$A\beta_{1-42}$ and $A\beta_{1-40}$ induce tau phosphorylation in human neurons.

by Caroline Bélair
Neurology and Neurosurgery Department
McGill University, Montréal

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RÉSUMÉ

La maladie d'Alzheimer est caractérisée par (1) la présence pathologique de plaques séniles formées de peptides d'amyloïde agrégés de 40 à 42 acides aminés ($A\beta_{1-40}$ et $A\beta_{1-42}$) provenant du métabolisme de la protéine précurseur de l'amyloïde, (2) de dégénérescence neurofibrillaire impliquant une dysfonction du cytoskelette provoquée par l'hyperphosphorylation de la protéine tau et (3) de dépôts d'amyloïde dans les vaisseaux sanguins cérébraux. L'objectif de ce projet est de déterminer quel est le lien entre les peptides amyloïdes et l'hyperphosphorylation de la protéine tau.

Nous avons traité des cultures primaires de neurones humains fétaux avec $A\beta_{1-40}$ et $A\beta_{1-42}$ (100nM). L'analyse des protéines neuronales démontre, par western blot, que l'épitope sérine 202 de la protéine tau est phosphorylé de façon cyclique. Grâce à un essai de la phosphorylation de tau par des protéines kinases, nous avons observé que les peptides $A\beta_{1-40}$ et $A\beta_{1-42}$ activent une ou plusieurs protéines kinases capables de phosphoryler la protéine tau et plus spécifiquement l'épitope sérine 202.

Nous concluons que des concentrations pathologiques de peptides synthétiques de $A\beta_{1-40}$ et $A\beta_{1-42}$ induisent l'activation de protéine(s) kinase(s) ce qui induit la phosphorylation de la protéine tau et plus spécifiquement de l'épitope sérine 202.

ABSTRACT

Alzheimer's disease is characterized by (1) senile plaques formed of aggregated amyloid peptides of 40 to 42 amino acids ($A\beta_{1-40}$ and $A\beta_{1-42}$) which are derived from the metabolism of the amyloid precusor protein, (2) by neurofibrillary tangles involving a dysfunction of the cytoskeleton due to the hyperphosphorylation of the tau protein, and (3) by amyloid-laden cerebral vessels. The goal of this project is to determine if there is a link between the presence of aggregated amyloid peptides and tau hyperphosphorylation.

We incubated the human fetal primary neuron cultures with $A\beta_{1-40}$ and $A\beta_{1-42}$ (100nM). The western blots show that serine 202 epitope of tau protein is phosphorylated in a cyclic manner. With the tau phosphorylation assay, we showed that $A\beta_{1-40}$ and $A\beta_{1-42}$ activate one or more protein kinase(s) able to phosphorylate tau protein and more specifically the serine 202 epitope

We conclude that pathological concentrations of aggregated peptides $A\beta_{1-40}$ and $A\beta_{1-42}$ activate protein kinases which induce the phosphorylation of tau protein and serine 202 epitope.

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LIST OF ABREVIATIONS

Aβ= Amyloid beta peptide
AD= Alzheimer's disease
AP buffer= Alkaline phosphatase buffer
APO E= Apolipoprotein E
APO E4= Apolipoprotein E4
APP= Amyloid precursor protein
ATP= Adenosine triphosphate
BCIP= 5-bromo-4-chloro-3-indolyl phosphate
BPDK= Brain proline-directed kinase
Cdc2= Cell-cycle dependent kinase 2
Cdk5= Cell-cycle dependent kinase 5
CNS= Central nervous system
dBCS= Decomplemented bovine calf serum
DNA= Deoxyribonucleic acid
ERK= Extracellular signal-regulated kinase
FAD= Familial Alzheimer's disease
FITC= Fluorescein isothiocyanate
GFAP= Glial fibrillar acidic protein
GSK-3β= Glycogen synthase kinase 3
GTP= Guanosine triphosphate
IPTG= Isopropyl-L-D-galactoside
KPI= Kunitz serine protease inhibitor
LDH= Lactate dehydrogenase
MAP2= Microtubule-associated protein 2
MAPK= Mitogen-activated protein kinase
MAPs= Microtubule-associated proteins
MBPs= Myelin basic proteins
NBT= Nitro blue tetrazolium chloride
NFTs= Neurofibrillary tangles
NF-H= High molecular weight neurofilament protein
NF-M= Middle molecular weight neurofilament protein
NGF= Nerve growth factor
NLCK= Neuronal cdc2-like kinase
NP-40= Nonidet P-40
PC12= Pheochromocytoma cells
PBS= Phosphate-buffered saline
PHFs= Paired helical filaments
PHF-tau= Paired helical filament tau
PNS= Peripheral nervous system
SDS-gel= Sodium dodecyl sulfate-polyacrylamide gel

SER= Serine
SER/THR-PRO= Serine- / threonine-proline
SPs= Senile plaques
TBST= Tris-buffered saline-tween
TCA= Trichloric acid
THR= Threonine
TPKI= Tau protein kinase I (or GSK-3β)
TUNEL= Terminal transferase-mediated dUTP-biotin nick end labeling

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A. INTRODUCTION

1- Alzheimer's Disease.

Alzheimer's disease (AD) is a heterogeneous neurodegenerative disorder that occurs in the aging population and is clinically characterized by a progressive loss of memory and cognitive functions resulting in dementia. Diagnosis is confirmed only in the post-mortem histological examination of the AD patient's brain. Gross observation reveals neuronal atrophy and three morphological features: neurofibrillary tangles (NFTs), senile plaques (SPs) and amyloid-laden cerebral vessels (reviewed in Glenner, 1988). The density of SPs and NFTs in cerebral cortex and hippocampus is used to positively diagnose AD.

The NFTs can be either extracellular or intracellular in abnormal neurons containing a cytoplasm filled with paired helical filaments (PHFs). PHFs are composed of hyperphosphorylated tau protein mainly associated with smaller amounts of neurofilament protein, microtubule-associated protein 2, ubiquitin and amyloid precursor protein (APP) (reviewed in Glenner, 1988; Katzman, 1986). As for the extracellular senile plaques, they are composed of proteinaceous deposits made mostly of amyloid-beta peptide (Aβ) (Glenner and Wong, 1984) associated with astrogliosis, microglia, and degenerating nerve terminals (dendrites and axons). These 2 structures are found only in the central nervous system (CNS), specifically in the cerebral cortex, hippocampus, entorhinal cortex, locus cereleus,

amygdala, anterior olfactory nucleus, prefrontal area, and in the nucleus basalis (reviewed in Katzman, 1986). The Aβ is also present in the blood vessels of the meninges, cerebral cortex, and hippocampus (reviewed in Katzman, 1986).

1.1- Classification of Alzheimer's Disease.

An AD patient can be classified in two groups: familial and sporadic. Only ten percent of the AD patients have a family history (FAD) and 90% have a sporadic form of AD. In FAD, there are early onset patients, younger than 65 years old, and late onset patients, older than 65 years old. There are 3 known genes associated with early onset FAD. **(1)** There are three types of mutations in the APP gene located on chromosome 21 (St George-Hyslop *et al*, 1990) which leads to an overproduction of the 4kDa Aβ peptide found in the senile plaques (Reviewed in LeBlanc, 1994). **(2)** Mutations of a newly discovered gene, presenilin I or S182 located on chromosome 14, are associated with a very aggressive form of early-onset FAD. This gene codes for a transmembrane protein with at least 7 hydrophobic membane spanning domains of unknown function (Sherrington *et al*, 1995). **(3)** Mutations in presenilin II or STM2 a homologue of S182, located on chromosome 1 are associated with rare cases of early onset FAD (Levy-Lahad *et al*, 1995a; b). The two proteins have three proposed functions in human: 1) receptors, 2) ion channels and 3) molecules involved in protein processing or trafficking (Tanzi *et al*, 1996) It is likely that there are other undiscovered mutant

genes, since mutations of APP, presenilin I and II can not explain all early onset FAD cases. There are also one or more unknown mutant genes associated with the late onset FAD.

The cause of sporadic AD is unknown but some risk factors are associated with the disease. Apolipoprotein E (APO E) on chromosome 19 is considered as a major risk factor for late-onset cases. APO E is associated with senile plaques, neurofibrillary tangles and cerebral vessel congophilic angiopathy. Specifically, it is the APO E4 allele which is significantly associated with sporadic AD (Corder *et al*, 1993; Poirier *et al*, 1993; Strittmatter *et al*, 1993). Many organs synthezise APO E which is implicated in lipid transport and metabolism. In the brain, APO E is involved in neuronal regeneration. Age, education, nonsmoking condition, being human, sex (woman), aluminum, viral infections, head trauma, hypothyroidism, and depression are just some of the other risk factors that could be involved in the pathology.

2- Neurofibrillary Tangles.

2.1- Description of the Neuronal Cytoskeleton.

Neurons posess a very special shape which allows them to have their unique properties. The cytoskeleton plays a major role in establishing and maintaining the regional specialization within neurons by providing the structural substrate for the

direct transport of materials into axons and dendrites, and by interacting with membrane proteins to restrict their localization to appropriate domains. The cytoskeleton is a counterplay between stability and dynamics of the differents components (reviewed in Vale *et al*, 1989).

The microtubules, neurofilaments and actin microfilaments are the fibrillar proteins composing the cytoskeleton. Microtubules and neurofilaments are cross-linked together whereas the actin filaments form a cortical network under the surface membrane of axons. In neurons, microtubules and neurofilaments are found throughout the cytoplasm whereas actin filaments are mostly at the periphery of axons and in growth cones. The ability of the neuronal cytoskeleton to be stable as well as to be dynamic is given by the monomeric subunit of the microfilaments and the microtubules, respectively the actin and the tubulin. The assembly (or the disassembly) of the cytoskeleton components is carefully controlled by the cell under normal conditions and vary in response to physical signals (reviewed in Schwartz, 1991).

The microtubules posess their own mechanisms for the regulation of assembly (cross-linked microtubules to each other and to other filaments), for the transport and the stability: the microtubule-associated proteins (MAPs). MAPs control microtubule growth, perhaps by keeping the balance between the dynamic and the stable forms. MAPs' distribution vary between the axon and the dendrites as well

as the type of filaments (reviewed in Vale et al, 1989). MAPs are regulated by phosphorylation: hyperphosphorylation decreases their ability to promote assembly and stability of microtubules which leads to their depolymerization and eventually to the cytoskeleton's problems (reviewed in Schwartz, 1991).

2.2- The Neurofibrillary Tangles.

Although the neurofibrillary tangles (NFTs) as well as the senile plaques have been known since the beginning of the 20th century, the molecular analysis of their components has started only 10 years ago and researchers have only begun to understand these pathological processes.

NFTs formation is a late intracellular event of AD and their abundance correlates well with the extent of neuronal loss and the degree of dementia. Neurofibrillary lesions are found in degenerating neurons. Precisely, they are located in cell bodies and apical dendrites as NFTs, in distal dendrites as neuropil threads and in the abnormal neurites associated with senile plaques. Extracellular insoluble deposits of NFTs (called ghost tangles) appear after the death of a neuron because of their relative insolubility (reviewed in Bondareff *et al*, 1990; Goedert, 1995; Goedert *et al*, 1995; Trojanowski and Lee, 1994; Trojanowski *et al*, 1995).

NFTs contain mostly paired helical filaments (PHFs) and a minor fibrous component, the straight filaments. The straight filaments are structural variants of the PHFs because they possibly contain the same subunit, but their relative arrangement differs. There are also several other proteins associated with the NFTs: the microtubule-associated proteins: MAP2 and tau, neurofilament proteins, tubulin, ubiquitin, alpha1-antichymotrypsin, actin, vimentin, heparan sulfate proteoglycans, APO E and APP proteins including Aβ as well as trace elements like aluminum (reviewed in Bondareff *et al*, 1990; Goedert, 1995; Goedert *et al*, 1995; Morishima-Kawashima *et al*, 1995; Trojanowski *et al*, 1995). It seems that the formation of the NFTs involves many proteins playing different roles in the neuron. Then, we can conclude that the formation of the NFTs is a complex event, and probably many mechanisms are impaired in AD affecting the different proteins already mentioned. Therefore, it can be assume that a cause of AD initiates a chain or a cascade of events leading to the formation of NFTs.

2.2.1- The Paired Helical Filaments.

The PHFs have an unusual morphology consisting in 2 strands of 10nM filament subunits which twist around one another in a helical fashion. Their width is between 8 to 20nm with an apparent periodicity of 80nm (reviewed in Goedert, 1993; Goedert *et al*, 1995; Morishima-Kawashima *et al*, 1995). The difference between them are their respective solubility and their sensitivity to proteases: PHFs

in tangle fragments are insoluble in sodium dodecyl-sulfate and in guanidine, and show a pronase-resistant core. This core is formed by the 2 strands of the PHFs (reviewed in Bondareff *et al*, 1990; Goedert, 1993; Goedert *et al*, 1995).

The major subunit of the PHFs is the tau protein in an hyper-phosphorylated form known as PHF-tau or A68 (Greenberg *et al*, 1990; Lee *et al*, 1991). The formation of PHFs has at least 2 different stages: **(1)** PHF-tau accumulates as PHF, and is gradually cleaved off at its amino-terminus followed by ubiquitination; **2)** the carboxyl-terminal portion of tau appears to be removed in ghost tangles (or extracellular tangles), representing the final stage of PHF (reviewed in Morishima-Kawashima *et al*, 1995).

There is no consensus on how tau proteins assemble into PHFs. The phosphorylation of tau protein does not appear to be the cause even though it probably plays a major role in the development of AD. A novel hypothesis proposes that the cysteine 322 of one tau protein can form intermolecular disulfide bonds with another tau protein which when oxidized can lead to PHF assembly. The dimers could form an initial core functioning as an initiator of the PHFs (Schweers *et al*, 1995).

2.2.1.1- The Tau Protein.

Tau protein is an abundant microtubule-associated protein expressed in neurons of both peripheral (PNS) and central nervous system (CNS). In the CNS, tau is concentrated in the axons (reviewed in Bondareff *et al*, 1990; Goedert, 1993; Goedert *et al*, 1995; Morishima-Kawashima *et al*, 1995). The function of tau is to promote the assembly of the microtubules by increasing their growth rate and decreasing the rate of depolymerization (reviewed in Trojanowsky and Lee, 1994).

There are 6 tau isoforms (352 to 441 amino acids) generated from a 13 exon-containing gene by alternative mRNA splicing (figure 1). The resulting tau proteins differ from each other by the presence or the absence of inserts of 29 or 58 amino acids (3 maximum) at the amino-terminal part and of 3 or 4 tandem repeats located in the carboxy-terminal half which represent the microtubule-binding domain. Adult tau proteins on SDS-gel appear as 6 bands with molecular weight varying between 50 and 68kDa (reviewed in Ksiezak-Reding *et al*, 1995).

The expression and the phosphorylation of the different isoforms are developmentally regulated. Only the smallest isoform with only 3 inserts, called fetal tau, is found in fetal human brain whereas all the 6 isoforms are found in adult human brain. Also fetal tau is abundantly phosphorylated, whereas adult tau proteins are phosphorylated at only 4 sites (figure 2) (reviewed in Bondareff *et al*,

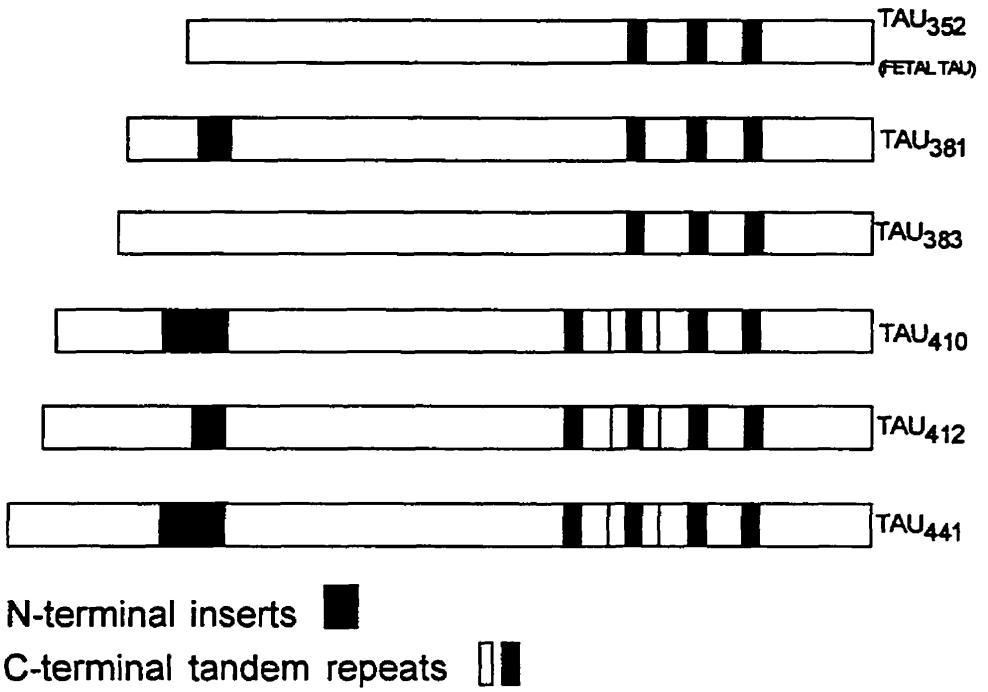


Figure 1. Schematic representation of the 6 human tau isoforms. The 6 tau isoforms arise from alternative splicing and vary in length. They also have different number of inserts at the N-terminal (big black square) and the different number of tandem repeats at the C-terminal part.

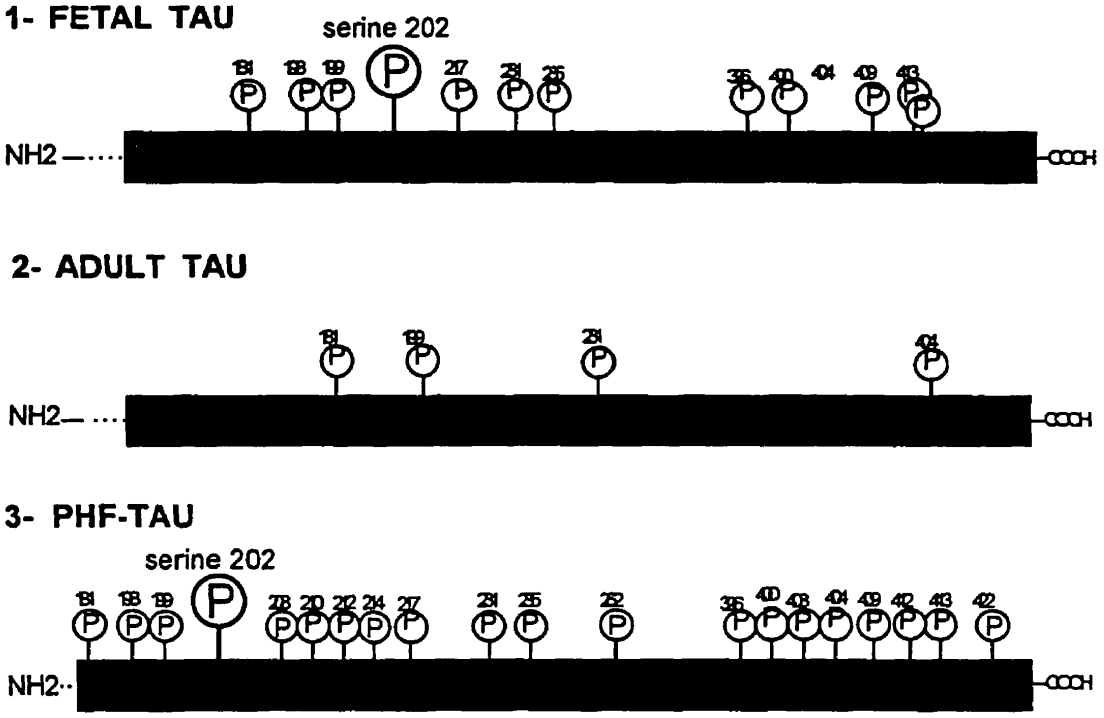


Figure 2. Phosphorylation sites of tau protein. Tau protein is phosphorylated on 12 different epitope early in the development (fetal tau). As we aged, the adult tau protein becomes dephosphorylated (only 4 epitopes are phosphorylated). In the brain of patients with AD there is PHF-tau which possesses 19 phosphorylated epitopes.

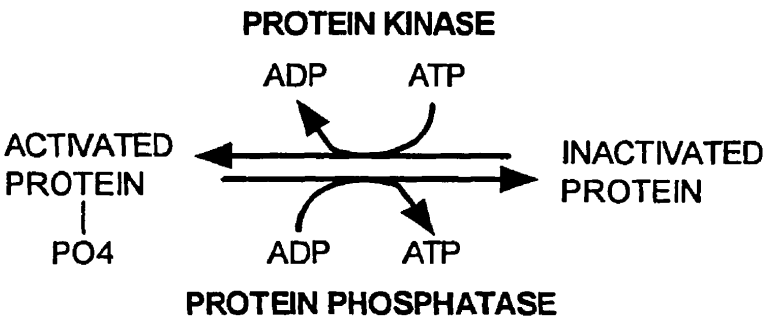


Figure 3. Phosphorylation reaction. Number of proteins are activated by the addition of a phosphate (the opposite is also true, some proteins become activated by the removal of phosphate). The protein kinases have the ability of taking a phosphate from the ATP and giving it to a protein whereas the protein phosphatases take the phosphate from a protein.

1990; Goedert, 1993; Goedert *et al*, 1995; Morishima-Kawashima *et al*, 1995). Fetal tau is preferentially dephosphorylated shortly after birth and stays dephosphorylated as adult tau. Moreover, the 6 isoforms of tau protein are sulfated on cysteine 322 for the isoforms with 3 repeats and on cysteine 291 and 322 for the 4-repeats isoforms (Schweers *et al*, 1995). Others have suggested a possible role for glycosylation in the formation of PHF, as this modification is present (Alonso *et al*, 1996). The mechanism of how the glycolsylation will affect the normal tau is still unknown.

2.2.1.2- The Role of Tau Protein in Paired Helical Filaments.

Normally, tau promotes tubulin assembly and stabilizes microtubules in vitro, but PHF-tau has a greatly reduced ability to bind microtubules; this functional impairment is proposed to result entirely from abnormal phosphorylation, since dephosphorylated PHF-tau binds as well to microtubules as does normal tau (Bramblett *et al*, 1993). However, Although Wille and his colleagues (1992) showed *in vitro* reassembly of PHFs from fragmented tau protein lacking most of the phosphorylation sites. The reduced binding of PHF-tau to microtubules coupled with reduced levels of normal tau probably destabilizes microtubules in AD, resulting in the impairment of vital cellular processes, such as rapid axonal transport, and leading to the degeneration of the affected nerve cell (reviewed in Goedert, 1995; Goedert *et al*, 1995; Trojanowski *et al*, 1995).

One surprising feature of PHF-tau is its distribution in the neuron. Normally, tau protein is located in the nerve cell axons whereas in AD brain, the tau protein is found in nerve cell bodies, axons and dendrites (Binder *et al*, 1985).

One major question to answer in the AD field is whether tau is hyperphosphorylated because of increased phosphorylation or of decreased dephosphorylation. This question is difficult to answer because the direct approaches to study PHF-tau are problematic to use because **(1)** human brain samples are often hard to obtain due to ethical reason, **(2)** post-mortem intervals lead to artifactual results, **(3)** the tau protein itself is considered as heterogeneous because of its 6 isoforms and, finally **(4)** PHF-tau are insoluble. The indirect methods to study the involvement of PHF-tau in the progression of AD are not representative of the events occuring in the human brain. They are principally based on tau protein from normal porcine or bovine brain (in which tau is not abnormally modified) or on recombinant tau expressed in bacteria where no phosphorylation occurs. To study PHF-tau in those conditions, the protein must be modified *in vitro* into PHF-tau by phosphorylation on ser-pro or thr-pro motifs as it is *in vivo* (Biernat *et al*, 1993). Thanks to the technological advances, PHF-tau can be distinguished from normal tau by 3 hallmarks: **(1)** the mobility shift on SDS-gel: PHF-tau migrate as a higher molecular weight protein than normal tau. PHF-tau runs as 3 bands of 60, 64, and 68kDa whereas normal adult tau migrates as 6 bands between 54 and 60 kDa, **(2)** PHF-tau is highly insoluble, and **(3)** certain

antibodies such as AT8, Alz50, PHF-1 react only with PHF-tau (reviewed in Biernat *et al*, 1993).

3- Tau Phosphorylation.

Tau proteins are mostly phosphorylated on serine (ser) and threonine (thr) residues followed by a proline (ser/thr-pro) and a given tau protein could be phosphorylated at some but not all of these sites. The sites of fetal tau phosphorylation are serines 198, 199, 202, 217, 235, 396, 400, 404, 409 and 413 and threonines 181 and 231 residues (according to the longest tau isoform) (figure 2). Adult tau proteins are phosphorylated only at serines 199 and 400 and threonines 181 and 231 residues. PHF-tau are phosphorylated on thr181, ser198, ser199, ser202, ser 208, ser210, ser212, ser214, ser217, thr231, ser235, ser262, ser396 ser400, ser403, ser404, ser409, ser412, ser413 and ser422 (reviewed in Goedert, 1995; Goedert *et al*, 1995; Trojanowski *et al*, 1995). Comparatively to normal adult tau protein, PHF-tau possess 6 other sites of phosphorylation.

3.1- The Phosphorylation Reaction

Phosphorylation regulates various enzymatic activities in a cell. This modification consists in the addition of a phosphate group to a specific hydroxyl group of a serine, tyrosine or threonine residue in the enzyme. The ATP provides

the phosphate and a protein kinase catalyses the reaction (figure 3). The addition of phosphate causes a structural change because of its negative charges: this can modify the activity of the ligand-binding site. The activities of the phosphoproteins in eukaryotic cells are controlled by an equilibrating rate of phosphorylation. Therefore, the phosphorylation reaction can be reversed by a protein phosphatase that remove the phosphate from the enzyme. The protein kinase uses ATP as the phosphate donor, while a phosphatase uses non-ATP as phosphate donor. Protein phosphatases, just like protein kinases, can be very specific or nonspecific to a protein.

3.2- The Phosphorylation of Tau Protein.

It has been demonstrated that several protein kinases are able to phosphorylate tau protein on different epitopes *in vitro*: mitogen-activated protein kinase (MAPK) (Goedert *et al*, 1992; Drewes *et al*, 1992), GSK-3β (Ishiguro *et al*, 1992; Singh *et al*, 1995), cyclin-dependent kinase 5 (cdk-5) (Paudel *et al*, 1993; Kobayashi *et al*, 1993; Baumann *et al*, 1993), cell-division-cycle-2 or cyclin-dependant-kinase-2 (cdc2) (Ledesma *et al*, 1992; Baumann *et al* 1993), cAMP-dependent protein kinase (Scott *et al*, 1993; Litersky and Johnson, 1992), casein kinase II (Masliah *et al*, 1992a) and protein kinase C (Correas *et al*, 1992). There is also a novel non identified serine/threonine protein kinase of 32kDa that phosphorylates tau in bovine brain (Takahashi *et al*, 1995).

3.2.1- The Phosphorylation of Epitope Serine 202 of Tau Protein.

Serine 202 is a serine residue followed by a proline and is not located in the microtubule-binding region. Fetal tau and PHF-tau are phosphorylated on the epitope serine 202 but normal adult tau is not (reviewed in Goedert, 1995; Goedert *et al*, 1995; Trojanowski *et al*, 1995). As demonstrated by Yoshida and Ihara (1993) tau-1 (an antibody reactive with dephosphorylated serine 199/202 epitopes of tau) lack immunoreactivity to PHF-tau, meaning that serine 202 epitope is highly phosphorylated in PHF-tau. Fetal tau immunoreacts weakly with tau-1 antibody. Goedert and his collaborators (1993) with the antibody AT8 that recognizes only the serine 202 phosphorylated epitope, demonstrated that in human fetal brain serine 202 is phosphorylated at 20, 23 and 39 weeks of gestation but not in neonatal or adult brain. They concluded that phosphorylation of serine 202 ceases abruptly around birth. They also showed the existence of kinase activity in adult rat brain extract for the epitope serine 202 of tau protein.

The phosphorylation of the epitope serine 202 by a protein kinase is an important event in both fetal and PHF-tau, possibly meaning that some developmental mechanisms could be reactivated in AD brain. Therefore, the study of serine 202 phosphorylation is required for the understanding of AD pathology.

For the purpose of my study, I will investigate the phosphorylation of only one epitope of tau protein, ser202 which could be phosphorylated by 4 protein kinases: MAPK, cdk5, cdc2 and GSK-3β. Only 3 of these kinases are neuronal: MAPK, cdk5, and GSK-3β. Cdc2 is not present in terminally differentiated post-mitotic neurons, but is expressed at early stages of embryogenesis (Tsai *et al*, 1993).

3.2.1.1- Cell-Cycle Dependent Kinase 5.

Usually, cell-cycle dependent protein kinases are involved in the process of division. They are normally expressed in dividing cells, but surprisingly cdk5 is found in neurons which are post-mitotic terminally differentiated cells. The expression of cdk5 occurs when the neuron is terminally differentiated, and this corresponds to the arrest in the expression of cdc2 which is active during the division of the neuronal cells (Tsai *et al*, 1993; Lew and Wang, 1995; Lew *et al*, 1995).

Cdk5, also called NLCK, BPDK, or cdc2-like kinase, is a heterodimer of cdk5 of 33kDa and a regulatory subunit of 25kDa, called p25, which is proteolytically derived from p35, a neuron-specific protein (Tsai *et al*, 1994; Lew *et al*, 1994). The complex is only active in the brain, even if cdk5 is found in most tissues at varying levels (Tsai *et al*, 1993).

Phosphorylation of a substrate by cdk5 is highly dependent of the proline residue at the carboxyl terminal to the phosphorylated serine or threonine. The phosphorylation of the epitope thr14 and tyr15 of cdk5 inactivates it, whereas phosphorylation of ser 159 activates it. The protein kinases responsible for the phosphate addition are wee1-like and thr14 kinase (reviewed in Qi *et al*, 1995; Lew and Wang, 1995). The cdc25 is a phosphatase that removes the phosphates on epitope thr14 and tyr15 (reviewed in Lew *et al*, 1995; Qi *et al*, 1995). This shows that the activation of cdk5 is highly regulated.

There is a number of neuron-specific proteins like synapsin, tau (Kobayashi *et al*, 1993; Paudel *et al*, 1993), middle and high molecular weight neurofilament proteins (NF-M and NF-H) (Lew *et al*, 1992; Hisanaga *et al*, 1993) that are phosphorylated *in vivo* by cdk5. These findings tend to confirm the function of cell-cycle-like kinases in nonproliferative cells and in processes other than cell-cycle control. It also implicates a role for cdk5 in cytoskeletal dynamics.

The phosphorylation of tau into an Alzheimer-like state by cdk5 was demonstrated *in vitro* by the use of purified substrate and enzyme (Baumann *et al*, 1993; Hosoi *et al*, 1995; Kobayashi *et al*, 1993; Paudel *et al*, 1993). All these experiments were done from bovine or porcine brain extracts, and nothing has been done yet in human neurons.

3.2.1.2- Mitogen-Activated Protein Kinase.

MAPKs, also called extracellular signal-regulated kinase (ERK) are a family of kinases that require phosphorylation on both a threonine and a tyrosine residue to be activated. These cytoplasmic proteins can translocate into the nucleus to activate gene transcription. There are 2 well-known MAPKs activated by mitogenic signals: the 42kDa and the 44kDa. They are expressed at high levels in the developping and adult CNS. MAPK p42 is expressed in both neuronal (cell bodies and dendrites) and glial cells and is highly expressed in the neocortex, the hippocampus, the striatum and the cerebellum (Fiore *et al*, 1993). Once MAPK is activated, it can phosphorylate a great number of substrates: myelin basic proteins (*in vitro*), the $p90^{rsk}$ which phosphorylates the ribosomal protein S6 associated with the G0-G1 transition of the cell cycle (*in vitro* and probably *in vivo*), c-Jun and other immediate early genes (reviewed in Crews *et al*, 1992).

MAPK (p42) is one of the kinases that phosphorylate the tau protein on serine/threonine-proline motifs and transforms it into an Alzheimer-like state *in vitro* (Drewes *et al*, 1992). This is a very important finding because we know that MAPK is activated by extracellular signals, which could be in AD, the Aβ peptides. Moreover, it is known that Aβ peptides stimulate the activation of tyrosine kinases that can activate focal adhesion kinase (FAK) (Zhang *et al*, 1994). FAK and MAPK can be activated by trophic signals, reenforcing the possible existence of a link

between MAPK and Aβ peptides. The real involvement of MAPK in the *in vivo* phosphorylation of tau protein is still debated because some researchers have failed to induce hyperphosphorylation of tau by MAPK in *in vitro* systems (Latimer *et al*, 1995).

3.2.1.3- Glycogen Synthase Kinase 3β.

GSK-3 consists of 2 related proteins GSK-3β and GSK-3α. They were primarily described as modulators of glycogen metabolism (Alberts, 1994). GSK-3β is able to phosphorylate a certain number of proteins like tau. Ishiguro and collaborators (1992) demonstrated that the "tau protein kinase I" (TPKI) is able to phosphorylate tau into PHF-tau. TPKI appears to be GSK-3β whose presence in neurons was demonstrated by immunostaining of AD brains (Ishiguro *et al*, 1993). Several studies are showing that GSK-3β is one of the kinase responsible for the transformation of normal tau into the hyperphosphorylated form in brain extracts (Mulot *et al*, 1994; Singh *et al*, 1995; Sperber *et al*, 1995).

Several authors think that GSK-3β is the unique kinase involved in the Alzheimer-like transformation of tau. To support this idea, they based their experiments on serine 262 which is an important microtubule-binding site and they showed that GSK-3β phosphorylates tau at ser262 (the presence of heparin or tubulin is required for the reaction) (Moreno *et al*, 1995). Moreover, Takashima and

collaborators (1993) have published that GSK-3β could mediate Aβ toxicity in neurons. These experiments will be described in the section 5 of the introduction.

Therefore, GSK-3β as well as cdk5 and MAPK could likely be involved in the phosphorylation of serine 202 epitope of tau protein possibly as a result of a signal transduction induced by Aβ peptides.

4- Amyloid Beta Protein.

4.1- Amyloid Precursor Protein.

4.1.1 Expression of the Amyloid Precursor Protein.

The APP gene is normally expressed in brain cells and in peripheral tissues (Tanzi *et al*, 1987; Ponte *et al*, 1988). By differential splicing, APP gives 5 mRNAs containing the Aβ domain. The difference between them is based on 3 domains: Kunitz serine protease inhibitor (KPI) domain of 57 amino acids (Schilling *et al*, 1991), J-domain (which has an unknown function) and Aβ domain. The longest mRNA is APP_{770} and it has three intact domains. The APP_{751} possesses only the KPI domain whereas APP_{714} has the J domain. APP_{695} with only the Aβ domain, is the major isoform in the brain whereas all the isoforms are expressed in most tissues (Tanzi *et al*, 1987; Ponte *et al*, 1988). APP_{695} is most abundant in neurons which is consistant with its high expression in the brain, whereas APP_{770} and APP_{751}

are expressed at lower concentrations in glial cells and meninges (LeBlanc *et al*, 1993 and LeBlanc *et al*, 1997).

4.1.2. Functions of the Amyloid Precursor Protein.

APP is suspected to have 4 functions: it may function as a receptor, an adhesion molecule, in synaptogenesis and in neurite extension. **(1)** The APP is a membrane protein that resembles a glycosylated cell-surface receptor because it contains an anionic domain that is able to bind positive ions or polycations that may be involved in the electrical activity of the neurons (Kang *et al*, 1987). Also, APP contains a Go binding domain, a GTP-binding protein in the brain (Nishimoto *et al*, 1993). **(2)** The APP localized in the plasma membrane has a modulatory role in cell-cell and cell-substratum adhesion (Shivers *et al*, 1988; Breen *et al*, 1991). The APP molecule, through amino acid sequence RERMS (Chen and Yankner, 1991; Ghiso *et al*, 1992) interacts with type IV collagen and laminin (Narindrasorasak *et al*, 1995) to promote adhesion. Another domain of APP close to the N-terminal part is a heparin-binding domain in hippocampal rat neurons (Small *et al*, 1994). This domain probably has an important role in the pathology, because several infusions of $A\beta_{1-40}$ and heparan sulfate proteoglycans in rat neurons promote extracellular amyloid deposits (Snow *et al*, 1994). Moreover, membrane-associated APP increases neurite length and branching in PC12 cells (Milward *et al*, 1992). **(3)** Transgenic mice expressing low and moderate levels of human APPs show an increase in synaptophysin

immunoreactive presynaptic terminal and in GAP-43 expression which means that APP may mediates neuritic outgrowth (Mucke *et al*, 1994). These results suggest a role in the formation and the maintenance of the synapses which is consistent with the presence of APP in growing neurites of neonatal rat brain (Masliah *et al,* 1992b; Loffler and Huber, 1992) and its upregulation during brain development (Fisher *et al*, 1991). **(4)** Secreted APP has mitogenic properties on fibroblast Swiss 3T3 cells. Isoforms APP_{770} and APP_{751} containing Kunitz protease inhibitor are more mitogenic than APP_{695} (found mostly in the brain) (Schubert *et al*, 1989). Therefore, APP is an important protein because it plays fundamental roles in neurons.

4.1.3. Metabolism of the Amyloid Precursor Protein.

APP is metabolized by 3 different pathways (Figure 4). The **secretory pathway** gives a large secreted N-terminal fragment which is called secreted APP (sAPP) (Weidemann *et al*, 1989; Sisodia *et al*, 1990) and a 10 kDa cell-associated fragment which can be further processed into a nonamyloidogenic 3kDa containing only part of the Aβ domain (Haass *et al* 1992b; Buscoglio *et al*, 1993; Haass *et al* 1993). This pathway is the most used by cells. Metabolism of APP through the **endosomal-lysosomal pathway** gives 5 C-terminal fragments. The largest fragment contains the entire Aβ domain (Chen *et al,* 1990) and is therefore potentially amyloidogenic (Estus *et al*, 1992; Golde *et al*, 1992). The **4kDa-Aβ producing pathway** that generates Aβ found in senile plaques and in cerebral vessels involves reinternalization of the APP in the endosomes. The

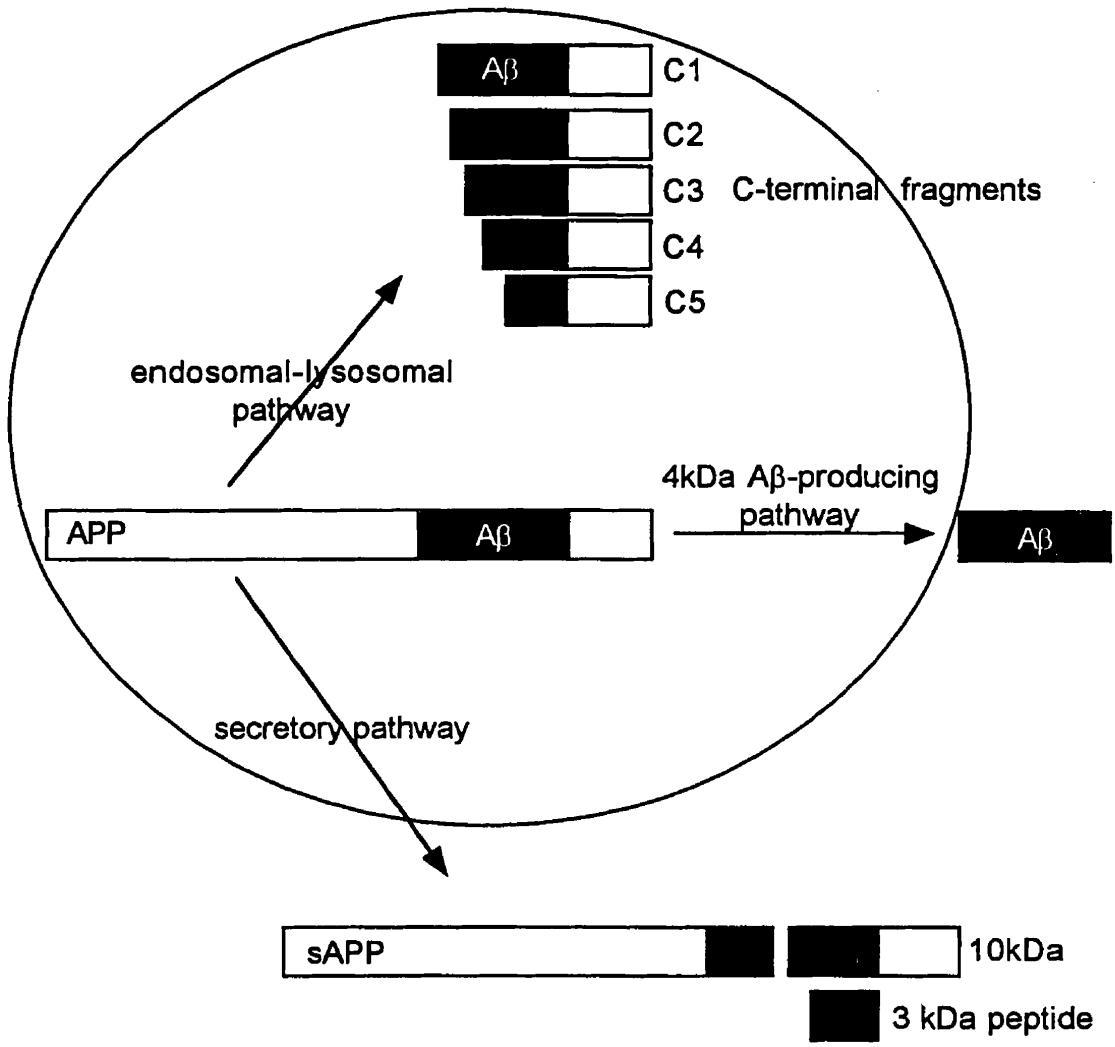


Figure 4. Amyloid precursor protein metabolism. The APP protein can be metabolized by three different pathways. The secretory pathway produces the secreted APP (sAPP) and a 10kDa which can be further processed into a 3kDa. The endosomal-lysosomal pathway produces 5 different C-terminal fragments (C1-C5) and the bigger fragment (C1) containing the entire Aβ domain and could be process into the 4kDa Aβ peptide. The 4kDa Aβ-producing pathway produces the Aβ peptide found in the senile plaques and the cerebral blood vessels.

cell-surface APP is its direct precursor and the C-terminal part of APP is required for Aβ production (Haass *et al,* 1992a; Koo and Squazzo, 1994; LeBlanc and Gambetti, 1994; Seubert *et al*, 1992; Shoji *et al*, 1992). In AD, Aβ production is increased in both familial and sporadic forms.

4.2- Amyloid Beta-Induced Apoptosis.

The Aβ peptide is a small protein of 39 to 43 amino acids. The toxicity of Aβ was first demonstrated in pheochromocytoma cells (PC12) and fibroblasts (NIH 3T3) (Yankner *et al*, 1989). PC12 cells were transfected with 3 different constructs of APP gene: entire APP gene, Aβ domain with a few amino acids at the N-terminal part and Aβ domain with the C-terminal portion. The PC12 cells were induced by the nerve growth factor to differentiate into a terminally differentiated post-mitotic cells. Seven days after the induction, the cells transfected with Aβ domain and the C-terminal part were dead in a vast majority. Moreover, conditioned media from 3T3 cells transfected with Aβ and the C-terminal portion induces the degeneration of terminally differentiated post-mitotic PC12 cells and of hippocampal rat neurons (Yankner *et al*, 1989; 1990). Aβ peptide exerts its toxic effect throught the amino acids 25-35 (Yankner *et al*, 1990). It's highly hydrophobic region (between amino acids 29 and 35) forms stable aggregations which are more toxic to cells than soluble Aβ (Pike *et al*, 1993; 1995).

Toxicity of 25-35 fragment was investigated on hippocampal, neocortical rat and mouse neurons. A dramatic loss of neurons occurs with chronic treatment of $A\beta_{25-35}$, increasing at high concentrations of Aβ. The neurons seem to die by apoptosis. The Aβ-treated neurons presented all the different characteristics of apoptosis: morphologic (DNA laddering, chromatin condensation) and biochemical (low release of LDH and slight decrease in mitochondrial activity) (Forloni *et al*, 1993; Loo *et al*, 1993). In these papers, apoptosis is induced at very high concentrations of Aβ (25μM), which is not physiological since the concentrations Aβ are around 4nM in cerebrospinal fluid (Gravina *et al*, 1995; Seubert *et al*, 1992). Therefore the experiments do not confirm that apoptosis really happens in AD brains or that *in vivo* Aβ peptides are toxic. It could be that pathological concentrations of Aβ has several disturbing effects on neurons that will eventually lead to cell death.

Aβ exerts its toxic effects by disturbing cellular components which could lead to cell death. Human cortical neurons treated with synthetic peptides (1-38 and 25-35) are more vulnerable to glutamate-mediated excitotoxicity. This vulnerability is dependent on calcium influx which is destabilized by Aβ (Mattson *et al*, 1992; Loo *et al*, 1993). Also, Aβ can induce formation of free radicals which disrupt the cell membrane (Behl *et al*, 1994; Shearman *et al*, 1994). Aβ has been shown to compete with nerve growth factor (NGF) for the low-affinity nerve growth factor receptor. This could possibly explain the higher susceptibility of certain neurons

that expressed high levels of low-affinity nerve growth factor to Aβ peptides (Rabizadeh *et al*, 1994).

Recently, Smale and collaborators (1995) found the first *in vivo* evidence of apoptosis in postmortem brain tissue (hippocampus and para hippocampal gyri) from AD patients. They used TUNEL (terminal transferase-mediated dUTP-biotin nick end labeling) staining which consists in the revelation of the increased number of hydroxyl-end-groups in the cleaved DNA (characteristic of apoptosis). They showed that the neurons from hippocampus and para hippocampal gyri stained with TUNEL but several astrocytes too, meaning that apoptosis is not restricted to neurons in AD.

Since Aβ was demonstrated to be toxic for neurons *in vitro* and *in vivo*, one of the main goals in the field of Alzheimer's disease is to find if there is a relationship between the extracellular Aβ and the intracellular neurofibrillary tangles.

5- The Effect of Amyloid Beta on Tau Phosphorylation.

The formation of senile plaques and neurofibrillary tangles is still a mystery in the sense that researchers do not know what exactly causes their formation and they do not know which precedes the other.

In the past 3 years, research has provided new clues showing that Aβ peptide may promote the formation of NFTs through mechanisms that are still unknown. Takashima and collaborators (1993) showed that 20μM of Aβ peptides 1-43 or 25-35 are toxic for primary cultures of embryonic rat hippocampus via the activity of the tau protein kinase I (TPK I) or GSK-3β. Using antisense oligonucleotides of TPK I, they demonstrated that the toxicity of Aβ peptides can be reduced by inhibition of TPK I (they assayed the toxicity by measuring the lactate dehydrogenase release and the cell survival). By immunocytochemistry they demonstrated that incubation of neuronal cultures with 20μM of $A\beta_{1-43}$ peptide for 24 hours are immunoreactive for Alz-50 epitope. They also showed that treatment of neuronal rat cell cultures with 20μM of Aβ peptides increase the tau phosphorylation activity of 1.6 times compared to control cultures. These results were the first concrete data showing a relationship between Aβ and tau phosphorylation. If Aβ really promotes tau phosphorylation, this suggests that extracellular Aβ may act through a transduction pathway to exert an effect on tau protein and consequently lead to a loss of microtubule binding, destabilizing the cytoskeleton and affecting the entire neuron as a final result.

In 1995, Busciglio and colleagues also using 20μM of Aβ, showed that in human and rat neuronal cultures, aggregated Aβ but not soluble Aβ, causes an increase in the immunoreactivity of phosphate-dependent antibodies to tau protein and the staining is increased in the somatodendritic compartment instead of being in the

axon as it is the case in normal neurons. Co-immunostaining with propidium iodide, a dye that stains the fragmented DNA, demonstrated that tau phosphorylation precedes neuronal apoptosis. They showed that an increase in tau phosphorylation due to Aβ treatment results effectively in the loss of microtubule binding capacity.

These two papers (Busciglio *et al*, 1995; Takashima *et al*, 1993) support the involvement of Aβ in the phosphorylation of tau protein and NFT formation occuring in AD. As opposed to these findings, Davis and collaborators (1995) found that 100μM of $A\beta_{25-35}$ has no effect on the phosphorylation of tau protein of 10 day-old rat cortical neuronal cultures even if there is an evident toxic effect (necrosis). In the three papers, the researchers used very high concentrations of Aβ which are known to be toxic for the neurons. Moreover, Davis and his colleagues (1995) used a concentration much higher which can possibly explain why they did not observe the same phenomenon (Aβ inducing tau phosphorylation). They were very far from the physiological concentrations of Aβ.

The major goal of this study is to examine the effects of physiological concentrations of Aβ on the phosphorylation of tau in the human. Therefore, we are very concerned about the concentrations of Aβ used in the experiments mentioned above and about the cell cultures used. We propose a human primary fetal neuron cultures as a system to study the effect of Aβ on the phosphorylation of tau protein, which is the best system to study AD. We also propose the use of 100nM of Aβ

peptides, a concentration that is close to the pathological concentrations of Aβ found in AD cerebrospinal fluid (Gravina *et al*, 1993; Seubert *et al*, 1993). We also used Aβ peptides that are found *in vivo*. $A\beta_{1-40}$ was chosen because it is the major form of Aβ found in the senile plaques. $A\beta_{1-40}$ is found in the cerebrospinal fluid of the AD brain, at a concentration of 73nM Hence, the 100nM concentration we used represents almost physiological and pathological conditions (Gravina *et al*, 1995; Seubert *et al*, 1992). One hundred nM of $A\beta_{1-42}$ is seven times less than what is found in AD brain but 250 times more than found in normal adult CSF. Also, $A\beta_{1-42}$ is perhaps more toxic than $A\beta_{1-40}$ since it is more hydrophobic and is able to aggregate more easily. Usually in the studies on the effect of Aβ peptides, $A\beta_{25-35}$ is used because this sequence was found to be the toxic part of Aβ peptides (Yankner *et al*, 1990), but it is not found under this form in human brain. As a control, we used the reverse sequence peptide for $A\beta_{1-40}$, named $A\beta_{40-1}$. We thought that it will be a very good control to determine if the addition of the peptides themselves would have an effect on the phosphorylation of tau protein in human neurons. Giordano and his colleagues (1994) showed that $A\beta_{1-40}$ and $A\beta_{40-1}$ have the same toxic effect in aged rats and in SH-5YSY differentiated cells, but only $A\beta_{1-40}$ was able to produce Alz50 staining of SH-5YSY. The three peptides were aggregated for the experiments because it is known that the toxicity is dependent on the aggregated state of the peptides (Pike *et al*, 1993; 1995).

The principal goal of this project is to investigate the role of Aβ on protein kinases in human neurons, we investigated if Aβ peptides can activate one or more protein kinase(s) that is (are) able to phosphorylate tau protein leading to the same type of hyperphosphorylated tau seeing in AD (figure 5).

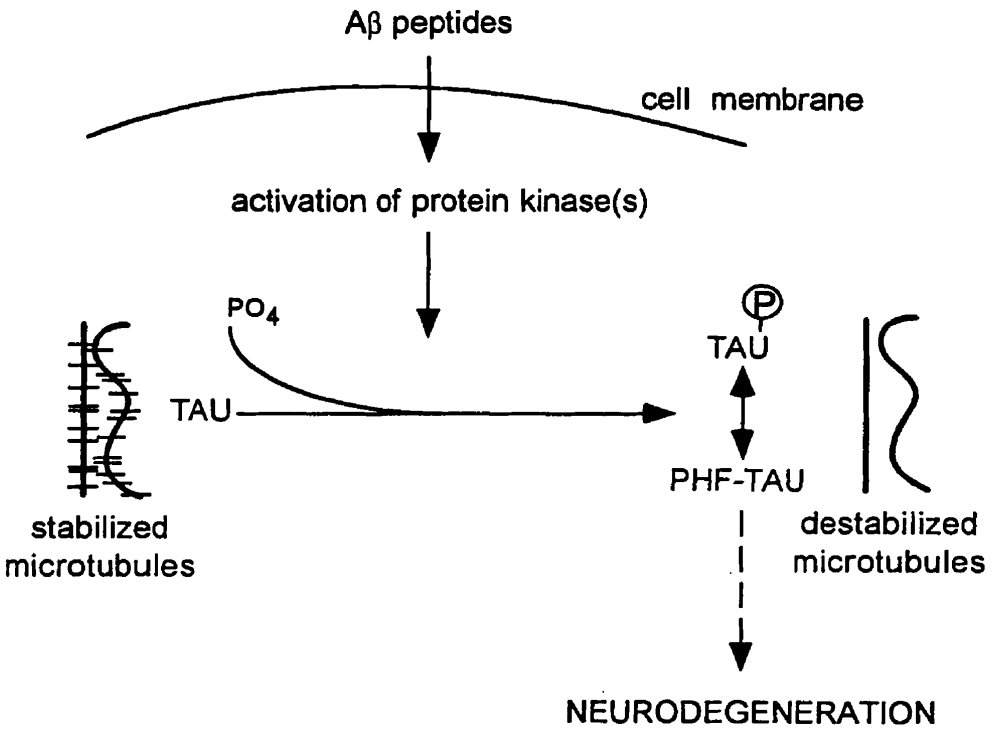


Figure 5. Effect of amyloid beta on tau phosphorylation. The Aβ peptides, through an unknown signal, activate a protein kinase which phosphorylates tau protein. The addition of phosphates on tau transforms it into PHF-tau which are unable to bind to the microtubules, leading to the degeneration of the neuron.

1- Human Fetal Primary Neuron Cultures.

Human fetal cultures (10-18 weeks of gestation) are prepared in accordance with guidelines established by MRC and NIH and approved by JGH ethical committees and McGill Institutional Review Board. The fetal brain is sterily dissociated in small pieces in a petri dish with 1X PBS after the removal of the meninges and the blood vessels. The tissue is placed in a Falcon tube with 0.25% trypsin (Gibco; 15090-046) to dissociate the cells for 15 minutes at 37^{0}C followed by treatment with 1mg\mL DNAse I (Boerhinger Mannheim; 1284 932). The action of the trypsin is inhibited by adding 5% decomplemented bovine calf serum, dBCS (Hyclone; A-2151). The serum is decomplemented by heating at 56^{0}C for 30 minutes. Then, the tissue is passed through 2 nylon meshes of 130µm and 70µm (Fisher; 2350) to separate the cells. The cells are spun and resuspended in MEM (Gibco 11700-010) supplemented with 0.225% sodium bicarbonate (Gibco; 25080-094), 1mM sodium pyruvate (Gibco; 11360-013), 2mM L-glutamine (Gibco; 25030-016), 1X penicillin-streptomycin (Sigma; 15240-021), 0.1% glucose (Sigma; G-7021) and 5% dBCS. The cells are seeded at a density of 3x10^{6}cells\mL of media on 10µg\mL poly-L-lysine (Sigma; P-6282) coated flasks. After 3 days in culture, 1mM fluoro-deoxyuridine (Sigma; F-0503), an antimitotic, is added to the media to

prevent growth and differentiation of dividing cells. The cells are fed every 3 days and on the 11th day, the cells are ready for experimentation.

2- Characterization of the Cultures by Immunocytochemistry.

$1.5x10^4$cells are seeded on an aclar coverslip coated with 10µg/mL poly-L-lysine. After 11 days in cultures, the coverslip is washed 2 times in 1X PBS and fixed in 5% acetic acid: 95% ethanol. Then the gel is incubated 1 hour in 10% goat serum (Gibco; 16210-015) in 1X PBS. The coverslip is incubated in a humid chamber with the first antibody for 1 hour at room temperature. The polyclonal antibody tau which stains the axons of normal neurons, is a kind gift from Dr. Paudel (McGill University) and the antibody to glial fibrillary acidic protein (GFAP) (Sigma; G-3893) stains the astrocytes. The dilution used is 1:500 (the antibody is diluted in 10% goat serum in 1X PBS). The coverslip is washed again in 1X PBS and incubated with goat anti-rabbit IgG (H+L) FITC-linked (Jackson; 02-15-06), 1:1000 in 10% goat serum in 1X PBS, for 1 hour at room temperature. The coverslip is washed 3 other times and mounted in glycerol:1X PBS (1:1) on a slide. The fluorescence is observed with a fluorescent microscope at 20X magnification.

3- Treatment of Human Fetal Primary Neuron Cultures with Amyloid Beta Peptides.

Peptides of $A\beta_{1-40}$ and $A\beta_{1-42}$ from Bachem (H-1194 and H-1368) and $A\beta_{40-1}$ from Sigma are solubilized in sterile nanopure water at a concentration of 25µM. The peptides are incubated 5 days at 37°C to promote aggregation (Pike *et al*, 1993; 1995). The day of the experiment, the aggregated peptides are solubilized in complete neuronal MEM media at a concentration of 100nM. The old media is removed and the complete media containing Aβ peptides is added to the six-well dishes. The neurons (3×10^6cells per dish) are incubated for 0, 1, 3, 6, 12, 24, 48 and 72 hours. After the incubation time, the media is removed and the cells are washed 2 times with 1X PBS containing protease and phosphatase inhibitors: 1% AESFB (ICN; 193503), 10µg\mL aprotinin (ICN; 190382), 10µg\mL leupeptin (ICN; 151553), 10µg\mL TLCK (ICN; 152152), 10mM sodium fluoride (Sigma; S-1504), 1mM sodium orthovanadate (Sigma; S-6508) and 0.1µM okadaic acid (Sigma; O-8010). Neurons are recuperated in 1X PBS containing protease and phosphatase inhibitors and spun down 10 minutes at 1100 rpm. The supernatant is removed and the cells are lysed in 30µL NP-40 (50mM NaCl; 50mM Hepes pH 7.3; 1mM $MgCl_2$; 0.5mM DTT; 1mM EDTA; 5mM EGTA; 0.1% NP-40) containing also protease and phosphatase inhibitors at the same concentrations. The cell lysate is sonicated 30 seconds and spun again 10 minutes at maximal speed

in a microfuge in the cold to remove insoluble proteins. Aliquots of 10µL are frozen at -70°C until the day of the experiment.

4- Immunoblotting of Neurons Treated with 100nM Amyloid Beta Peptides.

To 10µL of cell lysates (1 x 10^6 cells) in NP-40, 2.5µL of 4X loading sample buffer (BioRad: 0.5M Tris-HCl pH 6.8; Glycerol; 10% (w/v) SDS; 2-mercaptoethanol; 1% (w/v) bromophenol blue) is added. The samples and the low-molecular weight prestained marker (BioRad; 161-0305) are boiled 5 minutes and loaded on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-gel, the recipe used for SDS-gel is from Bio-Rad) and run for about 1 hour 30 minutes at 100 volts. The gels are transferred for 1 hour at 200 milliamperes on Immobillon-P membranes (Millipore, Canada). The membranes are blocked in blotto A (Santa Cruz, California) (5% (w/v) low fat milk in TBST (20mM Tris pH 8.0; 100mM NaCl; 1% Tween-20)) with gentle shaking. After 1 hour 30 minutes, the membrane are incubated overnight at 4°C in a humid chamber with the first antibody diluted in TBST, either with the polyclonal antibody tau 1:500 (from Dr. Paudel, McGill U.) or the monoclonal antibody 5ng\mL tau-1 (Boerhinger Mannheim; 1289977). The next morning, the membranes are washed 3 times for 10 minutes in TBST and incubated for 1 hour 30 minutes in a humid chamber with the secondary antibody, 1:5000 goat anti-rabbit IgG (H+L) (Jackson; 075-1506) or 1:1000 goat anti-mouse IgG (H+L) (Jackson; 115-055-046) conjugated to alkaline phosphatase, also diluted in TBST.

The membranes are washed again 3 times in TBST and 10 minutes in AP buffer (100mM Tris-Cl pH 9.5; 100mM NaCl; 5mM $MgCl_2$) and they are developed in 10mL AP buffer containing 66uL of 50mg/mL NBT (dissolve in 70% dimethylformamide) (Fisher; BP108-1) and 33uL of 50mg/mL BCIP (dissolve in 100% dimethylformamide) (Fisher; BP114-25).

5- Purification of Recombinant Tau_{441} from E. coli.

Escherichia coli bacteria, containing an expression contruct encoding the longest isoform of tau protein, recombinant tau_{441}, under an inducible promotor, T7 RNA polymerase promoter (Goedert and Jakes, 1990), are grown in 0.1mg\mL ampicillin\LB broth overnight at 37°C with strong agitation. The culture is then diluted 10 times in 1 litre of ampicillin\LB broth and incubated at 37°C with strong agitation until the optical density 600 (OD_{600}) reaches 0.5-0.8. At that point, IPTG was added to the final concentration of 0.2mM to the media to induce promotor activity. When the OD_{600} reaches 1-1.5 the bacterial suspension is centrifuged at 6500 rpm for 15 minutes and the pellet is resuspended in piperazine-N,N'-bis[2-ethane-sulfonic acid], PIPES buffer (100mM PIPES pH 6.4; 1mM $MgCl_2$; 1mM EGTA) containing protease inhibitors (10ng\mL AEBSF, 1ng\mL pepstatin, 1µg\mL leupeptin and 5mg\mL benzamidine). The amount of buffer is 1mL\100mL of culture. Twenty µg\mL lysosome is added and the suspension is incubated at room temperature for 15 minutes to break the cell membrane. After, 1% triton-100X and

10mM β-mercaptoethanol are added to reduced the disulfide bonds. The bacterial culture is sonicated 3 times using a probe sonicator for 15 seconds each and cooled on ice. The suspension is centrifuged 15 minutes at 15000rpm and the supernatant is incubated 20 minutes in boiling water and recentrifuged for 20 minutes. The supernatant is loaded onto a Q-sepharose (Pharmacia; 17-0510-01) column equilibrated with PIPES buffer pH6.4. The negative charge of the beads will elute the tau protein which is negatively charged at this pH. The suspension is collected in the flowthrough which is loaded directly onto a S-sepharose column equilibrated in PIPES buffer. The beads contained in the column are positive and the tau protein is caught in the column. The column is washed with at least 10X volume of PIPES buffer. Then the recombinant tau_{441} is eluted from the column with 0.2M NaCl in PIPES buffer. The peak of proteins is found by measuring OD_{280}. The fractions containing the proteins are combined and the concentration of proteins is determined by protein dosage using BCA assay with BSA protein as standard as described by the manufacturer (Pierce, Canada). The purity is assayed by Coomassie blue gel, where 10µL of proteins is loaded and run on a 10% SDS-gel and stained with Coomassie blue (dissolved in 90mL of methanol: H_2O (1:1 v:v) and 10mL of glacial acetic acid) for 2 hours with gentle shaking. The gel was destained by soaking in 90mL of methanol: H_2O (1:1 v:v) and 10mL of glacial acetic acid for 3 hours with many solution changes. Immunostaining with polyclonal tau antibody (as described above) is also performed on the fractions to verify if the major protein correspond to the recombinant tau_{441} protein.

6- Hot Tau Phosphorylation Assay.

The literature describes several tau phosphorylation protocols depending on the kinase studied. The conditions used to detect kinase activity on serine 202 epitope of tau protein by cdk5, MAPK and GSK-3 are summarized in figure 6. We chose the average conditions described to detect all 3 kinase activities (figure 7). We first tried our tau phosphorylation buffer on non Aβ-treated neuronal cell lysates in NP-40 as decribed in section 3. The reaction mix is the following: 10mM Hepes pH 7.3; 5mM $MgCl_2$; 0.1mM cold ATP; 1μCi ^{32}-P (Dupont NEN; BLU/NEG/502A); 0.5mM DTT; 0.25mg\mL recombinant tau_{441}; 0.1mM EGTA; 0.1mM EDTA; 10μg\mL TLCK; 10μg\mL AESBF; 10μg\mL leupeptin; 10μg\mL pepstatin; 5μM okadaic acid; 0.1mM sodium orthovanadate and 10mM sodium fluoride and 10μL of cell lysate at 30°C in a total volume of 40μL. The time of incubation required to observe the phosphorylation of tau is optimized by assesing phosphorylation reaction at 0, 1, 3, 6, 12 and 18 hours. The reaction is stopped with 1X loading sample buffer (BioRad) and the samples are boiled 5 minutes. The proteins are loaded onto a 10% SDS-gel for 1 hour 30 minutes at 100 volts. The gels are dried and exposed on X-ray film. To dry the gels, we used a DryEase Mini-Gel Dryer System (Novex; N12387). The gel is soaked 3 times 2 minutes in H_2O and 20 minutes in gel-dry drying solution (Novex; LC4025). Then the gel is placed between two sheets of cellophane and sealed between the 2 frames. The frames are clipped together and the gels are dry at room temperature.

ENZYME	MAPK		CDK5			GSK-3	
	1	2	1	2	1	2	3
reference	Drewes et al. 1992.	Goedert et al. 1992	Baumann et al. 1993	Paudel et al. 1993	Singh et al. 1995	Mulot et al. 1994.	Mulot et al. 1995.
Buffer	40mM Hepes pH 7.4	25mM Tris pH 7.0	40mM Hepes pH 7.4	50mM MOPS pH 7.2	40mM Hepes pH 7.	20mM Hepes pH 7.5	20mM Hepes pH 7.5
$MgCl_2$	3mM	10mM Mg acetate	3mM	10mM	6mM	5mM	5mM
ATP	2mM	2mM	2mM	0.2mM	0.1mM	3mM	3mM
gamma ^{32}-P ATP	10-20Ci/mmol	X	25-50Ci/mol	X	X	X	X
DTT	2mM	X	2mM	X	X	0.5mM	0.5mM
Mercaptoethanol	X	X	X	X	10mM	X	X
[Tau]	0.25mg/mL	1uM	0.2mg/mL	0.5mg/mL	0.15mg/mL	8µg	X
EGTA	5mM	0.1mM	5mM	0.1mM EDTA	X	X	X
PMSF	0.2mM	0.2mM	0.5mM	X	X	1mM	1mM
leupeptin	X	X	X	X	X	10µM	10µM
pepstatin	X	X	X	X	X	1µM	1µM
okadaic acid	5µM	X	X	X	X	5µM	5µM
$NaVO_4$	X	0.1mM	X	X	X	1µM	1µM
time (hours)	16	24	16	1.5	6	24	24
temperature	37°C	30°C	37°C	30°C	30°C	30°C	30°C

Figure 6. Summary of the different tau kinase buffers for MAPK, GSK-3B and cdk5. A review of the literature on serine 202 epitope of tau protein phosphorylation demonstrates that some solutions are require for maximale kinase activity.

Concentration in working solution	Working solution	Final concentration in reaction mix
40mM Hepes pH 7.3	1.2μL of 1M	30mM
5mM $MgCl_2$	0.15μL of 1M	3.75mM
0.1mM ATP	0.3μL of 10mM	0.075mM
gamma ^{32}-P ATP	0.1μL	0.1μCi
0.5mM DTT	0.75μL of 20mM	0.375mM
0.25mg/mL Tau	1.5μL of 5mg/mL	0.19mM
0.1mM EGTA	0.15μL of 20mM	0.075mM
0.1mM EDTA	0.6μL of 5mM	0.075mM
10μg/mL TLCK	0.3μL of 1mg/mL	7.5μg/mL
10μg/mL PMSF	0.3μL of 1mg/mL	7.5μg/mL
10μg/mL leupeptin	0.3μL of 1mg/mL	7.5μg/mL
10μg/mL pepstatin	0.3μL of 1mg/mL	7.5μg/mL
5μM okadaic acid	1.5μL of 0.1mM	3.75mM
0.1mM $NaVO_4$	0.3μL of 10mM	0.075mM
10mM NaF	0.75μL of 400mM	7.5mM
sample		10μL
water		21.2μL
time (hours)		6
temperature		30^{0}C

Figure 7. Tau phosphorylation buffer used to perform kinase assay. This buffer is the buffer we made to have the maximale activity from the three protein kinases: cdk5, MAPK and GSK-3β to phosphorylate tau protein. 51

The Aβ-treated samples in NP-40 from section 3 are incubated in the same tau phosphorylation buffer at 30°C for 6 hours and are also run on 10% SDS-gel, dried and exposed the same way.

7- Cold Tau Phosphorylation Assay.

Ten µL of neuronal soluble proteins (in NP-40 as described in section 3) treated with $A\beta_{1-40}$, $A\beta_{1-42}$ and $A\beta_{40-1}$ for 0, 1 and 24 hours are incubated in tau phosphorylation buffer (10mM Hepes pH 7.3; 5mM $MgCl_2$; 0.1mM cold ATP; 0.5mM DTT; 0.25mg\mL recombinant tau_{441}; 0.1mM EGTA; 0.1mM EDTA; 10µg\mL TLCK; 10µg\mL AESBF; 10µg\mL leupeptin; 10µg\mL pepstatin; 5µM okadaic acid; 0.1mM sodium orthovanadate and 10mM sodium fluoride at 30°C for 6 hours for a total volume of 40µL. The reaction is stopped with 1x loading sample buffer (BioRad) and boiled 5 minutes. Ten µL of the reaction mix is loaded on 10% SDS-gel and run for about 1 hour 30 minutes at 100 volts. The gels are transfered for 1 hour at 200 milliamperes on Immobillon-P membrane. The immunoblotting is then performed as described in section 4 with the same antibodies.

8- Histone H1 Protein Phosphorylation.

The phosphorylation site of the three kinases we are investigating for are S/T $XXXS^P$ for GSK-3; PX(S/T)P for MAPK and S/TPA/KKAA/KA/K for cdk5. The

histone H1 protein used for the assay can be phosphorylated only by cdk5. Therefore, if we observe an activity, it will show that cdk5 is one of the possible activated kinase phosphorylating tau on serine 202.

The neurons are treated for 0 and 24 hours with 100nM and 20μM of $A\beta_{1-40}$, $A\beta_{1-42}$ and $A\beta_{40-1}$ in complete MEM media and recuperated the same way as describe in section 3 of Material & Methods. As a positive control, cdk5 purified enzyme (gift from Dr. Paudel McGill University) in NP-40 lysis buffer containing protease and phosphatases inhibitors is used and a NP-40 only sample is used as negative control. Nine μL of soluble proteins in NP-40 are incubated 3 hours at 30^0C in 18μL reaction mix (50mM Hepes pH 7.2; 0.1mM EDTA; 10mM $MgCl_2$; 0.167mM sodium orthovanadate; 10mM sodium fluoride; 0.5mM DTT; 1μCi ^{32}P-ATP) containing 3μg of histone H1 protein (Boerhinger Mannheim) in a total volume of 30μL per assay. The reaction is stopped with trichloroacetic acid (TCA) to a final concentration of 20% and 15μL of the reaction is spotted on phosphocellulose paper. The paper is washed 3 times for 10, 15 and 30 minutes in 5mL H_3PO_4 in 500mL of tap water. The paper is placed in scintillation vial (1 paper\vial) and the radioactivity counted in a scintillation counter.

9- Densitometric Scanning and Quantification of Tau Protein Levels.

The intensity of the immunostaining of the human fetal neuron cultures with the antibodies tau and tau-1 is quantified by densitometric scanning of immunoblots (Molecular Dynamics Inc.). The intensity of the bands was determined by the scanner, and the digitized values were then used to determine the relative amounts of the proteins. Each western blot is corrected against the background and the number of pixels obtained is considered as the amount of total tau protein in the case of staining with the polyclonal antibody against tau and as the amount of dephosphorylated serine 202 epitope of tau protein with tau-1 antibody. The fetal tau band at 54kDa is quantified for the purpose of the study. The amount of serine 202 phosphorylation is obtained by dividing the pixels of total tau protein by the number of pixels of the dephosphorylated serine 202 epitope. The numbers are corrected for the zero time point and for the $A\beta_{40-1}$ control. A student T-test with a two-tailed distribution is performed to determine whether the results are significatively different from the control ($p < 0.01$).

The amount of phosphorylation of recombinant tau_{441} protein is quantified by phosphorimager (Molecular Dynamics Inc.). The quantity of radioactivity for each autoradiograph is corrected against the background and the relative values are also corrected for the zero time point. A student T-test with a two-tailed distribution is

performed to determine whether the results are significatively different from the control ($p < 0.1$).

C. RESULTS

1- Characterization of Human Fetal Primary Neuron Cultures.

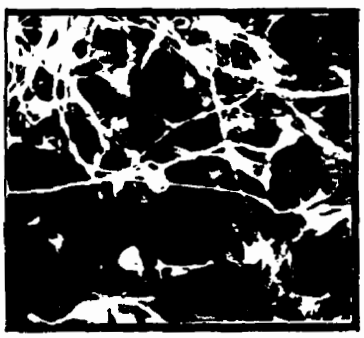
1.1- Phase Contrast and Immunocytochemistry.

To study problems related to AD, a human pathology, the use of a human system is the first step to be as close as possible of the reality. Therefore the human fetal primary neuron cultures establish a very good base to study the effect of Aβ on human neurons. The very first step is to characterize the cultures.

After 11 days *in vitro* and 7 days of treatment with 1mM of fluorodeoxyuridine, human fetal primary neuron cultures showed a high proportion of neurons as seen by phase contrast and immunocytochemistry (figure 8). Within 3 days of culture, the neurites were already extended. We characterized the cultures by immunocytochemistry using several cell specific antibodies. To perform immunocytochemistry, the cells were seeded on coverslips, fixed and permeabilized before immunostaining. The polyclonal antibody against tau, an abundant neurocytoskeleton protein, was used to identify neurons (figure 8b) and the glial

a) PHASE CONTRAST

b) TAU



c) GFAP

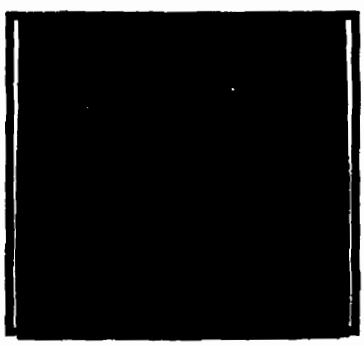


Figure 8. Human fetal primary neuron cultures. (a) Phase contrast (original magnification 200X). (b) Immunocytochemical staining of human neurons with the polyclonal antibody tau bound to the secondary antibody FITC-linked (original magnification 200X). (c) Immunocytochemical staining of human neurons with the monoclonal antibody GFAP bound to the secondary antibody rodamine-linked (original magnification 200X).

fibrillar acid protein (GFAP) (figure 8c) was used to recognize the astrocytes. LeBlanc (1995) had shown that the neurons constitute 90% of our cultures, whereas the remaining 10% cells are astrocytes. We routinely confirmed the purity of the cultures by repeating the immunostaining for tau and GFAP.

2- Phosphorylated Serine 202 Epitope of Tau Protein in Neurons Treated with Amyloid Beta Peptides.

In AD, tau protein is hyperphosphorylated and aggregates to form PHFs and eventually the NFTs. Senile plaques formed of aggregated Aβ peptides, $A\beta_{1-42}$ and $A\beta_{1-40}$, are found as well in AD brain. To understand if there is a link between the aggregated extracellular Aβ and the hyperphosphorylation of tau protein in human neurons, we treated the neuron cultures with 100nM $A\beta_{1-40}$ which is 25 times the physiological concentration of Aβ in cerebrospinal fluid; 100nM of $A\beta_{1-42}$ and the control reverse peptide $A\beta_{40-1}$.

We exposed the human fetal primary neuron cultures for different incubation times and collected the neuronal lysates in protease and phosphatase inhibitors to conserve the integrity of the tau protein phosphorylation state. In order to measure the effect on tau gene expression and protein phosphorylation, we performed western blots with the following antibodies: anti-tau protein polyclonal antibody and monoclonal tau-1 against the dephosphorylated serine 202 epitope.

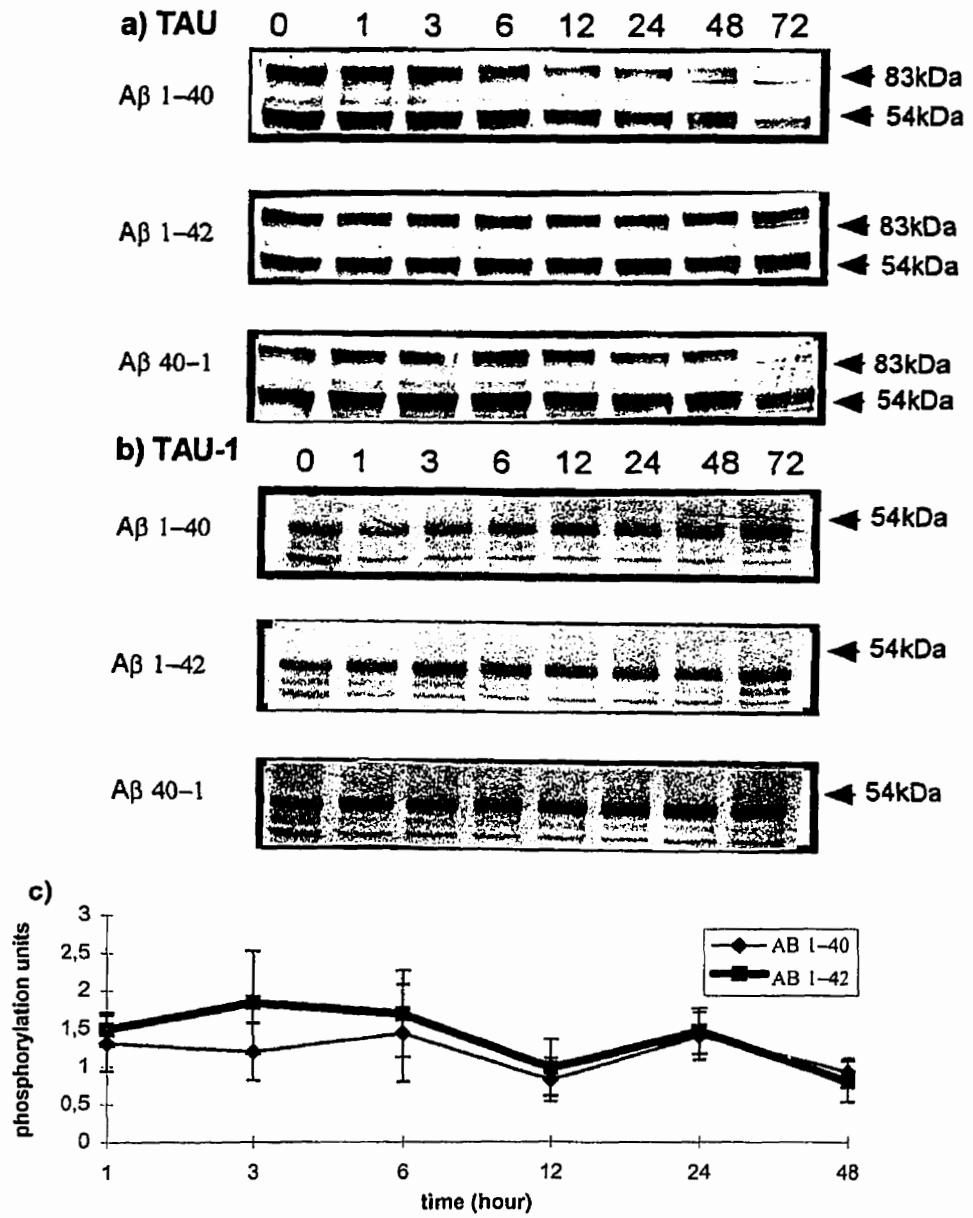


Figure 9. Western blot showing immunostaining of (a) tau protein detected with the polyclonal antibody and, (b) dephosphorylated serine 202 epitope of tau protein detected with the monoclonal antibody tau-1, in human neurons treated with Aβ 1-40, 1-42 and 40-1 (n=3). Secondary antibodies conjugated to alkaline phosphatase and NBT/BCIP substrates are used to detect immunoreactivity (c) Phosphorimaging quantification demonstrating phosphorylation of serine 202 epitope by measuring the ratio tau/tau-1. The results are standardized to the zero time point and to the Aβ 40-1 control to obtain phosphorylation units.

Western blots stained with the polyclonal antibody against tau (figure 9a) show two major bands at 54 and 83kDa (arrows). The lower molecular weight band corresponds to the molecular weight of fetal tau, around 54kDa and runs as a doublet. The doublet band has been observed before by Yoshida and Ihara (1993) and they speculate that the upper band of the doublet could correspond to a hyperphosphorylated form of fetal tau. This would explain the single band seen at 54KDa when immunoblotted with tau-1 (figure 9b). The upper band at 83kDa is a cross contaminating band that could be the microtubule-associated protein 2 (Yoshida and Ihara, 1993). In the 72 hour time point (figure 9a), we observe slightly lower molecular weight tau and lower levels of tau than at other time points. The increased mobility on SDS-gel could be explained by N and C-terminal truncation of the tau protein after 72 hours of incubation with either of the Aβ peptides ($A\beta_{1-40}$, 1-42 and 40-1).

For the neurons treated with $A\beta_{1-40}$, the western blot stained with the polyclonal antibody tau (figure 9a) shows that the staining of total tau protein decreases with time of treatment for 3 independent cultures. Every experiment showed decreased staining with time but the effect was slightly variable between three independent cultures studied. However, we observe that the staining with tau-1 antibody increases with time of treatment with $A\beta_{1-40}$ (figure 9b). The neurons treated with

$A\beta_{1-42}$ show equal staining with the polyclonal antibody against tau until 12 hours and slightly decreases after (figure 9a). The staining with tau-1 shows that there is an increase in the staining at 3 and 6 hours (figure 10b). These results indicate increased tau serine 202 phosphorylation in $A\beta_{1-40}$ and $A\beta_{1-42}$ treated neurons.

Neurons treated with the control peptide, $A\beta_{40-1}$, show that for up to 12 hours the staining with tau antibody is the same. The staining decreases slightly at 24 hours and continue to become lighter at 48 and 72 hours. $A\beta_{40-1}$ treated neurons show equivalent levels of tau-1 immunoreactivity for 0 to 12 hours (figure 9b). Staining increases indicating increased dephosphorylation of phosphoserine 202 residue with time of $A\beta_{40-1}$ treatment.

In order to quantify the amount of serine 202 phosphorylation changed in Aβ-treated neurons, we performed densitometric scanning of the western blots. The ratio tau\tau-1 pixels was calculated for each time of incubation with $A\beta_{1-40}$, $A\beta_{1-42}$ and $A\beta_{40-1}$-treated neurons. Then to control for variability in baseline levels of tau in independent cultures, the data were corrected to the zero time point. The ratio of $A\beta_{1-40}$ and $A\beta_{1-42}$ were compared to control reverse peptide. The results obtained represent the average of 3 independent cultures. The results obtained in $A\beta_{1-40}$ and 1-42 treated neurons are not statistically different from the control, $A\beta_{40-1}$, as determined by a student T-test with a two-tailed distribution. Nevertheless, $A\beta_{1-42}$-treated neurons consistently give higher tau phosphorylation than $A\beta_{1-40}$ between

1 and 12 hours of treatment but not at 24 and 48 hours, indicating that 100nM of the peptide $A\beta_{1-42}$ is a more potent effector for tau phosphorylation in human neurons than 100nM of $A\beta_{1-40}$ (figure 9c). The degree of phosphorylation of phosphoserine 202 residue shows a cyclic phosphorylation, the phosphorylation is high at three hours and decreases at 12 hours and then increases again for 24 hours and decreases at 48 hours.

3- Determination of Tau Phosphorylation.

3.1- Purification of Recombinant Tau_{441} from E. coli.

Knowing that $A\beta_{1-40}$ and $A\beta_{1-42}$ have an effect on the phosphorylation of serine 202 epitope, we tried a series of experiments to determine whether Aβ activated a protein kinase(s) resulting in increased serine 202 epitope phosphorylation. Protein kinase(s) activation was determined by performing: **(1)** a hot tau phosphorylation assay and **(2)** a cold tau phosphorylation assay. Since we are using fetal neurons that express only the smallest isoform of tau protein, tau_{352}, we needed an isoform of tau protein that could be distinguish from the fetal tau. We chose the longest isoform (441 amino acids), called tau_{441}, that could be easily differentiated from fetal tau on SDS-gel based on their respective molecular weights.

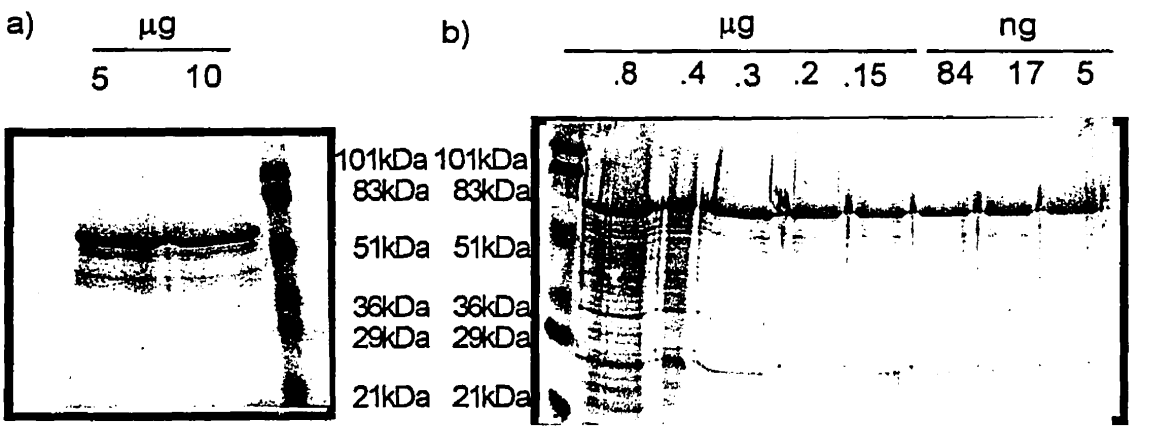


Figure 10. Purity of recombinant tau_{441}. (a) Coomassie blue gel with 10ug of recombinant proteins loaded on a 10% SDS-gel. The gel was stained for 1 hour and destained for 3 hours. (b) Western blot showing immunostaining of decreasing amount of recombinant tau_{441} with a polyclonal antibody against tau.

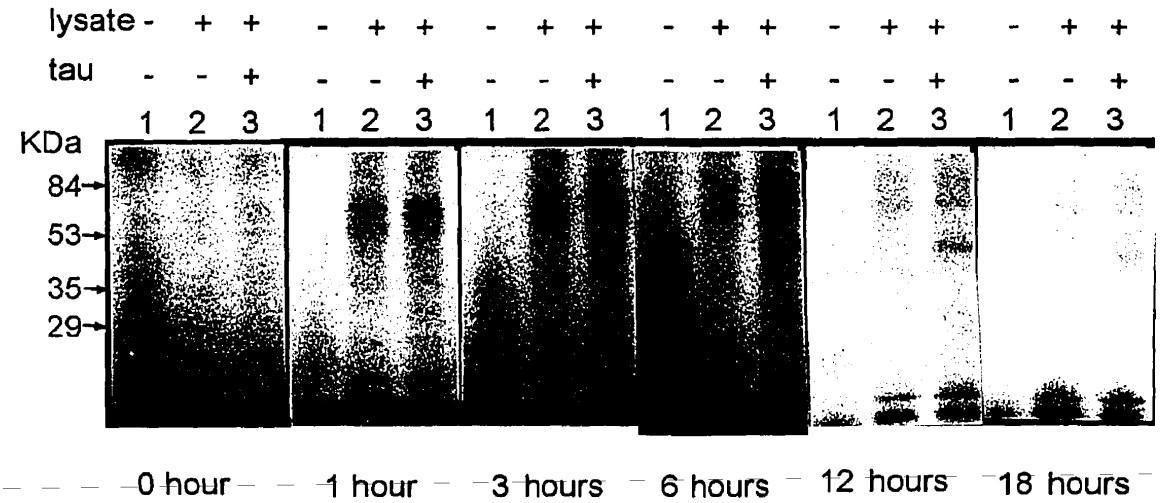


Figure 11. Conditions for tau phosphorylation assay. The samples are incubated at different times 0, 1, 3, 6, 12 and 18 hours in reaction mix at 30°C. 20µL of samples are loaded on a 10% SDS-gel and run for 1 hour. The gels are dried and exposed on X-rays film overnight. Lane 1: reaction mix only; lane 2: human neuronal lysate treated with reaction mix lacking of recombinant tau; lane 3: human neuronal lysate treated with reaction mix containing recombinant tau_{441}.

Recombinant tau_{441} , coupled to the inducible T7 RNA polymerase promoter in E. coli (Goesdert and Jakes, 1990) is grown overnight. After ther induction of the promoter, the bacteria are grown up to 1-1.5 of $O.D._{600}$. Then after several centrifugations, the bacterial proteins are loaded onto 2 chromatography columns, eliminating most of the contaminating proteins. The fractions containing the proteins are pooled and we obtained 1720.8µg/litre of culture, as determined by protein assay. Coomassie Blue gel of the preparation shows the expected strong band of 70kDa for tau_{441} indicating that the recombinant tau is the dominant protein of this preparation (figure 10a). The identity of this band as tau is confirmed on western blot with decreasing amounts of the tau protein preparations. We observe that 5ng of total protein is enough to be detected with an antibody against tau, also testifying to the purity of the recombinant tau_{441} preparation (figure 10b).

3.2- Hot Tau Phosphorylation Assay.

3.2.1- *In Vitro* Protein Kinase Assay in Human Neurons: Optimization of the Incubation Time.

To determine if protein kinase(s) are activated by Aβ treatment, we made a reaction mix based on different papers reporting activation of protein kinases: cdk5, MAPK, and GSK-3β (figure 6). To set up the conditions of the assay, we used untreated human neurons. The neurons were recuperated in PBS containing

protease and phosphatase inhibitors and solubilized in NP-40 and incubated with the kinase reaction mix for different incubation times: 0, 1, 3, 6, 12 and, 18 hours at 30°C. As expected, no phosphorylation can be detected when there is no cell lysate, only reaction mix (figure 11). In the reaction mix containing the neuronal lysate without the recombinant tau_{441}, endogenous phosphorylation of a protein around 54kDa which could be fetal tau is evident. In the presence of neuronal cell lysate, 2 proteins are phosphorylated. The higher molecular weight band likely represents the recombinant tau_{441} migrating at molecular weight slightly higher than 70kDa because the addition of phosphates on tau protein decreases its mobility in SDS-gel. The second band has the same molecular weight, 54kDa, than in the sample without recombinant tau_{441}, suggesting this is endogenous tau.

3.2.2- Activation of Human Neuronal Protein Kinase(s) Phosphorylating Recombinant Tau_{441}.

To determine if the human neurons treated with Aβ peptides have increased tau kinase activity, we performed the assay as described in the section 3.2.1 of the results with 4 independent neuronal cultures. The incubation time of 6 hours, was chosen in function of the results obtained at the section 3.2.1, since, after 6 hours the phosphorylation of recombinant tau appears to be maximal (figure 11).

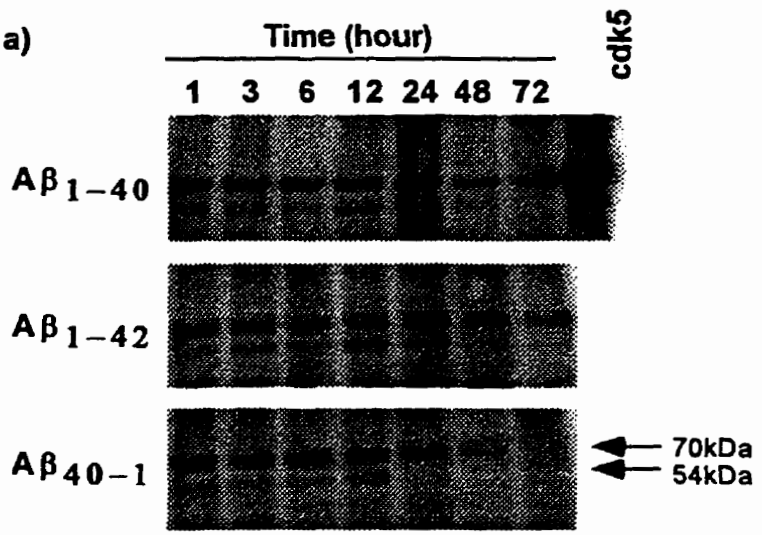


Figure 12. Hot tau phosphorylation assay with neurons treated with $A\beta_{1-40}$, 1-42 and 40-1.

A) Human neurons are incubated for 6 hours at 30°C in reaction mix containing recombinant tau_{441}. 20mL of the samples are loaded on a 10% SDS-gel. The gels are dried and exposed on X-rays film overnight.

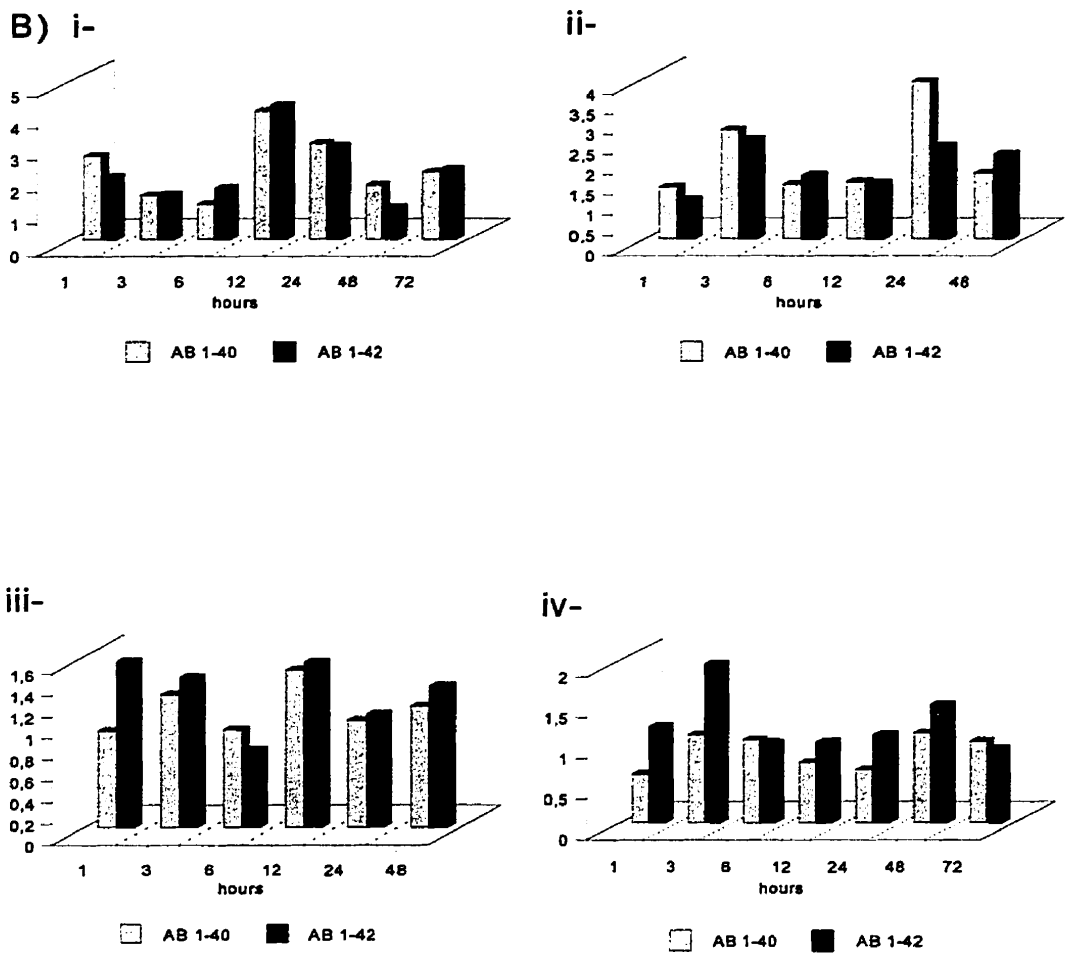


Figure 12. Hot tau phosphorylation assay with neurons treated with Aβ 1-40, 1-42 and 40-1.
B) Phosphorimaging quantification demonstrating phosphorylation of recombinant tau_{441} in 4 different fetal neuron cultures. The phosphorylation units represent the number of pixels obtained by phosphorimager.

The autoradiography of the M51HF experiment shows 2 radioactive bands at 54 and 70kDa molecular weight (figure 12a), again indicating the phosphorylation of recombinant tau_{441} and possibly fetal tau_{352}. Cdk5 purified enzyme (kind gift from Dr. Paudel) acts as a positive control for the reaction and as seen in the last lane, induces high levels of recombinant tau_{441} phosphorylation. Observation of the autoradiograph, shows that at 1 hour neurons treated with the 3 peptides, $A\beta_{1-40}$, 1-42 and 40-1, there is already high phosphorylation of endogenous fetal tau and recombinant tau_{441}. We can presume that the change of the MEM media or the addition of the peptides in the media have an effect on the phosphorylation of tau. However, in neurons treated with $A\beta_{40-1}$, the phosphorylation decreases at 48 hours whereas it stays up in $A\beta_{1-40/1-42}$-treated neurons. Therefore, $A\beta_{1-40}$ and 1-42 have a specific effect on tau phosphorylation because the phosphorylation stays high for a longer time.

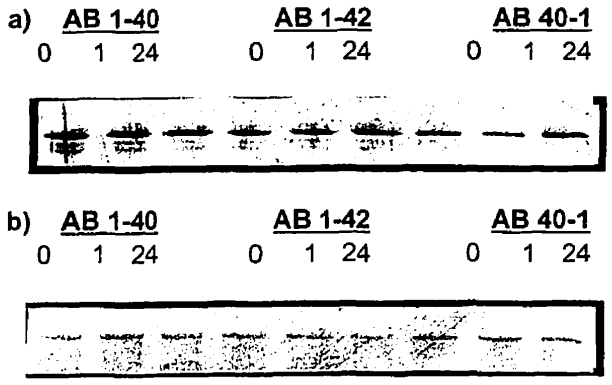
To quantify the effect of the Aβ peptides on the phosphorylation of recombinant tau_{441}, we performed phosphorimaging of the gels (we decided not to quantify the so-called endogenous fetal tau band because we have no proof of its identity). The results are from 4 independent neuronal cultures and the results are in comparison to the $A\beta_{40-1}$ treated neurons . The phosphorylation activity of the first neuron cultures (figure 12B i) shows that there is no difference for neurons treated with either $A\beta_{1-40}$ and neurons treated with $A\beta_{1-42}$. We observe the same phenomenon with the second culture (figure 12B ii), although we observe a peak in the

phosphorylation of neurons treated with $A\beta_{1-40}$ at 24 hours and decreases thereafter. In the figure 12B iii and iv, the phosphorylation of recombinant tau_{441} is higher in $A\beta_{1-42}$-treated neurons compared to $A\beta_{1-40}$ treated.

These data indicate that $A\beta_{1-40}$ and $A\beta_{1-42}$ activate a protein kinase(s) that phosphorylate the recombinant tau_{441} *in vitro* in some neuron cultures. However, the amount of phosphorylation is variable with each culture. The variability may be due to the genetic heterogeneity of the culture.

3.3- Activation of Human Neuronal Protein Kinase(s) Phosphorylating Recombinant Tau_{441} on Serine 202.

In section 2 of the results, we showed that $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides have an effect on the phosphorylation of the phosphoserine 202 residue of the endogenous tau protein. In the results section 3.3.2., we demonstrated that the Aβ peptides have increased a human neuronal protein kinase(s) ability to phosphorylate tau. We wanted to investigate if Aβ activates a protein kinase(s) that has been shown to phosphorylate the phosphoserine 202 residue of tau protein. The known neuronal protein kinases capable of phosphorylating tau at the phosphoserine 202 residue are GSK3β, cdk5 and MAPK. The increase or the decrease that we observed is only due to kinase activity because the phosphatases were inhibited as described previously.



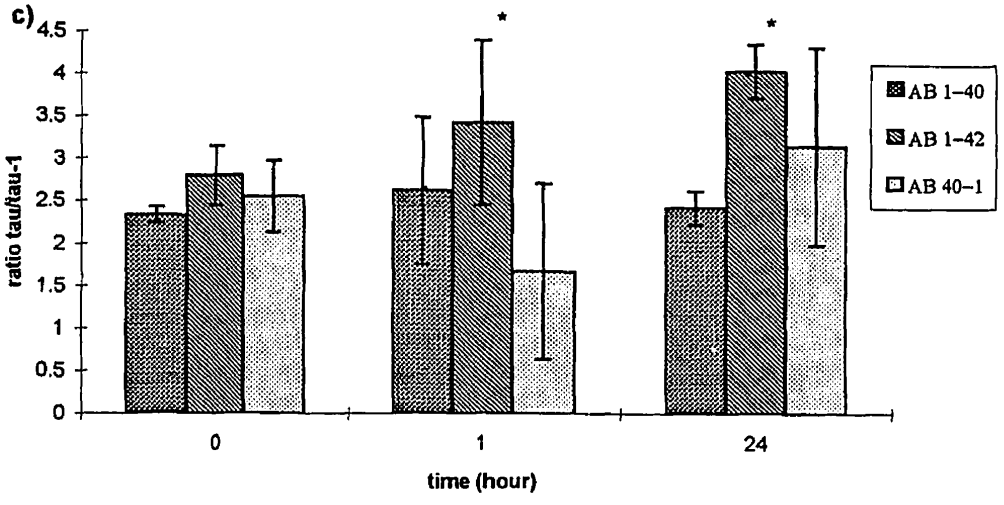


Figure 13. Western Blot with neurons treated with Aβ 1-40, 1-42and 40-1 for 0, 1 and 24 hours (n=4). Human neurons are incubated for 6 hours at 30°C in reaction mix containing recombinant tau_{441}. 20µL of the samples are loaded on a 10% SDS-gel. Western blot showing immunostaining of (a) tau protein with a polyclonal antibody and, (b) dephosphorylated serine 202 epitope of tau protein with the monoclonal antibody tau-1. Secondary antibodies are detected with alkaline phosphatase and NBT/BCIP substrates. (c) Phosphorimaging quantification demonstrating phosphorylation of serine 202 epitope of recombinant tau_{441} (ratio tau/tau-1). The asterisks show the results statistically differents ($p<0.01$).

As shown by western blot, staining with the polyclonal antibody tau presents only 1 band with the molecular weight corresponding to the recombinant tau_{441}, 70kDa (figure 13a). The staining is of the same intensity all through the experiments for neurons treated with $A\beta_{1-40}$, 1-42 and 40-1 except for the 1 hour time point in $A\beta_{40-1}$ treated neurons (figure 13a). It is not possible to see the endogenous fetal tau on the western blot because the protein is too diluted.

The staining with the antibody tau-1 (against the dephosphorylated phosphoserine 202 residue) is ligther for the 1 and 24 hours time points of $A\beta_{1-42}$-treated neurons compared to neurons incubated with $A\beta_{1-40}$ and $A\beta_{40-1}$ (figure 13b), indicating increased phosphoserine 202 residue phosphorylation in these cells.

We quantified the western blots of 4 different cultures by densitometry to look at the phosphorylation of phosphoserine 202 residue of tau protein when the protein phosphatases are inhibited. The ratio tau\tau-1 given by the densitometer shows that the phosphorylation of phosphoserine 202 residue is higher in neurons treated with $A\beta_{1-42}$ compared to the control $A\beta_{40-1}$. Indeed, the increase is of 35% at 1 hour and of 142% at 24 hours. Neurons treated with $A\beta_{1-40}$ do not increase significantbly the phosphorylation of the tau at phosphoserine 202 residue.

4-Determination of cdk5 Activation in Human Fetal Neuron Cultures Treated with 100nM and 20μM of $A\beta_{1-40}$ and $A\beta_{1-42}$.

To have an indication of which kinase(s) phosphorylating the phosphoserine 202 residue of the tau protein, is activated by $A\beta_{1-40}$ and $A\beta_{1-42}$, a histone H1 protein assay is performed. The histone protein can be phosphorylated only by cdk5 and not by MAPK and GSK-3β. The neurons are treated with the Aβ peptides for 24 hours, recuperated as previously described and incubated in a reaction mix containing the histone H1 protein for 3 hours at 30°C.

For this histone H1 protein assay, we decided to look at 2 different concentrations of Aβ peptides, 100nM because all the study is done with this concentration and 20μM because Takashima and his colleagues (1993), showed a kinase activity with 20μM $A\beta_{1-43}$ and 25-35. We can observe that there is a slight background obtained with the no histone H1 control (figure 14). The untreated control shows phosphorylation of the histone H1 protein which is almost 5 times above background indicating that there is a great amount of phosphorylation in untreated neurons. The results in neurons treated with 100nM $A\beta_{1-40}$, 1-42 and 40-1 show the same amount of phosphorylation. We can conclude that it is unlikely that cdk5 or other kinases are activated in human fetal neurons treated with 100nM of $A\beta_{1-40}$ and 1-42 or that there is no specific activation of cdk5. There also could be other kinases activated by 100nM of Aβ.

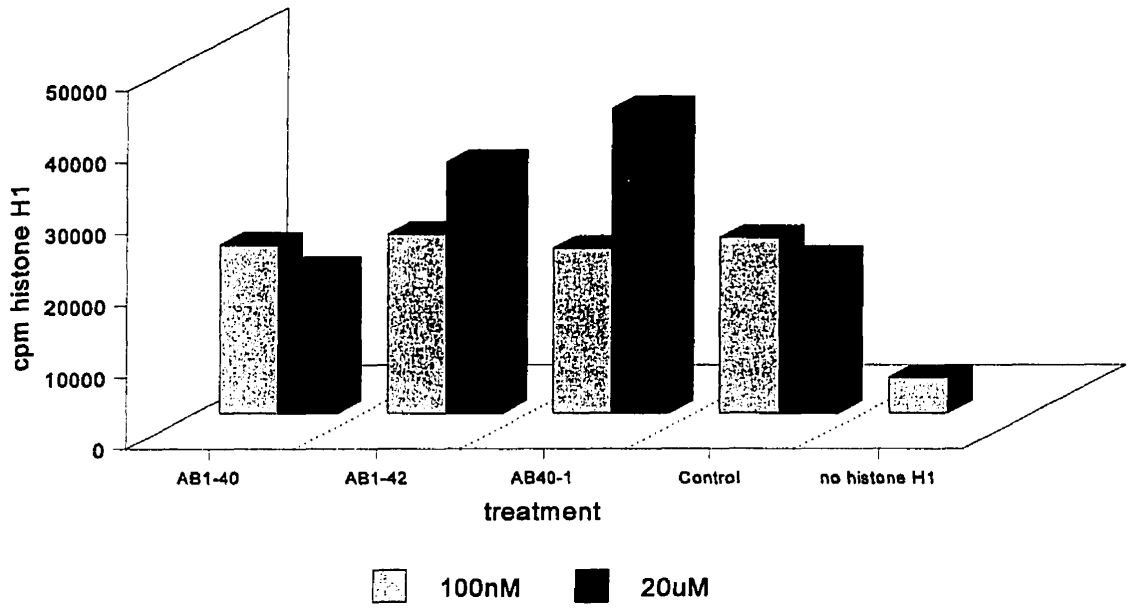


Figure 14. Histone H1 protein assay with neurons treated with 100nM and 20μM of $A\beta_{1-40}$, 1-42 and 40-1 for 24 hours. Human neurons are incubated for 6 hours at 30°C in reaction mix containing 1μg of histone H1 protein. The reaction is stopped with 20% of TCA. 15μL of samples is spot on phosphocellulose paper and washed 3 times in 500mL of tap water containing 5mL of H_3PO_4. The radioactive counts are read by Scherenkov counting.

In contrat to our expectations, it seems that 20μM of Aβ40-1 has an activating effect on a histone H1 kinase because we observe higher phosphorylation. Therefore, the kinase activity observed in neurons treated with $A\beta_{1-42}$ is not specific to the Aβ peptide. Therefore, we also conclude that 20μM of $A\beta_{1-40}$ and 1-42 do not have a specific activating effect on histone H1 kinase activity and in consequence, cdk5.

D. DISCUSSION

Alzheimer's disease is defined pathologically by the presence of neurofibrillary tangles, senile plaques and cerebrovascular amyloid in specific areas of the brain that are responsible for normal memory processes. The association between neurofibrillary tangles and senile plaques is not clear and neither is the exact role of these pathological lesions in the disease. Recent evidence indicates that Aβ plays a primary role in Alzheimer's disease. Mutations of the amyloid precursor protein and presenilin I genes responsible for at least 50% of familial Alzheimer's disease cases increase the production of Aβ, especially of the $A\beta_{1-42}$ (Sheuner *et al*, 1996). Down Syndrome individuals who invariably develop AD during aging also show elevated production of $A\beta_{1-42}$ (Teller *at al*, 1996). Transgenic animals overexpressing either Aβ , normal or mutant APP develop pathological features of AD such as senile plaques, neurodegeneration, gliosis and memory impairment (Games *et al*, 1995; Hsiao *et al*, 1995; Hsiao *et al* 1996; LaFerla *et al*, 1995). Therefore, it has been proposed that the presence of neurofibrillary tangles in the AD brain is a consequence of the overexpression of Aβ. Neurofibrillary tangles contain high and abnormally phosphorylated tau. It has been shown that the hyperphosphorylated tau cannot perform the normal function of stabilizing microtubules resulting in cytoskeleton breakdown (Bramblett *et al*, 1993). Busciglio and his colleagues (1995) have shown that fibrillar Aβ induced the phosphorylation of tau at epitopes 202 and 396/404 in rat and human cortical neuron cultures. They

showed that hyperphosphorylation of tau resulted in its redistribution from the normal axonal compartment to the somato-dendritic compartment, an effect which was reversed upon dephosphorylation of tau. Therefore, a clear link appears etablished between Aβ and tau hyperphosphorylation. However, these experiments were done with very high, non-physiological concentrations of Aβ (25-100μM). The concentration of Aβ in normal human fetal and adult CSF is 4nM. The object of our study was to determine if a 25 fold increase in circulating Aβ, which is more likely representative of the non-deposited Aβ of AD brains, could also cause hyperphosphorylation of tau. In this study, I showed that 100nM concentrations of aggregated $A\beta_{1-42}$ and $A\beta_{1-40}$ on human fetal neurons cause a hyperphosphorylation of tau at epitope 202 within 3 hours treatment. The results indicate that aggregated Aβ activates a signal transduction pathway involving a protein kinase or kinases that results in the phosphorylation of tau protein.

We observed that $A\beta_{1-40}$ and more significantly $A\beta_{1-42}$ treatment of human neuron cultures increased the phosphorylation of the serine 202 of tau protein in a cyclic manner. There are many possible explanations for this cyclic phosphorylation which has not been observed before. First, it is possible that Aβ turns over after the activation of tau phosphorylation signal pathway and disappearance of receptors from the cell surface down-regulate this pathway. In time, receptors for Aβ would be replenished at the cell surface and after reaching a critical mass, allow reactivation of the signalling pathway by the Aβ. Second, different neuronal cell

types respond at different times after exposure to Aβ. We know that these neuronal cultures contain cholinergic CHAT-positive and dopaminergic tyrosine hydroxylase positive neurons (LeBlanc, unpublished results). Other types could also be present. The region specificity of AD lesions would support this mechanism. Third, Aβ may mediate a temporally regulated cascade of events including tau phosphorylation by different kinases which would be activated at different times. Many more experiments would be required to test these different possibilities.

We found that $A\beta_{1-42}$ was more potent than $A\beta_{1-40}$. This is interesting since it is the increase of $A\beta_{1-42}$ which is highly suspected to be the primary Aβ problem in AD. The $A\beta_{1-42}$ is increased significantly early in Down Syndrome individuals and also in AD (Iwatsubo *et al*, 1994; Teller *et al* ,1996; Russo *et al,* 1996). The $A\beta_{1-42}$ has more potential for aggregation due to its additional two C-terminal hydrophobic amino acids and has been proposed to act as a seeding mechanism for the deposition of $A\beta_{1-40}$(Jarrett *et al,* 1993). Our finding that $A\beta_{1-42}$ has a more potent effect on the phosphorylation of tau at epitope 202 indicates additional detrimental effect of $A\beta_{1-42}$.

Although the results of our experiments confirm and extend those of Yankner and his colleagues (Busciglio *et al*, 1995), one of the problems that we had was the variability of the results. Each preparation of neurons was genetically different and how can we control for genetic difference between each culture? This variability

cannot be resolved since every human being posseses a different genetic background. Therefore, the variability of the responses (within a certain range) has to be considered as the reflection of genetic heterogeneity. We found that despite an activation of tau phosphorylation in each experiment, the time, the level of induction and the duration of induction varied between the 4 experiments. The variations are possibly due to the different sensitivity of neurons to the toxicity of Aβ. Some preparation of neurons seem more resistant than others, a situation which is also possible *in vivo*. Since we have absolutely no control over this variable, we would need to repeat these experiments in a much higher number of different neuron preparations in order to determine the percentage of the individual neuronal preparations sensitive to Aβ as far as the effect of tau phosphorylation is concerned. However, the results contain an interesting although speculative message that possibly there is an individual response to Aβ dependent on the genetic make-up of the neuron. This would indicate that certain individuals have the capacity to resist higher doses of Aβ while others may be more vulnerable.

The increase in the phosphorylation in independent experiments was around 1.5 to 2.5 fold, which is not very high. Usually, kinase activation is expected to result in high, although transient increases in protein phosphorylation. One possibility to explain our results is that the fetal tau is already highly phosphorylated. In fact, fetal tau normally is phosphorylated at 12 sites, adult tau at 4 sites and hyperphosphorylated AD tau at 19 sites. We do not know if these sites are

conserved in the neuronal cultures. The tau-1 antibody which detects dephosphorylated epitope 202 stains proteins extracted from untreated neuronal cultures indicates that all sites of fetal tau phosphorylation are not occupied in the cultured neurons. Since phosphorylation is a balance between kinase and phosphatase activity, it is possible that there is always a proportion of sites which are dephosphorylated. Another possible explanation for the low levels of phosphorylation is that the kinases involved in tau phosphorylation are highly regulated in human neurons and not subject to very high transient activation. Alternatively, phosphatases are very active in neurons in order to regulate in a very sensitive manner the kinase activity.

To determine if the low levels of phosphorylation were due to the already high phosphorylation level of fetal tau, we used recombinant bacterial tau which is not phosphorylated as the substrate for possible Aβ-activated kinases. The results showed similar levels of phosphorylation as seen with endogenous tau (figure 12). The phosphorylation of serine 202 is detected within 1 hour of Aβ treatment and sustained for 24 hours in $A\beta_{1-42}$ but not in $A\beta_{1-40}$-treated neurons (figure 13). The low levels of Aβ-induced tau phosphorylation have also been observed by Takashima and his colleagues (1993). They showed that treatment of embryonic rat hippocampal neurons cultures with 20μM $A\beta_{1-43}$ or $A\beta_{25-35}$ increased tau phosphorylation by 1.6 fold through glycogen synthase kinase (GSK-3β).

We determined that one of the tau epitopes phosphorylated in Aβ-treated neurons is serine 202 tau epitope by detection with tau-1 antibody (figure 13). Busciglio and colleagues (1995) also claimed phosphorylation of serine 202 in human neuron cultures based on a shift on polyacrylamide gels. However, the reactivity to tau-1 did not decrease as expected since tau-1 is directed at the dephosphorylated epitope of serine 202 suggesting that in their system, serine 202 was not phosphorylated. The difference in our results may be explained by the concentration of Aβ used in our experiments as mentioned above.

Three neuronal kinases are known to phosphorylate the tau phosphoserine 202 residue: GSK-3β, neuronal cell cycle dependent kinase (cdk5) and microtubule associated protein kinase (MAPK) (Goedert *et al*, 1992; Drewes *et al*, 1992; Ishiguro *et al*, 1992; Singh *et al*, 1995; Paudel *et al*, 1993; Kobayashi *et al*, 1993; Baumann *et al*, 1993). Preliminary experiments used in our laboratory failed to identify activation of MAPK by direct gel assays (Douillard and LeBlanc, unpublished observations). Since Aβ-mediated activation of GSK-3β was already identified in the rat neurons, we opted to investigate the possible role of cdk5 in Aβ-mediated serine 202 tau phosphorylation. Using histone H1 which is a substrate for cdk5 but not MAPK or GSK-3β, we did not detect increased kinase activity in neurons treated with 100nM Aβ peptides and control reverse $A\beta_{40-1}$ peptide. In contrast, cells treated with 20mM $A\beta_{1-42}$ but not $A\beta_{1-40}$ showed a kinase activity capable of using histone H1 as a substrate. The effect was not specific to $A\beta_{1-42}$

since the control reverse peptide also had a similar activity. Therefore, high concentrations of the $A\beta_{1-42}$ and $A\beta_{1-40}$ induce a kinase activity which could be cdk-5. The reason for the $A\beta_{40-1}$ effect has not been clarified but could be dependent on the conformation of this control peptide or the ability of $A\beta_{40-1}$ to bind to a receptor which activates the same pathway as $A\beta_{1-42}$. The fact that the effect is only observed in non-physiological high concentrations indicates that these peptides do not act through cdk-5 kinase activation for the phosphorylation of tau. We also have to keep in mind that the histone H1 assay is not specific for cdk-5 activity and can act as a substrate for other kinases. If we had detected increased phosphorylation of histone H1 with 100nM $A\beta_{1-42}$ or $A\beta_{1-40}$, we would have pursued the experiments and conclusively showed that it was cdk-5 that was activated by using peptide substrates specific to cdk-5 in the presence and absence of inhibitors. Since neither MAPK nor cdk-5 appear to be activated by Aβ, it is possible that GSK-3β is the kinase involved in the phosphorylation of the phosphoserine 202 residue. Future experiments will determine if this is true.

The mechanism by which Aβ causes tau hyperphosphorylation is not known. The effect is highly suspected to take place by interaction of the Aβ with a receptor. The work of Zhang and colleagues (1994) has demonstrated the activation of the focal adhesion kinase protein (FAK) by Aβ. The activation of FAK is normally mediated through integrin receptor binding and indicates that Aβ may interact with

these receptors as well (figure 15). Integrin-mediated signal transduction has been well characterized and is shown to be linked to the Ras pathway through FAK activation and leads to activation of microtubule associated protein kinase. We were unable to detect MAPK activation by either peptide phosphorylation or direct gel kinase assays using myelin basic protein as a substrate (results not shown) suggesting that Aβ in human primary neuron cultures either acts through a different pathway or that the integrin pathway is mediated differently in neurons.

Two receptors for Aβ have been demonstrated. One is the age-dependent glycation end product receptor (RAGE) (Du Yan *et al*, 1996). This receptor is especially relevant to the toxic effect of Aβ in aging individuals since these glycation end products increase during aging and especially in AD. Since the neurons that we used are of fetal origin and are used shortly after being cultured, it is unlikely that the action of Aβ is through this receptor. The other known receptor that binds Aβ is the scavenger receptor, which is expressed on microglia (El Khoury *et al*, 1996), but until now it has not been demonstrated to be on neurons. Since, we don't have microglia in our cultures, the chances are that this receptor is not involved in our system.

The treatment of neurons with Aβ peptides and mostly $A\beta_{1-40}$ and 1-42, is of major importance in the understanding of the disease. Aβ peptides are toxic for human neurons as demonstrated by Dr. LeBlanc and colleagues (1996).

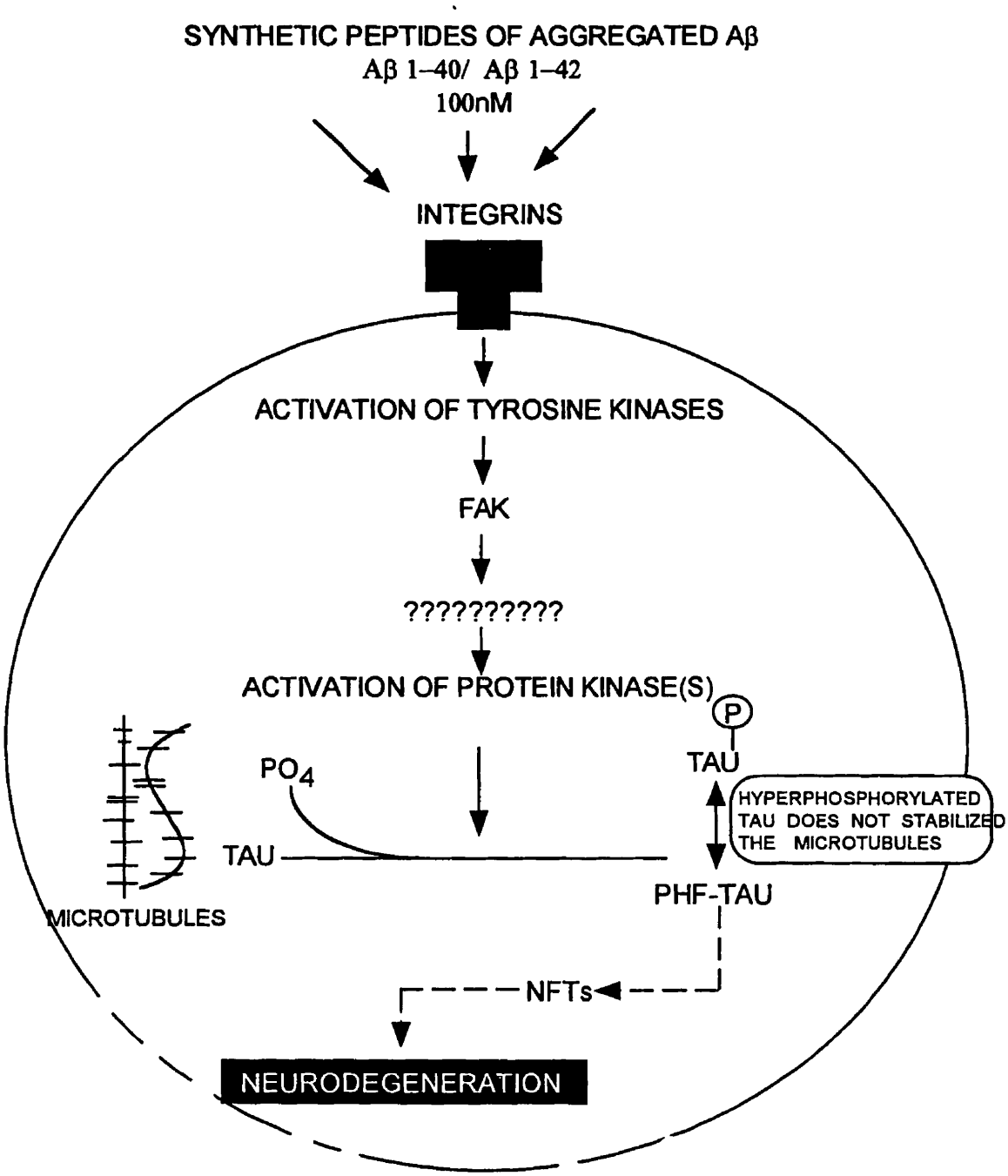


Figure 15. Model proposed for Aβ action in human neurons. The aggregated Aβ peptides at 100nM act through the integrin proteins to activate the tyrosine kinases. The tyrosine kinases will activate the FAK, leading to a serie of unknown events. The last event of the cascade is the activation of a protein kinase(s) which is able to phosphorylate tau protein. The tau protein binds to the microtubules and stabilize them but the addition of phosphates on tau transformes it into PHF-tau which are unable to bind to the microtubules, leading to the formation of NFTs and to the degeneration of the neuron.

At 100nM concentration, Aβ peptides provoke the down-regulation of an important protein Bcl-2 which plays a role in protecting the cell against apoptosis. In addition, $A\beta_{1-42}$ up-regulates the Bax protein which promotes cell death. The treatment of human neurons with Aβ peptides is not sufficient to provoke apoptosis at short term but it certainly deregulates essential mechanisms that could lead to neurodegeneration.

E. CONCLUSION

In conclusion, we have shown that physiological concentrations of Aβ peptides increase the phosphorylation of tau at serine 202. It is of major importance since serine 202 is an epitope highly phosphorylated in AD (Goedert *et al*, 1993). The Aβ peptides probably activate many other kinases that can phosphorylate all the epitopes of tau protein. It is possibly happening gradually, resulting in PHF-tau and eventually NFTs, leading to the usual neurodegeneration seen in AD.

The variability of individual neuronal responses may reflect the different sensitivity of humans to AD. It is known that certain brains obtained from very lucid old individuals contain abundant levels of plaques and Aβ deposits. Yet for some reason, these individuals are protected from AD. Our results indicate that possibly the genetic make up of a person protects against some of the detrimental effects of Aβ. In time, it may be interesting to identify genetic differences between individuals that are susceptible to AD and those that are not.

For the future, immunoprecipitation of cdk5 in neurons treated with $A\beta_{1-40}$ and $A\beta_{1-42}$ should be perform to really confirm its involvement in the increase in the phosphorylation of tau protein and of phosphoserine 202 residue. Also, we should work on different assays to find out how many other protein kinases are activated by aggregated Aβ peptides. Finally, a study on protein phosphatases (by inhibition

of protein kinases) should be perform to discover first, if there are really some protein phosphatases deactivated by Aβ peptides, and second, to know which phosphatases are deactivated.

The goal of the project was to demonstrate the existence of a link between the extracellular aggregated Aβ peptides $A\beta_{1-40}$ and $A\beta_{1-42}$ and the increased phosphorylation of tau protein, specifically on phosphoserine 202 residue, a characteristic features of AD pathology. We were able to show that Aβ peptides activate a signal transduction pathway leading to the phosphorylation of tau protein by a protein kinase. However, this effect may be mediated by non-physiological concentrations and the delayed activation of tau phosphorylation indicates that Aβ-mediated tau phosphorylation is a late effect of Aβ.

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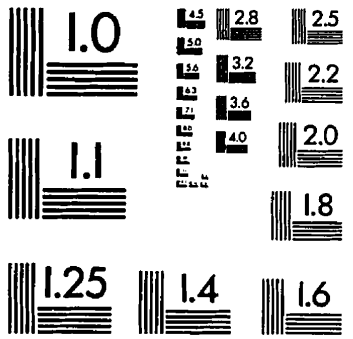

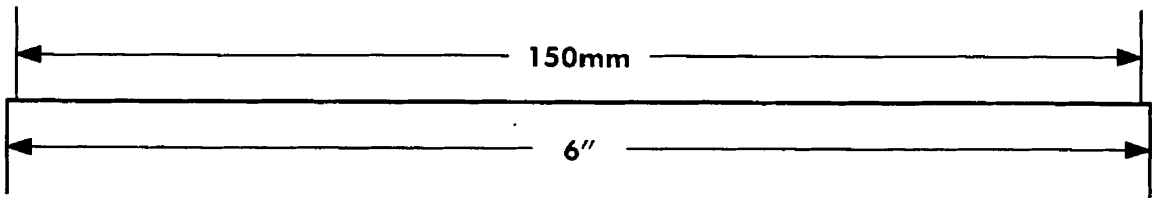

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