not appear that the expression of GAP-43 is regulated by this family of transcription factors. Numerous studies in the literature have characterized the GAP-43 promoter and its regulatory sequences, and at the present time, AP-1 consensus sequences have not been reported. Thus, it appears that the regenerative response is part of an uncharacterized pathway that leads to the expression of GAP-43 in apoptotic neurons.

A possible pathway involves PKC. We speculate that PKC activity is stimulated during apoptosis in neurons, which could increase GAP-43 protein levels, since PKC activity has been shown to increase GAP-43 mRNA levels by increasing the stability of the mRNA (Perrone-Bizzozero et al., 1993). Furthermore, PKC activation leads to the phosphorylation of GAP-43 (Chan et al., 1986), which stimulates GAP-43 activity (Strittmatter et al., 1991). Activation of a PKC-related pathway could also account for the c-Jun expression that we observed, since PKC activity can mediate c-Jun phosphorylation and is transactivation potential (reviewed by Angel and Karin, 1991); an event that we have discussed previously that could stimulate *c-jun* expression itself. Thus, we propose that a PKC-mediated pathway is activated during apoptosis in human neurons and via independent pathways, affects the activities of c-Jun and GAP-43. Therefore, determining the molecular events affecting PKC activation during apoptosis in human neurons is warranted.

We observed the expression levels of synaptophysin decreased to 90% of original levels by 48h of serum deprivation, indicating that the microcircuitry and synaptic transmission was likely to have been dramatically altered in neurons dying by apoptosis. The pathways regulating the expression of synaptophysin are unknown at the present time, but it is clear that the process of apoptosis affects the expression of synaptophysin, and likely, the function of synapses. Taken together, our results show that the synaptic pathology found in the AD is mimicked in primary human neuron cultures induced to undergo apoptosis.

In summary, given the changes in Aβ, tau, GAP-43, and synaptophysin that occurred in primary human neurons undergoing apoptosis parallel changes for the same proteins in the AD brain, we believe, in light of the *PS-I/PS-II* and FAD-APP mutations capable of inducing apoptosis (Vito et al., 1996; Wolozin et al., 1996; Yamatsuji et al., 1996a; Yamatsuji et al., 1996b), our study substantiates the contention that apoptosis is likely the mechanism of neuronal cell death and degeneration in AD. Our findings are summarized in Figure 15.

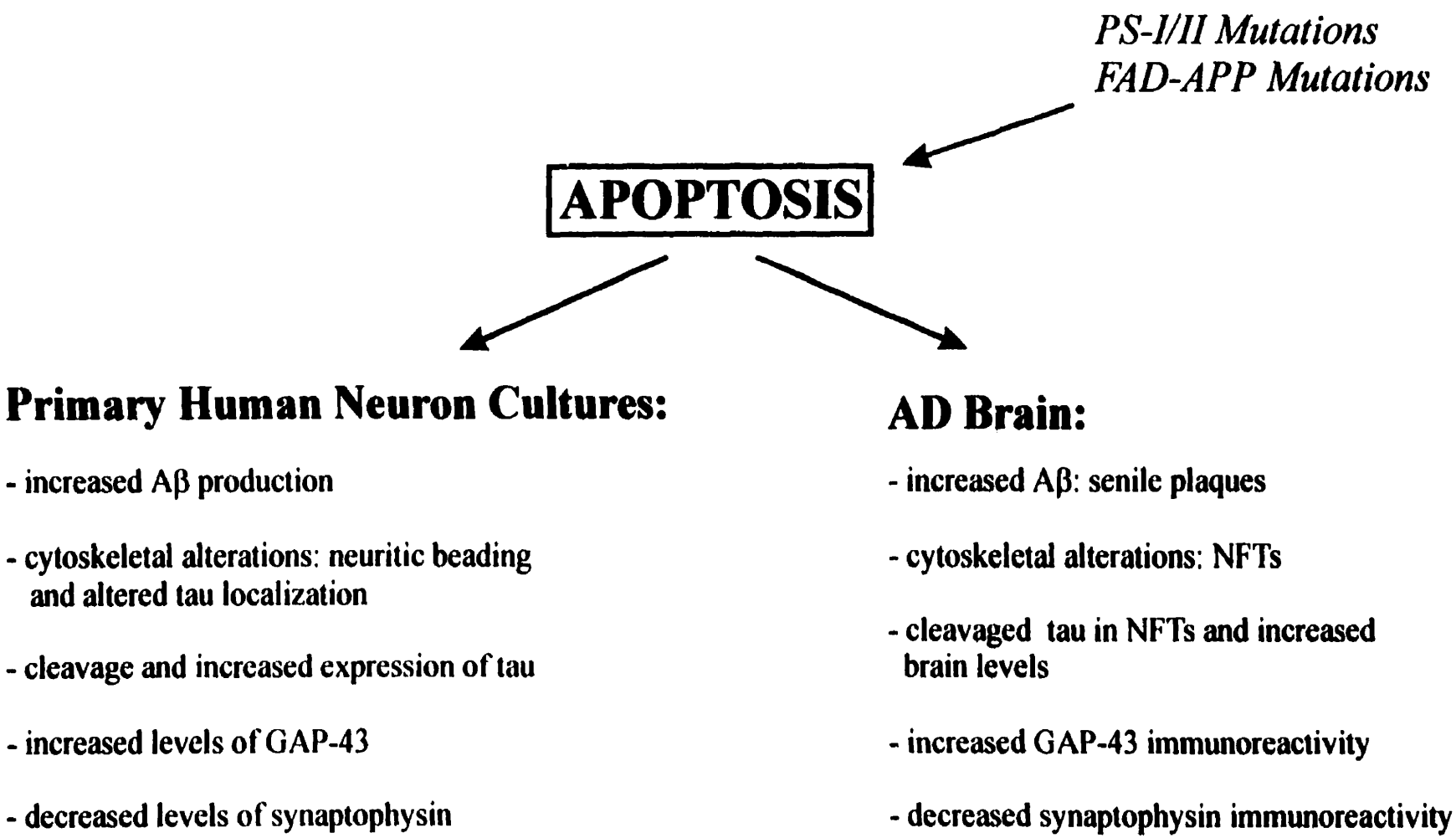


Figure 15. - Similar Pathologies Between Apoptotic Neurons and the AD Brain

V Conclusions

In conclusion, we have studied some of the molecular events that occur in primary human fetal neuron cultures undergoing apoptosis by serum deprivation. During apoptosis, the expression of *c-fos* and *c-jun* is induced at the mRNA level, and c-Jun at the protein level. Whether the expression of these immediate early genes is a necessary requirement for apoptosis to occur in human neurons in *vitro* and *in vivo* remains to be further elucidated. The expression of these immediate early genes does not apparently increase the level of APP expression. Thus, the increased production of Aβ previously observed in these cultures may not result from an overexpression of APP, but possibly from the aberrant metabolism of APP. Immunohistological analysis of tau clearly showed alterations in the neuronal cytoskeleton during apoptosis. Furthermore, degradation products and an increase in the expression of tau were observed. These results indicate that tau is a target of cytoskeletal alterations during apoptosis in human neurons, and suggest that the degeneration of the cytoskeleton during the pathogenesis of AD may be caused by apoptosis. The increase in the levels of GAP-43 and the decrease in the levels of synaptophysin during apoptosis in primary human neuron cultures parallels the synaptic pathology observed in the AD brain. Taken together, the observations from this study demonstrate that cultured human neurons induced to undergo apoptosis can develop features similar to those found in the AD brain, and substantiate the validity of human primary neuron cultures as an *in vitro* model of human CNS neuronal cell death.

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