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Role of P70 S6 Kinase in the Formation of Tau Pathologies in Alzheimer's Disease

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To my family with love

"Whatever you can do or dream you can, begin it.
Boldness has genius, power, and magic in it."

-- Johann Wolfgang von Goethe

ABSTRACT

One of the important neuropathological features of Alzheimer's disease (AD) is the tau pathology seen as accumulation and hyperphosphorylation of this protein. Evidences showed that tau could be phosphorylated mostly at serine/threonine (S/T) residues by many kinases, including protein kinase B, glycogen synthase kinase (GSK)-3β, extracellular signal-regulated kinase (ERK1/2), ERK1/2 kinase (MEK1/2), c-Jun N-terminal kinase (JNK), and p38. However, there is a lack of evidence regarding tau accumulation caused by deregulation of tau protein translation. Since 70-kDa ribosomal protein S6 kinase (p70SK) is a S/T kinase that also plays a crucial role in the regulation of protein translation, it is of great interest to investigate whether or not this kinase is involved in the formation of tau-associated pathologies in AD brain, such as tau hyperphosphorylation, accumulation and assembly.

Paper I investigated whether p70S6K activation is associated with PHF-tau accumulation in AD. By immunohistochemistry, we found that levels of phosphorylated (p) p70S6K at T389 or at T421/S424 were increased in accordance with the progressive sequence of neurofibrillary changes according to Braak's criteria. Confocal microscopy showed that in AD brain, p-p70S6K appeared especially in neurons that are known to later develop NFTs. By indirect enzyme-linked immunosorbent assay, the levels of p-p70S6K (T389 or T421 / S424), total tau, and PHF-tau were found to significantly increased in AD as compared to control cases. Levels of p-p70S6K (T421/S424) showed significant correlations with both total tau and PHF-tau. Regression analyses revealed a significant dependence of total tau or PHF-tau on p-p70S6K at T421/S424 sites rather than at the T389 site. Levels of ribosomal protein S6 as well as levels of markers for the proteolytic systems were also significantly increased in AD as compared to control brains. In both SH-SY5Y neuroblastoma cells and primary cultured neurons, we found that zinc sulfate could induce p70S6K phosphorylation and activation, which results in an increased expression and phosphorylation of tau. Pretreatment of cells with rapamycin (an inhibitor of FRAP/mTOR, which is the immediate upstream kinase of p70S6K), attenuated zinc-induced effects.

Paper II investigated whether a decrease in protein phosphatase (PP)-2A activity could induce activation of the mitogen-activated protein kinase (MAPK) pathway togther with tau phosphorylation. Rat brain slices kept under metabolically active conditions in oxygenated artificial cerebral spinal fluid were treated with 1.0 μM okadaic acid for 1h at 33^{o}C. Under this condition, PP-2A activity was decreased to ~35% of the vehicle-treated control slices, and activities of PP-1 and PP-2B were not affected. In the OA-treated slices, a dramatic increase of p-ERK1/2, p-MEK1/2 and p-p70S6K was observed both immunohistochemically and by Western blots. Treatment of 6-μm sections of the OA-treated slices with purified PP-2A reversed the phosphorylation / activation of these kinases. Tau hyperphosphorylation at several sites seen in AD brain was also affected.

Paper III investigated the influences of various components of phosphatidylinositol 3-kinase (PI3K)-and MAPK pathways on p70S6K and GSK-3β phosphorylation. We found that 100 μM zinc could induce an increase of p-p70S6K, p-PKB, p-GSK-3β, p-ERK1/2, p-JNK, and p-p38, especially in long-term treatment (4 to 8h) in serum-deprived SH-SY5Y cells. Treatment with different inhibitors including rapamycin, wortmannin, LY294002, and U0126, and their combinations indicated that p-p70S6K and p-GSK-3β are regulated by rapamycin-dependent, PI3K and MAPK pathways. Furthermore, phosphorylation of p70S6K and GSK-3β affected levels of tau at Tau-1 and PHF-1 sites, and p70S6K phosphorylation affected total tau level.

Paper IV investigated whether or not p70S6K mediates tau synthesis and phosphorylation. We found that: 1) p70S6K can directly phosphorylate tau at S262, S214, and T212 sites, and inhibit recombinant tau assembly *in vitro*; 2) the epitope T421/S424 of p-p70S6K is associated with tau phosphorylated at the S262 or S396/S404 sites; 3) over-expression of tau requires p70S6K phosphorylation / activation; 4) elevated levels of p-p70S6K (T421/S424) are significantly correlated with tau phosphorylated at S262, S214 and T212 sites in AD brains.

These data suggest that p70S6K not only acts as a new tau kinase that directly phosphorylates tau at S262, S214, and T212 sites, but also mediates tau accumulation by up-regulation of tau translation. Activated p70S6K in NFT-bearing neurons might be caused by the aberrant regulation of PI3K and MAPK pathways, as well as the reduced activity of PP-2A in AD brain.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numerals.

I. **An WL**, Cowburn RF, Li L, Braak H, Alafuzoff I, Iqbal K, Grundke-Iqbal I, Winblad B, Pei J-J. Up-regulation of phosphorylated/activated p70 S6 kinase and its relationship to neurofibrillary pathology in Alzheimer's disease. Am J Pathol, 2003, 163,591-607

II. Pei J-J, Gong CX, **An WL**, Winblad B, Cowburn RF, Grundke-Iqbal I, Iqbal K.Okadaic-acid-induced inhibition of protein phosphatase 2A produces activation of mitogen-activated protein kinases ERK1/2, MEK1/2, and p70 S6, similar to that in Alzheimer's disease. Am J Pathol, 2003, 163,845-858

III. **An WL**, Bjorkdahl C, Liu R, Cowburn RF, Winblad B, Pei J-J. Mechanism of zinc-induced phosphorylation of p70 S6 kinase and glycogen synthase kinase 3beta in SH-SY5Y neuroblastoma cells. J Neurochem, 2005, 92,1104-15

IV. **An WL,** Zhou X-W, Nishimura T, Norberg J, Benedikz E, Winblad B, Götz J, Pei J-J. P70 S6 kinase mediates tau phosphorylation and synthesis. (Submitted).

CONTENTS

LIST OF ABBREVIATIONS

5'TOP	5'-Terminal oligopyrimidine tracts
5'UTR	5'-Untranslated region
AD	Alzheimer's disease
AD p-tau	Soluble hyperphosphorylated tau
CaMKII	Ca^{2+}/calmodulin-dependent kinase II
Cdk5	Cyclin-dependent kinase 5
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
GSK-3	Glycogen synthase kinase-3
HNE	4-Hydroxynonenal
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MAP	Microtubule-associated protein
MARK	Microtubule affinity regulating kinase
MBD	Microtubule-binding domain
MEK	Mitogen activated protein kinase kinase
mTOR	Mammalian target of rapamycin, also known as FRAP or RAFT1
NFTs	Neurofibrillary tangles
P70S6K	70-kDa Ribosomal protein S6 kinase
PDPK	Proline-directed protein kinase
PHF	Paired helical filament
PI3K	Phosphatidylinositol 3-kinase
PK	Protein kinase
PP	Protein phosphatase
PP-2A	Protein phosphatase -2A
S6K1	Ribosomal S6 kinase 1
SAPK	Stress-activated protein kinase
SP	Senile plaques
SF	Straight filament

INTRODUCTION

1. ALZHEIMER'S DISEASE

1.1. General introduction

Alzheimer's disease (AD) is a complex chronic disorder characterized by a progressive and hierarchic decline in cognitive function. Among the people who suffer from senile dementia, between 50-70% are affected by AD. The clinical diagnosis of this neurodegenerative disease is based on exclusive criteria, such as the International Classification of Disease, 10^{th} revision (WHO 1992) and the Diagnostic and Statistical manual of Mental Disorders (DSM-III-R) (American Psychiatric Association). Current criteria for the pathological diagnosis of AD are mostly based on (semi) quantitative assessment of senile plaques (SPs) and neurofibrillary tangles (NFTs) (Tierney et al., 1988). Other generally accepted pathological features of AD include decreased synaptic density and neuron loss (Gomez-Isla et al., 1996, Grace et al., 2002). A large number of AD cases are sporadic in which the underlying causes remain unknown. Only less than 10% of AD patients are due to mutations in the presenilin-1/2 and β-amyloid precursor protein genes.

1.2. Neuropathological hallmarks of Alzheimer's disease

The two neuropathological hallmarks of AD are SPs that are largely composed of Aβ deposits (reviewed in (Selkoe, 1996)), and NFTs that are bundles of PHFs whose main constituent is abnormally hyperphosphorylated tau (Grundke-Iqbal et al., 1986) (Fig.1). The abnormally hyperphosphorylated tau accumulates in neurons in the form of PHFs, twisted ribbons, and straight filaments (SFs) (Iqbal et al., 2005). Although emerging data showed that Aβ deposits are correlated with dementia in AD patients (Naslund et al., 2000, Religa et al., 2003), the number of NFTs shows a more consistent and stronger correlation with dementia severity (Braak and Braak, 1991, Arriagada et al., 1992, Bierer et al., 1995).

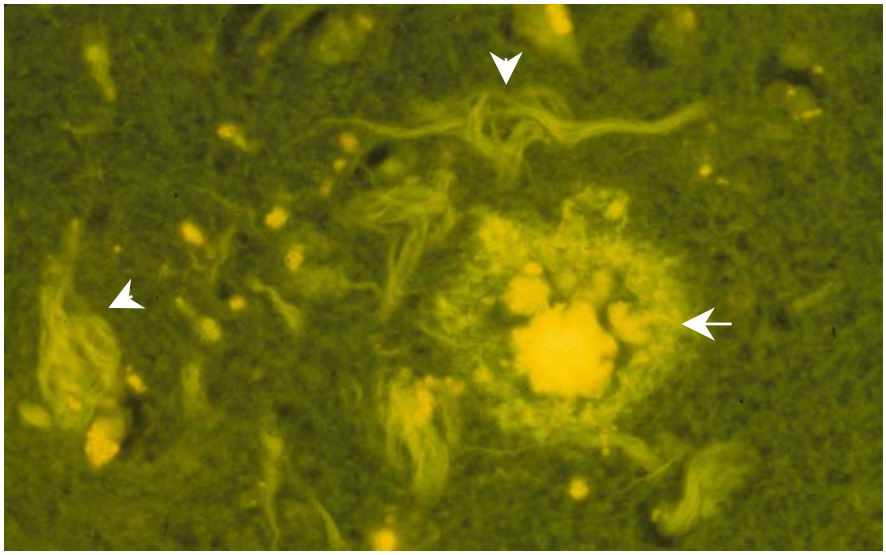

Fig.1. Senile plaques (SPs) and neurofibrillary tangles (NFTs) in Alzheimer's disease (AD) brain. Thioflavin S displays a typical plaque and several tangles with UV light (Robert D. Terry, University of California at San Diego, USA). SP (arrow) and NFT (arrow heads) are the two hallmarks of AD pathological changes. The former is largely composed of Aβ deposits, and the later is abnormally hyperphosphorylated tau that forms bundles of paired helical filaments.

2. MICROTUBULE ASSOCIATED PROTEIN TAU

2.1. Tau protein and its physiological function

Tau has four domains: the acidic N-terminal, the basic and proline-rich middle, the basic microtubule-binding repeats, and the C-terminal. In the central nervous system, six isoforms of tau exist. They are derived from a single gene by alternative splicing of tau mRNA, and vary in having 3 or 4 microtubule-binding repeat domains (MBDs) and in the number and size of the N-terminal inserts (Billingsley and Kincaid, 1997). MBDs are the regions that bind to microtubules and promote their assembly, the rest flanking domains protruding from MBDs (also called projecting domains) target tau to microtubules, and assist tau to stabilize them (Preuss et al., 1997). Interactions of tau with microtubules are regulated by the length and phosphorylation of MBDs (Morita-Fujimura et al., 1996).

Through promoting tubulin assembly and stabilizing microtubules, physiologically, tau plays significant roles in the development of neuronal processes, the establishment of cell polarity, and intracellular transport (Sato-Harada et al., 1996, Mandelkow and Mandelkow, 1998). Tau is also involved in signal transduction (Hwang et al., 1996, Flanagan et al., 1997, Jenkins and Johnson, 1998), anchoring

enzymes such as protein kinases (PKs) and protein phosphatases (PPs) (Reszka et al., 1995, Morishima-Kawashima and Kosik, 1996, Sontag et al., 1996, Liao et al., 1998) and interaction with the actin cytoskeleton (Cunningham et al., 1997) and plasma membranes (Brandt et al., 1995, Lee et al., 1998).

2.2. Post-translational modification of tau

Like other proteins, tau function is determined by different post-translational modifications, including phosphorylation (Grundke-Iqbal et al., 1986, Mandelkow and Mandelkow, 1998), glycosylation (Ledesma et al., 1994, Yan et al., 1994), ubiquitination (Mori et al., 1987), oxidation (Troncoso et al., 1993, Schweers et al., 1995), and truncation (Novak et al., 1993, Garcia-Sierra et al., 2003). Of these, phosphorylation has been most extensively studied.

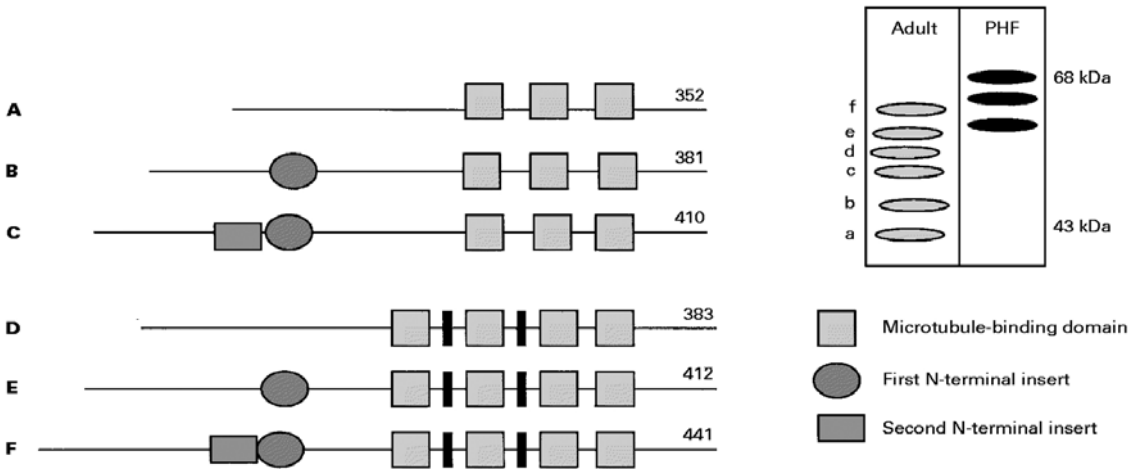

Fig. 2. Alternative splicing and domain structure of tau. The adult human brain expresses six isoforms of tau that are derived from a single gene via alternative splicing. Microtubule-binding repeats are located in the C-terminal half of tau. Two N-terminal inserts and inserts surrounding microtubule-binding domain 2 are characteristic of the larger isoforms. The patterns of isoforms seen in the adult brain following electrophoresis are shown in the box. PHF-tau consists of a characteristic triplet of peptides ranging between 60 and 68 kDa (Billingsley and Kincaid, 1997).

2.3. Tau phosphorylation

Tau is a phospho-protein, mainly located in neuronal axons. Abnormally hyperphosphorylated tau in AD brain is located in neuronal bodies as NFTs and in dendritic processes as dystrophic neurites or neuropil threads. Hyperphosphorylated tau seen in PHF is called PHF-tau, and in SF called SF-tau. About 5% of tau filaments in AD brain are SFs (Lau et al., 2002). PHF-tau is phosphorylated to a degree of ~8 Pi/mol compared to ~2 Pi/mol for normal tau (Kopke et al., 1993). The degree of phosphorylation of soluble hyperphosphorylated tau in AD (AD p-tau) is almost the

same as PHF-tau (Alonso et al., 2001). In AD brain, apart from AD p-tau and PHF-tau, there is also a considerable amount of normal tau (Pei et al., 2003). PHF-tau shows slower mobility shifts on immunoblots with three major bands of molecular masses 60, 64, and 68 kDa (Morishima-Kawashima et al., 1995b, Johnson and Hartigan, 1999). Dephosphorylation of PHF-tau disaggregates or disassembles PHF, recovering its mobility to normal levels on SDS-PAGE. Dephosphorylated PHF-tau can bind to microtubules and promote microtubule assembly (Alonso et al., 1994, Iqbal et al., 1994, Wang et al., 1995, Alonso et al., 1996).

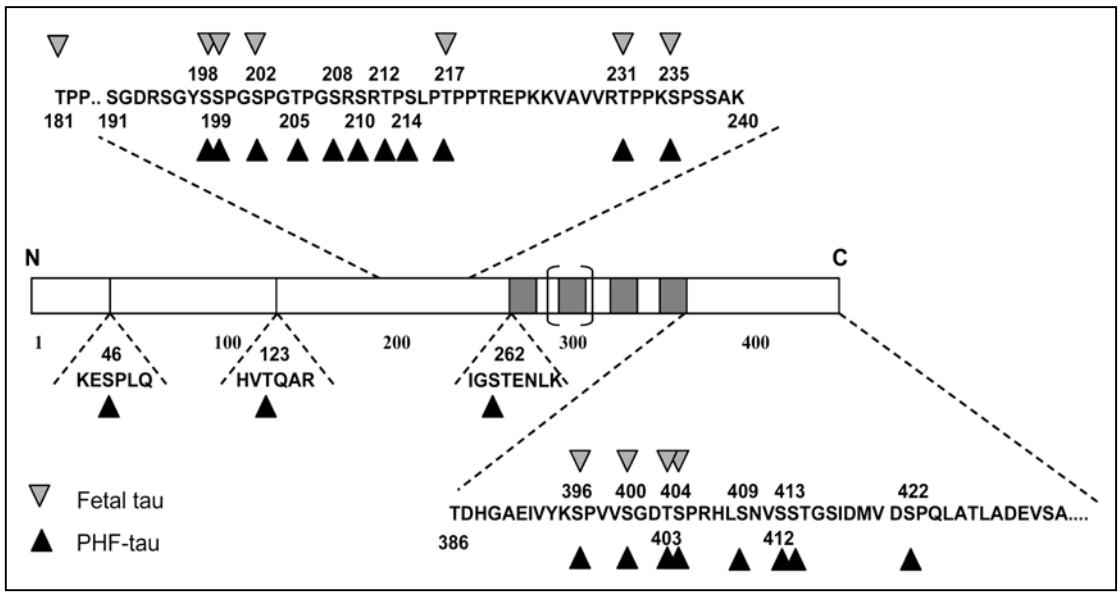

Fig. 3. Diagram of the phosphorylation sites on tau protein (modified from (Imahori and Uchida, 1997)). The phosphorylation of some sites occurs only in fetal brain tissues and mitotic cells, whereas some other sites occurring in fetal brain have also been found in AD brain. However, some sites are believed to be AD specific, such as T212/S214 sites recognized by the AT-100 antibody.

By using mass spectrometry, amino acid sequencing and phospho-site-specific tau antibodies, tau hyperphosphorylation at more than 30 sites has been identified (Hanger et al., 1998, Buee et al., 2000, Gong et al., 2005) (Fig.3).

2.4. Tau protein kinases and phosphatases

Tau protein can serve as a substrate of many kinases that can be grouped into two families: proline-directed protein kinases (PDPKs) and non-PDPKs (Morishima-Kawashima et al., 1995a). The PDPKs consist of glycogen synthase kinase 3 (GSK-3), cyclin-dependent kinase (cdk) 5, and mitogen activated protein kinase (MAPK) family (extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal protein kinase (JNK) and other stress kinases). The non-PDPKs mainly include second-messenger-

activated kinases, such as PKC, PKA, and Ca^{2+}/calmodulin-dependent kinase II (CaMKII), as well as microtubule affinity regulating kinase (MARK). PDPKs mainly modify S-P or T-P tau motifs in the flanking domains, whereas tau phosphorylation by non-PDPKs mainly occurs at the MBDs, where the S or T phosphorylation site is not followed by a proline. Different kinases may phosphorylate tau at different amino residues with overlap at certain sites. Except for a few tyrosine residues, such as Tyr (Y) 29, most of the phosphorylation sites on tau protein are at S/T residues, which can be dephosphorylated by protein phosphatases.

Two major families of protein phosphatases have been classified: S/T protein phosphatases and protein tyrosine phosphatases. Among these phosphatases, PP-1, PP-2A and PP-2B are believed to de-phosphorylate tau *in vitro* and/or *in vivo* with some degree of site overlap (Gong et al., 1994a, Gong et al., 1994b, Wang et al., 1995, Gong et al., 1996, Gong et al., 2000).

2.5. Tau-associated proteins

Tau binds to tubulin through an ionic interaction with MBDs (Serrano et al., 1985). As a sticky protein, tau has been found to bind to many proteins (Avila et al., 2004) including spectrin (Carlier et al., 1984), actin (Correas et al., 1990), ERK1/2 (Fiore et al., 1993, Reszka et al., 1995), cdk5 (Paudel et al., 1993, Paudel, 1997a), PP-1 and PP-2A (Liao et al., 1998, Sontag et al., 1999), presenilin 1 that also binds to GSK-3 (Takashima et al., 1998), α-synuclein (Jensen et al., 1999), phospholipase C-γ (Hwang et al., 1996, Jenkins and Johnson, 1998), fyn tyrosine kinase (Lee et al., 1998, Klein et al., 2002), and GSK-3β (Sun et al., 2002).

3. TAU ABNORMALITIES IN ALZHEIMER'S DISEASE

Tau hyperphosphorylation is the major tau abnormality in AD brain. Others include tau accumulation, tau assembly into PHFs in NFTs. These abnormalities are often accompanied by microtubule disruption in AD brain.

3.1. Tau hyperphosphorylation

Tau hyperphosphorylation is thought to be caused by an imbalance of the activities of protein kinases and phosphatases. Regulation of tau phosphorylation is complicated by prephosphorylation of tau at certain sites that expose others to kinases which are otherwise inaccessible to them, as well as by the synergistic effects of the dynamic activities of tau kinases and phosphatases. For instance, tau phosphorylation at T50 by stress-activated protein kinase (SAPK) 4/p38 can down-regulate tau phosphorylation at T181 (Feijoo et al., 2005). Tau prephosphorylation at the S214 site by PKB blocks PKA activity, whereas tau prephosphorylation by GSK-3β effectively

blocks PKB activity at the S214 site, although the S214 site cannot be directly phosphorylated by GSK-3β (Ksiezak-Reding et al., 2003). The double-phospho-epitopes AT100 (T212/S214) that is specific for PHFs but absent in biopsy-derived normal tau (Matsuo et al., 1994, Hoffmann et al., 1997), can be produced by a sequential phosphorylation first by GSK-3β at T212, then by PKA at S214 (Zheng-Fischhofer et al., 1998). However in rat brain, tau becomes a more favorable substrate for GSK-3 when it is prephosphorylated by PKA (Liu et al., 2004).

3.2. Tau accumulation

Although tau mRNA is increased in the brains of Down syndrome patients (Oyama et al., 1994), tau mRNA levels do not change in AD brain (Mah et al., 1992, Boutajangout et al., 2004), whereas the total tau protein level dramatically increases (Khatoon et al., 1992, Pei et al., 2003). This suggests that a significant amount of tau mRNA needed for synthesizing new tau might pre-exist in neurons at early stages of neurofibrillary degeneration. This is supported by data showing that in physiological conditions, the translational apparatuses such as ribosomes, adaptor and motor proteins are available for tau mRNA in the axon hillock, so that tau protein destined for slow transport down to the axonal terminals is synthesized (Black et al., 1996, Kempf et al., 1996, Billingsley and Kincaid, 1997). Tau protein synthesis appears to be regulated at the translational level.

The proteolytic systems for protein turnover mainly include the endosomal-lysosomal and ubiquitin-proteasomal systems. Alterations in lysosomal trafficking of tau and/or loss of lysosomal function may lead to aberrant processing of tau (Nixon et al., 2000). Levels of conjugated ubiquitin appeared to be dramatically increased in AD brain (Wang et al., 1991, Kudo et al., 1994). PHF-tau is resistant to degradation, and although more lysosomes, endosomes and ubiquitin are produced in AD brain (Sato-Harada et al., 1996, Ebneth et al., 1998), the up-regulated proteolytic system might not be sufficient to degrade over-produced and over-phosphorylated tau. An increased level of tau in AD might result from a decreased turnover and/or an up-regulation of translational capacity.

3.3. Tau assembly: tau-tau interactions

Tau is a highly soluble and natively unfolded protein, its biological functions such as binding to microtubules and promoting microtubule assembly are kept even when tau is treated with heat and denaturing agents such as acid (Weingarten et al., 1975, Lindwall and Cole, 1984, Friedhoff et al., 2000). However, tau forms insoluble aggregates in AD and related tauopathies.

By mapping the role of different regions of tau in self-assembly, it has been found that the N-terminal region decreases the ability of tau in self-assembly with an increased effect as the molecular size increases (Perez et al., 2001). The C-terminal region increases tau polymerization by providing a self-assembly surface (392-407 amino acids) even in the absence of polyanions (Perez et al., 2001). The MBDs are the scaffold for tau to be polymerized into PHFs (Wischik et al., 1988, Crowther et al., 1992, Wille et al., 1992, Perez et al., 1996), and tau assembly into PHFs is facilitated by the presence of anions (Goedert et al., 1996, Kampers et al., 1996, Perez et al., 1996).

It is hypothesized that tau assembly initiated by the conformational change of MBDs first forms the random coils, followed by formation of β-structures, and aggregation and elongation into helical structures (Hiraoka et al., 2004). This process is assisted by other regions of the tau molecule (Garcia de Ancos et al., 1993, Abraha et al., 2000, von Bergen et al., 2000, Perez et al., 2001). Tau dimerization and aggregation may occur prior to PHF formation (Garcia de Ancos et al., 1993). Johnson and Bailey proposed a process of tau filament formation (Johnson and Bailey, 2002) (Fig. 4). In normal brain, tau is mainly associated with microtubules. In AD brain, pathological events may lead to an increased phosphorylation in a site-specific manner, by which the pool of free monomeric tau increases. Soluble tau monomers form insoluble homodimers by oxidation of SH groups. These dimers represent the effective assembly species of PHFs. About 4-7 dimers can form nuclei, which then elongate into PHFs. Nucleation and elongation of PHFs are greatly accelerated by anionic co-factors (Friedhoff et al., 1998a, Friedhoff et al., 1998b, Friedhoff et al., 2000, Barghorn and Mandelkow, 2002).

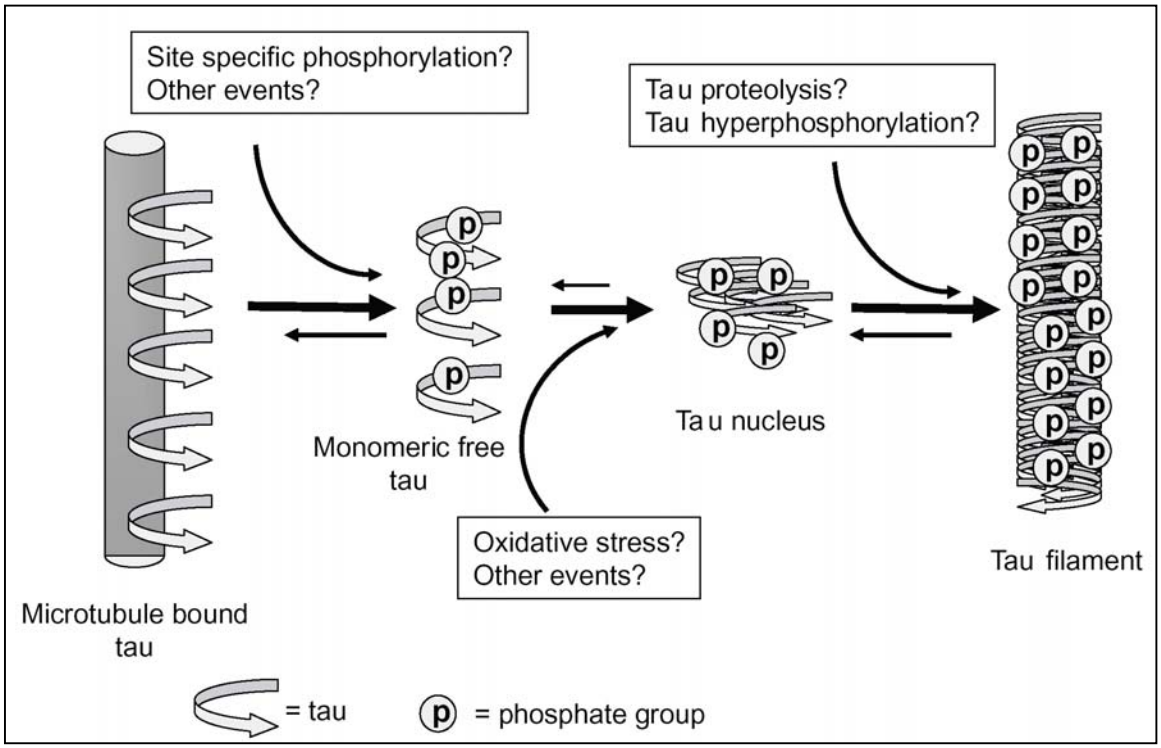

Fig.4. Hypothetical diagram of the process of tau filament formation (Johnson and Bailey, 2002)**.** In normal brain, the majority of tau is associated with microtubules. However, in AD pathological events may lead to an increase of tau phosphorylation in a site-specific manner, which increases the pool of free monomeric tau. Due to increased levels of free tau coupled with other facilitatory events, such as oxidative stress, tau aggregates into a nucleus that can then go on to "seed" tau filaments. This process may be facilitated by such events as cleavage of the C-terminal of tau and/or hyperphosphorylation.

3.4. Role of phosphorylation in tau assembly

Three types of aggregated tau: PHFs, twisted ribbons, and SFs are found in AD brain. How the three types of tau aggregates are formed is one of the major puzzles in the AD field. It was proposed that tau hyperphosphorylation promotes tau aggregation into PHFs (Grundke-Iqbal et al., 1986). Phosphorylation appears to precede NFT formation in degenerating neurons (Bancher et al., 1989, Braak et al., 1994). However, later studies from Goedert et al showed that tau assembly seems to be independent of tau phosphorylation, since each of the six isoforms of recombinant human tau forms PHF-like filaments in the presence of heparin (Goedert et al., 1996). The role of tau phosphorylation in formation of PHFs was further challenged by the evidence that: 1) tau MBDs are necessary and sufficient for PHF formation; 2) polyanions promote PHF formation *in vitro*; 3) dimerization and nucleation are the rate-limiting steps for PHF formation (Friedhoff et al., 1998b). Moreover, native tau is able to self-aggregate in the

absence of other molecules although it requires a high concentration (Wille et al., 1992, Crowther et al., 1994). Thus, Goedert et al pointed out that phosphorylation is not necessary for formation of PHF-like filaments.

To address the question, the effect of tau phosphorylation on its assembly was re-evaluated in Iqbal's lab (Alonso et al., 2001). In this study, they found that: 1) AD p-tau and all of the six isoforms of recombinant tau can self-assemble into PHF-like structure *in vitro* under physiological conditions (without heparin and any other tau assembly-promoting co-factors) within 90 min; 2) *in vitro* dephosphorylation of AD p-tau inhibits the self-assembly; 3) polymerization of tau into PHFs depends on the degree of tau phosphorylation. Thus, although tau phosphorylation is not a prerequisite for formation of PHFs, no doubt the process of tau assembly is dramatically enhanced by phosphorylation. However, at least tau phosphorylation at certain sites such as S262 and S214 strongly inhibits tau assembly (Biernat et al., 1993, Drewes et al., 1997, Schneider et al., 1999). Phosphorylation of tau at S262 and S214 releases tau from microtubules. The released tau sequesters normal tau and other high molecular weight microtubule-associated proteins (MAPs), causing on one hand microtubule disruption, and on the other hand the self-assembly of the hyperphosphorylated proteins into PHF and SF (Alonso et al., 2001). Moreover, pesudophosphorylation of double mutations from serine to glutamic acid at residues 396 and 404 ([396/404] S→E) significantly enhanced formation of tau filaments *in vitro* (Abraha et al., 2000), suggesting that conformational changes may also influence tau assembly.

Data from Avila's group showed that in *in vitro* cultured human neuroblastoma cells, it is the phosphorylated but not unphosphorylated form of tau that allows formation of tau filaments (Perez et al., 2000). Additionally, oxidative stress was found to be necessary for tau assemblies into PHFs. Tau dimers are important building blocks of PHFs. When tau molecules are cross-linked into dimers by an oxidized disulfide bridge at Cys322, tau assembly is enhanced (Wille et al., 1992, Schweers et al., 1995). Oxidative stressors especially the compounds derived from arachidonic acid by lipid peroxidation, such as 4-hydroxynonenal (HNE) (Sayre et al., 1997), are present in AD brain and are co-localized with NFTs (Smith et al., 1996, Sayre et al., 1997, Lovell et al., 2001, Nunomura et al., 2001). HNE can inhibit tau dephosphorylation in cultured hippocampal neurons (Mattson et al., 1997), and act as an adduct to phosphorylated tau, leading to formation of tau filaments (Perez et al., 2000).

In vitro PHF-like structures could be readily formed from soluble AD p-tau within 90 min in physiological conditions (Alonso et al., 2001). However, why a

significant pool of soluble AD p-tau exists in human brains obtained with postmortem delay from 3 to 8h that is much longer than the required reaction time of PHF-like filament formation *in vitro* (90 min) needs to be investigated. It is likely that a portion of AD p-tau from AD brain is oxidized when it is exposed to air during the processing of sample preparation and in *in vitro* experimental procedures, and that this portion forms tau dimers that facilitate tau assembly.

In summary, oxidative stress, hyperphosphorylation, and conformational changes may contribute to tau assembly. Other factors such as fatty acids (Wilson and Binder, 1997), heparin (Goedert et al., 1996, Perez et al., 1996), polyanions (sulfo-glycosaminoglycans) (Perry et al., 1991, Goedert et al., 1996) and RNA (Kampers et al., 1996)), polyglutamate (Friedhoff et al., 1998a), products of lipid oxidation (Uchida and Stadtman, 1993) were also thought to enhance tau assembly in NFTs.

3.5. Microtubule disruption: tau-microtubule interactions

Once tau binds to tubulin, it promotes microtubule assembly and enhances microtubule stability and stiffness (Matus, 1994). Tau (the longest human tau isoform with 441 amino acids) binds firmly to microtubules through repeat 1 (Q_{244}TAPVPMPDLKNVKSKIGSTENLKHQPGGGK$_{274}$), and weakly through repeat 2 (V_{275}QIINKKLDLSNVQSKCGSKDNIKHVPGGGS$_{305}$), repeat 3 (V_{306}QIVYKPV DLSKVTSKCGSLGNIHHKPGGGQ$_{336}$), and repeat 4 (V_{337}EVKSEKLDFKDRVQS KIGSLDNITHVPGGGN$_{368}$) (Minoura et al., 2005). The interaction between tau and microtubules is regulated by the status of tau phoshorylation (Biernat et al., 1993, Drewes et al., 1995).

The influence of phosphorylation on tau microtubule-binding ability is site-specific. Among the > 30 phosphorylation sites, S262 is the only residue located within the first microtubule-binding repeat (Morishima-Kawashima et al., 1995a). Phosphorylation at this site abolishes tau binding to microtubules (Drewes et al., 1995, Drewes et al., 1997), causing cytoskeleton disruption (Biernat et al., 1993, Seubert et al., 1995, Sengupta et al., 1998). However, phosphorylation at Tau-1 sites (S198/S199/S202/T205) during differentiation of SH-SY5Y cells does not affect tau microtubule-binding activity (Haque et al., 1999). Binding of tau to microtubules is also controlled by phosphorylation at the T231 and S235 sites (Seubert et al., 1995, Sengupta et al., 1998). Evidences have shown that tau phosphorylation at the S214 site or conversion of S214 to Asp strongly decreases tau-microtubule binding *in vitro*, suppressing microtubule assembly (Illenberger et al., 1998) or microtubule nucleation

(Leger et al., 1997). The T212 site on tau protein appears to have a relatively less significant role in regulating tau binding to microtubules (Ksiezak-Reding et al., 2003).

Tau phosphorylation at the S262 site is regulated by several kinases, including phosphorylase kinase (Paudel, 1997b), CaMKII (Sironi et al., 1998, Bennecib et al., 2001, Yamamoto et al., 2002), MARK (Schneider et al., 1999), and PKA (Sironi et al., 1998), but not by GSK-3β (Godemann et al., 1999). In addition, both CaMKII and PKA can phosphorylate tau at the S356 site, which is also located within the MBDs (Litersky et al., 1996). In contrast, if tau is prephosphorylated at T50 by SAPK4 / p38 which can down-regulate tau phosphorylation at T181, microtubule assembly could be up-regulated (Feijoo et al., 2005). Maintaining a dynamic interaction of tau and microtubules is vital for various cellular functions. Tau-mediated development of cell processes requires tau phosphorylation at S262/356 sites in the MBDs, and the development is inhibited by phosphorylation at the proline-rich flanking domains (Biernat and Mandelkow, 1999). Tau detachment mediated by S214 phosphorylation might be involved in regulation of synaptic strength (Wang et al., 2003). Microtubules serve as tracks for intracellular transport. Disruption of microtubules induced by tau hyperphosphorylation causes a breakdown of intracellular traffic (Mandelkow, 1999). Tau redistribution from axons to somatodendrites in AD indicates a defective transport of tau to the right compartment. Since the S262 site shows enhanced phosphorylation in AD (Morishima-Kawashima et al., 1995a), it is possible that loss of microtubules and breakdown of axonal traffic might result from tau hyperphosphorylation at the S262 site.

3.6. Toxicities of normal and abnormally hyperphosphorylated taus

Abnormal hyperphosphorylation of tau might also be toxic to neurons. AD p-tau loses its binding ability to tubulin, and instead binds to normal tau and other MAPs. This may cause disruption of microtubules and formation of tau filaments (Alonso et al., 1994, Alonso et al., 1996, Alonso et al., 1997). In normal dividing cells, tau is hyperphosphorylated at some residues (Illenberger et al., 1998). Site-specific hyperphosphorylated tau is seen in foetal brain (Seubert et al., 1995). A pseudohyperphosphorylation model revealed that in cultured cells tau hyperphosphorylation is detrimental to tau function, and contributes to neuronal death (Fath et al., 2002). In contrast, other evidence suggests a protective effect of tau hyperphosphorylation (Lesort et al., 1997). So, hyperphosphorylated tau appeared to have both toxic and protective roles, which are phosphorylation-site- and cell-type-specific.

Over-expression of human tau not only causes a significant inhibition of intracellular transport, but also neuronal neurofibrillary degeneration (Hall et al., 2001). As hypothesized by Mandelkow et al (Mandelkow et al., 2003), in a healthy neuron, the tau/tubulin ratio is usually low, so that a few tau molecules may suffice to initiate a growing neurite, and the effect of such few tau molecules on transport is negligible. However, the effect on transport could become noticeable if tau becomes elevated in degenerating neurons. When a traffic jam occurs, the net anterograde transport of vesicles and cell organelles such as mitochondria and peroxisomes is reduced. This then induces starvation of synapses and oxidative stress, which might happen long before tau detaches from microtubules and aggregates into AD NFTs (Mandelkow et al., 2003). Transport of the β-amyloid precursor protein is also retarded by blocking microtubule tracks when tau is elevated, which suggests a possible link between the two key abnormal proteins in AD. In addition to an intracellular traffic jam, tau protein at a high concentration is capable of self-aggregation *in vitro* even when in an unmodified form (Avila et al., 2002). Thus, it is possible that tau is beneficial to neurons at a physiological low concentration, but harmful at an elevated concentration.

4. RIBOSOMAL PROTEIN S6 KINASE

4.1. Structure

The 70-kDa ribosomal protein S6 kinase (p70S6K) is a S/T kinase that regulates the phosphorylation of the 40S ribosomal protein S6 (Ferrari and Thomas, 1994). It is one of the two isoforms of ribosomal S6 kinase 1 (S6K1) (Romanelli et al., 2002), the other isoform being p85S6K. S6K2 is highly homologous to S6K1, and has similar functions (Martin et al., 2001a, Martin et al., 2001b). The p70S6K and p85S6K are translated from the same transcript by two different start codons. The p70S6K is cytoplasmic, whereas p85S6K appears to be exclusively nuclear owing to an additional 23-amino-acid sequence in the N-terminus, and functions as a nuclear localization signal (Reinhard et al., 1992, Reinhard et al., 1994). P70S6K has acidic, catalytic, regulatory, and autoinhibitory domains (Fig. 5).

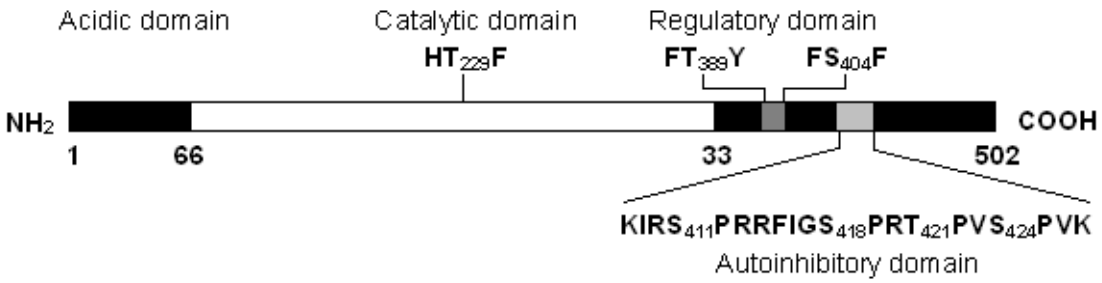

Fig. 5. Schematic representation of the functional structure of p70 S6 kinase. P70 S6 kinase (Genbank: AAA36411) is composed of four functional domains. The potential phosphorylation sites S411, S418, T421 and S424 locate in the autoinhibitory domain, T389 lies in the regulatory domain, and T229 in the catalytic domain.

4.2. Physiological function of p70 S6 kinase

Activated p70S6K up-regulates ribosomal biosynthesis, and enhances cell translational capacity through phosphorylating S6 and regulating mRNAs with 5'-terminal oligopyrimidine tracts (5'TOP) which generally encode ribosomal proteins and elongation factors (Terada et al., 1994, Jefferies et al., 1997). In addition to up-regulating cell translational capacity (Avruch et al., 2001), p70S6K activation plays a crucial role in cell size, cell growth, cell cycle control, cell differentiation and cell motility (Petritsch et al., 2000, Saucedo and Edgar, 2002). P70S6K is likely involved in production of a large number of proteins needed during re-entering the cell cycle for differentiated mature neurons under environmental stimuli, or during neuroblastoma cell mitosis.

4.3. Regulation of p70 S6 kinase activation by phosphorylation

Some of the phosphorylation sites on p70S6K have been found experimentally, of which, S411, S418, T421 and S424 are in the autoinhibitory domain of the C-terminus; S371, S404 and T389 in the linker domain; and T229 in the catalytic domain (Dennis et al., 1998).

Major conformational changes usually occur when a kinase switches between inactive and active states (Huse and Kuriyan, 2002). This activation is in several cases due to phosphorylation, which can generate an interactive network optimizing loop conformation (Fig.6.). Phosphorylation at the T421 and S424 sites in the autoinhibitory domain leads to a large conformational change that mediates the first step of kinase activation. In turn, it facilitates the phosphorylation of the linker domain at T389, and then exposes the catalytic domain at T229, following the hierarchical fashion of p70S6K activation (Pullen and Thomas, 1997). Full activation of p70S6K depends on phosphorylation of the S411, S418, T421, and S424 sites of the autoinhibitory domain,

the T389 site of the linker domain, and the T229 site of the catalytic domain (Dufner and Thomas, 1999).

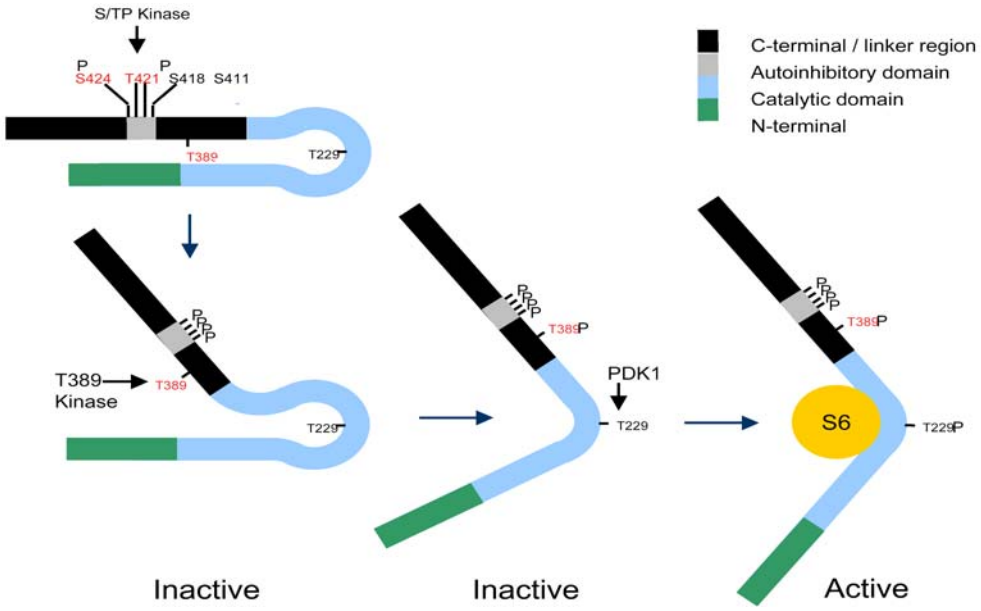

Fig. 6. Diagrams of sequential steps during the activation of wild type p70S6K (Dennis et al., 1998). The first step of kinase activation is mediated by phosphorylation of the S/T-P sites in the autoinhibitory domain. This step facilitates the next step, phosphorylation of T389 in a PI3K-dependent manner, disrupting the interaction of the amino and carboxy termini of the kinase, thereby allowing phosphorylation of T229 and activation of the kinase in the final step (Dufner and Thomas, 1999).

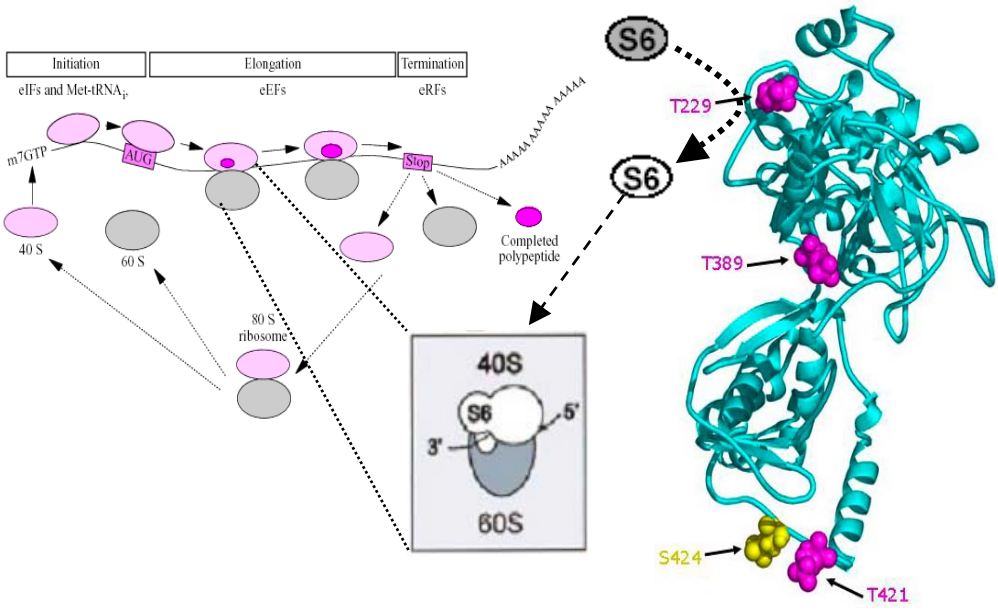

Fig. 7. Schematic representation of the regulation of protein translation through p70 S6 kinase and ribosomal protein S6. As one of the components of 40S subuint, ribosomal protein S6 can be phosphorylated by activated p70S6K, which then preferentially regulates 5'TOP mRNA translation through either direct interaction with the polypyrimidine tract, or alterating affinity of the 40S ribosome mRNA binding site for the polypyrimidine tract mRNA, or recognition proteins that directly bind to the polypyrimidine tract (Ferrari and Thomas, 1994). The 3D structure of p70S6K is predicated in **Paper IV**.

4.4. Regulation of protein synthesis by p70 S6 kinase via S6

The S6 protein is a component of the small 40S subunit of the eukaryotic ribosomes. The S6 protein has up to five serine residues in the C-terminus that can be phosphorylated in an orderly manner both *in vivo* and *in vitro*: S236-S235 or S240-S244-S247 (Bandi et al., 1993). In mammalian cells, p70S6K is considered as the major kinase for S6 phosphorylation at S235/236 (Flotow and Thomas, 1992, Kozma and Thomas, 1994). A number of other kinases have been reported to phosphorylate S6, including PKC and p90S6K (Flotow and Thomas, 1992, van den Brink et al., 2000). As demonstrated in Fig. 7., p-S6 can regulate translation of 5'TOP mRNAs through a number of potential mechanisms (Ferrari and Thomas, 1994).

In response to many mitogenic and nutritional stimuli, synthesis regulation of translational apparatuses (such as ribosomal proteins and elongation factors) is at the translational level, this gives rise to the speedy and readily reversible action to altering physiological conditions (Meyuhas, 2000). Upon shortage of amino acids or growth arrest, the speedy and readily reversible responses enable cells to rapidly repress the biosynthesis of translational machinery, which blocks unnecessary energy wasting. When nutrients are replenished or mitogenic stimulation is applied, cells can rapidly resume the biosynthesis of 5'TOP structure containing translational apparatuses.

5. THE POSSIBLE ROLES OF P70 S6 KINASE IN FORMATION OF TAU ABNORMALITIES IN ALZHEIMER'S DISEASE

As discussed previously, tau hyperphosphorylation plays a dramatic role in formation of tau pathologies in AD brain. It is known that tau could be phosphorylated at S/T residues by many kinases, such as PKB (Ksiezak-Reding et al., 2003), GSK-3β (Lucas et al., 2001), ERK1/2, JNK, and p38 (Reynolds et al., 2000), and phosphorylated tau could be dephosphorylated by PP-2A (Gong et al., 2000). However, how the abnormally hyperphosphorylated tau is accumulated in NFT-bearing neurons, accompanied by microtubule disruption in AD brains is still unclear. We hypothesized that continuous conversion of newly synthesized tau to the hyperphosphorylated form, and or insufficient degradation of the hyperphosphorylated tau contribute to the formation of tau pathologies and microtubule disruption in AD brains.

With antibodies against activated kinases now availabe, it was possible to study focal biochemical changes that precede formation of NFTs *in situ*. We found that PHF-tau associated pathologies positive for the tau kinases PKB, GSK-3β, ERK1/2, JNK, and p38 are distributed in the entorhinal cortices, the hippocampal CA1 area, and the

temporal cortices of brains following the sequential predicatable order described by Braak and Braak (Braak and Braak, 1991, Pei et al., 1997, Pei et al., 1999, Pei et al., 2001, Pei et al., 2002, Pei et al., 2003). Intracellularly, the active forms of these kinases accumulate in NFT-bearing neurons, especially in the pretangle neurons (Pei et al., 1997, Pei et al., 1999, Pei et al., 2001, Pei et al., 2002, Pei et al., 2003). This suggests that upregulation of both phosphatidylinositol 3 kinase (PI3K) and MAPK pathways are involved in formation of tau pathologies in AD.

P70S6K is a merging point of the PI3K and MAPK pathways in terms of regulation of protein translation through the S/T kinase p70S6K (Kim et al., 2000, Zhang et al., 2001). Zinc, leucine, insulin, H_20_2, and leptin can activate p70S6K (Kim et al., 2000, van den Brink et al., 2000, Greiwe et al., 2001, Tu et al., 2002). P70S6K is also regulated by a mammalian target of rapamycin (mTOR)-regulated phosphatase PP-2A, which can be co-immunoprecipitated with p70S6K (Charlton et al., 1999, Westphal et al., 1999).

Of the known activators of p70S6K, it has been shown that zinc is increased in brain regions such as the hippocampus, heavily affected by AD pathologies (Frederickson and Bush, 2001). Altered zinc metabolism in brains can accelerate Aβ deposition in SPs in AD (Atwood et al., 1998) and exacerbate neuron injury. Zinc can enhance Aβ aggregation that in turn induces tau hyperphosphorylation (Danscher et al., 1997, Cherny et al., 2001, Gotz, 2001). Zinc was shown to activate PI3K and MAPK pathways in a similar to insulin (Kim et al., 2000). It is thus tempting to speculate that local increases in zinc concentration in AD brain may in part be responsible for activation of PI3K and MAPK pathways seen in this disease and may also play a role in p70S6K regulation.

PP-2A is also an important regulator of p70S6K via mTOR. PP-2A can also reverse Ser9 phosphorylation of GSK-3β induced by insulin/IGF-1 in human embryonic kidney 293 cells overexpressing GSK-3β (Shaw et al., 1997). Protein tyrosine phosphatase (PTP) was suggested to regulate tyrosine phosphorylation of GSK-3β, since Chinese hamster ovary cells in which the insulin receptor is overexpressed showed partial dephosphorylation of Tyr216 and a deletion mutant lacking the N-terminal nine residues of GSK-3β could still be partially inhibited by insulin (Murai et al., 1996). It is known that phosphorylation at the Thr308/Ser473 of PKB, at the Ser217/221 of MEK1/2, and at the tripeptide dual motif (Thr-x-Tyr) of ERK1/2, JNK, and p38, dominates their activities. Owing to the reduced phosphatase

activities of PP-1, PP-2A and PTP-1B in AD brain (Gong et al., 1993, Gong et al., 1995), both the dephosphorylation of these kinases and the dephosphorylation of tau would be compromised, allowing tau to be abnormally hyperphosphorylated at multiple sites, and phosphorylated PKB, GSK-3, MEK1/2, ERK1/2, JNK, and p38 to be accumulated in NFT-bearing neurons (Pei et al., 1997, Pei et al., 1999, Pei et al., 2001, Pei et al., 2002, Pei et al., 2003).

Given what is known about the modulation of p70S6K and that this enzyme plays a crucial role in the regulation of protein translational capacity through phosphorylation of S6, we thought it was of great interest to investigate whether this kinase is involved in formation of tau-associated pathologies in AD brain and possible underlying mechanisms.

AIMS OF THE STUDY

The general aim of study was to investigate the role of p70S6K in tau pathologies in AD.

The specific aims included:

(1) To determine whether or not p70S6K activation is associated with abnormal hyperphosphorylation and accumulation of tau in AD brain.

(2) To investigate whether or not selective PP-2A inhibition can induce p70S6K activation and tau phosphorylation in metabolically active rat brain slices

(3) To investigate the mechanism of zinc-induced p70S6K activation and its effect on tau

(4) To investigate whether or not p70S6K mediates tau phosphorylation and synthesis

RESULTS AND DISCUSSIONS

1. RELATIONSHIP OF ACTIVATED P70 S6 KINASE WITH TAU PATHOLOGIES

Defects in a single neuron were described to start with tau phosphorylation by diffuse cytoplasmic stainings, which gives the definition of a pretangle stage (Bancher et al., 1989, Duong et al., 1993, Braak et al., 1994, Pei et al., 1997, Pei et al., 1999). It is thought that with the progression of neuronal degeneration, tau filamentous polymers start to be formed (nucleation), resulting in formation of cytoplasmic (intracellular) NFTs. As a consequence of this progression, neurons degenerate and die, leaving NFTs in the extracellular space (Bondareff et al., 1989, Goedert et al., 1999). Using confocal microscopy, we proposed an additional stage prior to pretangles, in which abnormal accumulation of a series of activated kinases including PKB, ERK1/2, JNK, p38, p70S6K, and GSK-3, but not hyperphosphorylated tau was found in neurons in AD brain (**Paper I**) (Pei et al., 1997, Pei et al., 1999, Pei et al., 2001, Pei et al., 2002, Pei et al., 2003).

Progression of filamentous tau pathologies in AD was described on the basis of the appearance of NFTs (Gallyas, 1971, Bancher et al., 1989, Duong et al., 1993, Braak et al., 1994, Pei et al., 1997, Pei et al., 1999). Braak proposed that neurofibrillary degeneration starts in the transentorhinal region, moves to the entorhinal region and the hippocampus, and then appears in the whole hippocampus and association cortices (Braak and Braak, 1991). Immunohistochemically, using antibodies to the active form of p70S6K, we found that the progression of number of neurons positive for active p70S6K coincided with tau pathologies in regions described by Braak and Braak (**Paper I**). The levels of active p70S6K were significantly increased in homogenates of medial temporal cortex of AD brains as compared to controls (**Paper I**). This increase showed a progressive pattern, which was significantly correlated with PHF-taus in AD brains (**Paper I**). These data suggested that a systematic activation of kinases as an early biochemical event occurs in neurons prior to tau hyperphosphorylation, and which might consequently induce tau hyperphosphorylation.

2. UP-REGULATION OF P70 S6 KINASE IN ALZHEIMER'S DISEASE BRAIN

Levels of both p-p70S6K and p-S6 were found increased in AD brain (**Paper I**), suggesting that increased p-p70S6K in AD brain is active. Concurrently, increases of total S6 and tau were found in AD brain, implying that translational control of

proteins such as tau is disturbed in AD brain (**Paper I**). It is possible that increased total tau might result from the up-regulated translation of tau mRNA in addition to decreased turnover in AD brain.

Translation initiation is a rate-limiting step, and is mainly influenced by elements within the 5' untranslated region (5'UTR) of the mRNA (Levy et al., 1991, Avni et al., 1994, Hornstein et al., 1999, Meyuhas, 2000). One element in 5'UTR in mRNAs encoding ribosomal proteins (such as S3, S6, S14) or translation elongation factors (such as eEF1A and eEF2) is the 5'TOP structure. The features of 5'TOP structure are: 1) located immediately adjacent to the cap (m(7)GpppN) structure; 2) with a C residue start at the cap structure, followed by an uninterrupted stretch of 4-14 pyrimidines; 3) followed by a CG-rich sequence; and 4) that its activity is strictly dependent on the integrity of the first 30 nucleotides (Meyuhas, 2000). The 5'TOP structure plays a critical role in translational control (Jefferies et al., 1997). Activation of p70S6K can enhance protein translational capacity through phosphorylating S6 and regulating mRNAs with 5'TOP structure, sequentially promoting the translation of proteins encoded by 5'TOP mRNAs including ribosomal protein S6.

P70S6K phosphorylation does not necessarily reflect the actual rate of overall protein synthesis, since it regulates the translation of a set of mRNAs rather than overall protein synthesis. Comparative analysis of the first 20 nucleotides of mRNAs encoding proteins such as p70S6K, S6 and tau, showed a 5'TOP-like structure in the 5'UTR of tau mRNA similar to that of S6 mRNA (**Paper IV**). This structure might enable tau mRNA to be translated into protein preferentially in response to p70S6K activation. Accumulation of total tau and S6, not total p70S6K, suggested that protein accumulation in AD brain is relatively selective (**Papers I, IV**). This phenomenon can also be seen in zinc-treated SH-SY5Y cells (**Paper IV**). Thus, it is logical to hypothesize that the accumulated tau in AD brain is at least in part caused by up-regulated translation of tau mRNA.

3. EFFECTS OF PP-2A INHIBITION ON P70 S6 KINASE AND TAU PHOSPHORYLATION IN METABOLICALLY ACTIVE RAT BRAIN SLICES

By using phospho-specific antibodies against activated ERK1/2, MEK1/2, and p70S6K, we found that ERK1/2, MEK1/2 or p70S6K was activated in most of the neurons containing AT8-positive/PHF-tau (Pei et al., 2002) (**Papers I and II)**. However, no significant immunostaining was found in control brains with these antibodies. These data suggest that the MAPK pathway is up-regulated in many

neurons undergoing neurofibrillary degeneration in AD brain (**Paper II**) (Pei et al., 2002).

Treatment of metabolically active rat brain slices with 1.0 μM okadaic acid (OA) for 1h can inhibit ~70% PP-2A activity, but does not change PP-1 or PP-2B activity (**Paper II**). Incubation of the OA-treated tissue sections with purified PP-2A dramatically reduced tau phosphorylation at S262/356, and OA-activated ERK1/2, MEK1/2, and p70S6K as compared to the sections stained in parallel but without PP-2A treatment (**Paper II**). These results indicated that PP-2A could dephosphorylate tau at S262/356 and the three kinases. These data indicated that the MAPK pathway might be regulated by PP-2A in rat brain, and the compromised PP-2A activity could result in activation of the MAPK pathway in selected neurons of AD brain as well as tau hyperphosphorylation at sites Tau-1, 12E8, PHF-1, and R145. Thus, the abnormal hyperphosphorylation of tau might result from a decrease in tau dephosphorylation by reduced PP-2A activity, as well as from an increase in tau phosphorylation by kinases such as ERK1/2, the activity of which is also regulated by PP-2A.

4. EFFECTS OF ZINC-INDUCED P70 S6 KINASE ACTIVATION ON TAU IN SH-SY5Y CELLS

In SH-SY5Y cells, we found that zinc can induce phosphorylation of p70S6K, PKB, and MAPKs in a two-stage manner (**Paper III**). Application of inhibitors including rapamycin (mTOR inhibitor), U0126 (ERK1/2 inhibitor), wortmannin and LY294002 (PI3K inhibitors) and their combinations revealed that regulation of p70S6K at the T389 and T421/S424 sites is more favored by the PI3K pathway, which is assisted by the ERK1/2 pathway. In parallel to p70S6K regulation, increased levels of p-S6 (S235/236) and total S6 induced by zinc are also modulated via the PI3K and MAPK pathways. These results suggested that zinc could induce p70S6K activation and increase protein translation (**Paper III**). In both primary cultured neurons and SH-SY5Y cells, zinc could increase levels of total tau and p-tau at the S396/S404 sites (**Papers I and III**). Although increased p-tau at S396/S404 sites could be blocked by rapamycin, our *in vitro* data showed that S396/S404 sites could not be directly phosphorylated by p70S6K (**Paper IV**). Zinc-induced phosphorylation of tau on S396/S404 sites might be not mediated by ERK1/2 as well, since blocking ERK1/2 with U0126 could not influence tau phosphorylation at S396/S404 sites induced by selective PP-2A inhibition (**Paper II**). Addition of zinc at concentrations (1-10 μM) in the same range that enhance Aβ amyloid aggregation (Bush et al., 1994)

could significantly stimulate formation of tau filaments induced by heparan sulfate (Hasegawa et al., 1997).

5. ROLE OF P70 S6 KINASE IN TAU PHOSPHORYLATION AND SYNTHESIS

Since most of the phosphorylation sites on tau are at S/T residues, and the fact that the S/T kinase p70S6K is associated with PHF-tau (**Paper I**), we hypothesized that p70S6K might induce a direct tau phosphorylation. Our *in vitro* data proved that p70S6K could directly phosphorylate tau at S262, S214, and T212, but not at S396/404 (**Paper IV**). By immunoprecipitation, we found that only tau protein phosphorylated at S396/S404 and S262, not at Tau-1 sites (dephosphorylated at S198/199/202/T205), can be co-precipitated with p-p70S6K (T421/S424), not p-p70S6K (T389) (**Paper IV**). We thought it is likely that an interaction of p-p70S6K (T421/S424) with tau phosphorylation sites (S396/S404 and S262), tau phosphorylation on S262, S214, and T212 could be carried out and/or enhanced. Evidence showed that the level of tau mRNA is not significantly changed in AD brain compared with controls (Mah et al., 1992, Chambers et al., 1999, Boutajangout et al., 2004). The requirement of p70S6K upregulation was demonstrated in SH-SY5Y cells transfected with wild type human tau as compared with the mock-transfected cells (**Paper IV**). Taken together, the data from Paper IV suggest that p70S6K mediates both tau phosphorylation and synthesis.

CONCLUSIONS AND GENERAL SUMMARY

1. Immunohistochemically and biochemically, activated / p-p70S6K is closely associated with total tau and PHF-tau in AD brains (**Paper I**).
2. P-p70S6K (T421/S424) interacts with tau phosphorylated at S396/S404 and S262 sites (**Papers I and IV**).
3. Activated p70S6K and increased tau phosphorylation are caused by selective PP-2A inhibition in rat brain slices (**Paper II**).
4. P70S6K is regulated mainly by the PI3K pathway, which assisted by the MAPK pathway in SH-SY5Y cells (**paper III**).
5. P70S6K phosphorylates tau at S262, S214 and T212 sites, and mediates tau synthesis (**paper IV**).

Taken together, our findings suggest that activated p70S6K is involved in the genesis of tau abnormalities in AD brain (Fig. 8). P70S6K might be a promising target for therapeutic approaches for drug intervention of tau abnormalities in AD.

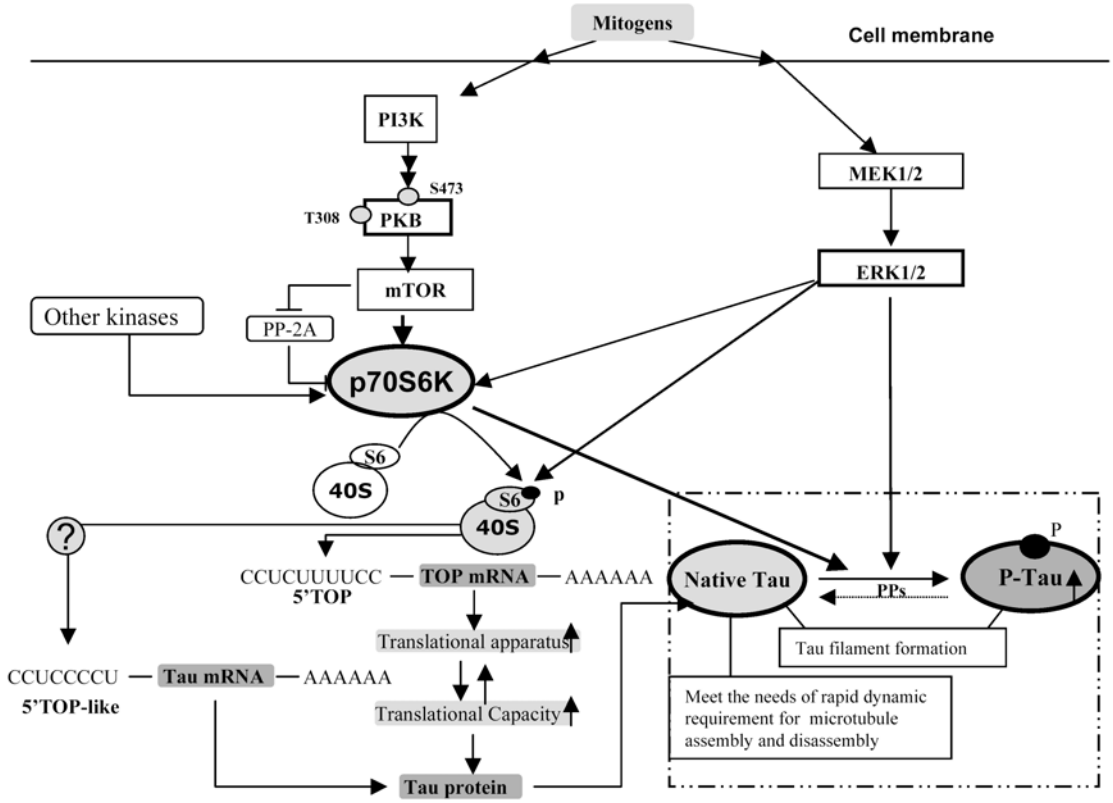

Fig. 8. Possible mechanisms of tau protein phosphorylation and translation mediated by p70S6K. Activated p70S6K through PI3K/PKB and MEK/ERK pathways by mitogens induces phosphorylation of ribosomal protein S6, which

preferentially facilitates translation of 5'TOP mRNAs. The 5'TOP-like structure of tau mRNA may give rise to the possibility that tau protein synthesis is regulated, at least, partially at the translational level in response to p70S6K activation. Under physiological conditions, there should be a balance between tau synthesis and tau degradation, and a balance between tau phosphorylation by kinases and dephosphorylation by protein phosphatases (PPs). A certain amount of normal tau protein can meet the needs of rapid dynamic requirement for microtubule assembly and disassembly as shown by the fact that tau is enriched on dynamic microtubules in the distal region of growing axons**.** Dysfunction of PPs and site-specific phosphorylation of tau, together with other factors such as oxidative stress and polyanions, promote formation of tau filaments in addition to compromising binding ability of tau to microtubules. Accumulation of phosphorylated tau and filamentous tau might drive degenerating neurons to generate native tau so as to compensate for the loss of function of hyperphosphorylated tau through activation of p70S6K. However, under pathological conditions that overwhelmed activation of protein kinases and inactivation of protein phosphatases, the newly synthesized native tau proteins can either be rapidly phosphorylated and assemblied into filaments or be sequestered into tau filaments. In addition, robust activation of p70S6K might enhance tau accumulation, accelerating formation of PHFs. Deposition of tau filaments in cell body, dendrites and axon reduces or even blocks the intracellular organelle transport.

FUTURE PERSPECTIVES

As we discussed in the previous sections, interactions between tau-microtubules and tau-tau are much more complex than expected. This thesis sheds some light on the role of p70S6K in the formation of tau pathologies in AD. To further understand the role of p70S6K, the following studies can be envisaged:

1) Clarifying all of the tau phosphorylation sites by p70S6K by 2-D gel and mass spectrometry
2) Crystallizing active p70S6K, and investigating the interactions of p70S6K with different segments of tau molecules
3) Using the model systems established in this thesis and future studies to screen new drugs that can affect p70S6K activity and tau phosphorylation

MATERIALS AND METHODS

1. MATERIALS

1.1. Reagents and some preparations

Fetal bovine serum (FBS) and F12/DMEM culture media from Invitrogen AB (Täby, Sweden) were used in **Papers I, II and III**. Zinc sulfate, protease inhibitor cocktail (**Papers I, III and IV**), tetrazolium salt 3, [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (**Papers III and IV**) were bought from Sigma-Aldrich (St. Louis, MO). Abnormally hyperphosphorylated tau (AD p-tau) and PHF-tau were isolated from AD brain in **Paper I**. The catalytic subunit of PP-2A and purified phosphorylase kinase was isolated from bovine brain (Cohen et al., 1988a), and from the skeletal muscle of White New Zealand rabbits by the method of Cohen (Cohen, 1973), respectively (**Paper II**). Inhibitor-1 was also from the rabbit skeletal muscle and phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (Sigma, St. Louis, MO) according to the method of Cohen et al (Cohen et al., 1988b) (**Paper II**). Extracts from non-treated and serum-treated NIH-3T3 cells were purchased from Cell Signaling Technology (Beverly, MA) (**Paper I**).

1.2. Inhibitors

Most of the inhibitors used in this thesis are listed in Table 1.

Table 1. Detailed information about inhibitors

Inhibitors	Abbrev.	Targets	Function	Concentration	Source
Rapamycin	R	MTOR	Inhibit p70S6K via mTOR	20ng/ml	A
Wortmannin	W	PI3K	Inhibit PI3K	200nM or 1μM	B
LY294002	LY	PI3K	Inhibit PI3K	50μM	B
U0126	U	MEK1/2	Inhibit ERK1/2 via MEK1/2	10μM or 50μM	B
Okadaic acid	OA	PP-2A	Inhibit PP-2A	1μM	C
Cyclosporin A	CsA	PP-2B	Inhibit PP-2B	1μM	D

A: Sigma-Aldrich, St. Louis, MO; B: Cell signaling technology, Beverly, MA; C: Calbiochem (San Diego, CA); D: Cyclosporin A from Alexis Corp. (San Diego, CA)

1.3. Antibodies

Antibodies used in this thesis are listed in Table 2.

Table 2. Primary antibodies used in the study

Antibody	Specificity [a]	Phosphorylation sites	Dilution	Sources	Papers
P70S6K	Total p70 S6K		1:100-1:500	A	I, III, IV
P70S6K (T389)	P, active p70/p85 S6K	T389	1:100-1:500	A	I, III, IV
P70S6K (T421/S424)	P, active p70/p85 S6K	T421/S424	1:100-1:500	A	I,II, III, IV
PDK1 (S241)	P, active PDK1	S241	1:500	A	III
PKB (T308)	P, active PKB	T308	1:500	B	III
PKB (S473)	P, active PKB	S473	1:500	B	III
P38 (T180/Y182)	P, active p38	T180/Y182	1:100-1:500	A	II, III
ERK1/2 (T202/Y204)	P, active ERK1/2	T202/Y204	1:100-1:500	A	II, III, IV
ERK1/2	Total ERK1/2	P-independent	1:100	A	II
MEK1/2 (S217/221)	P, active MEK1/2	S217/221	1:100	A	II
MEK1/2	Total MEK1/2	P-independent	1:100	A	II
JNK (T183/Y185)	P, active JNK	T183/Y185	1:500	A	II, III
GSK-3α/β (Y279/216)	P, active GSK-3α/β	Tyr279/216	1:800	B	II
S6	Total S6		1:250	A	I, III, IV
S6 (S235/236)	P, active S6	S235/236	1:250	A	I, III, IV
S6 (S240/244)	P, active S6	S240/244	1:250	A	I, III, IV
R134d	Total tau	P-independent	1:2500 or 5000		I, III, IV
92e	Total tau		1:5000		II
Tau-1	NP tau	S198/199/202/T205	1:20,000-40,000		I, II, III, IV
PHF-1	P tau	S396/404	1:200-1:400		I, III, IV
AT8	P tau	S202/T205	1:500-1:2000		I
p-tau (S262)	P tau	S262	1 :2000-5000	B	IV
p-tau (S214)	P tau	S214	1 :1000-10000	F	IV
p-tau (T212)	P tau	T212	1:2000-2500	F	IV
α-tubulin	tyrosinated	-	1:200		IV
AT100	P tau	T212/S214	1:100-500	G	IV
12E8	P tau	S262/356	1:500		II
R145	P tau	S422	1:3000		II
Rab5	Early endosomes	-	1:400	C	I, III
Lamp-1	Lysosomes	-	1:200	C	I, III
MAb NCL-UBIQm	Ubiquitin	-	1:10	E	I, III
Rabbit ubiquitin	Ubiquitin	-	1:20	E	I

[a] P, phosphorylated epitope; NP, non-phosphorylated epitope. A, Cell Signaling Technology, Beverly, MA; B, Biosource International, Camarillo, Calif. C, Transduction Laboratories; D, Stressgen Biotechnology; E, Novocastra Laboratories; F, Biosource Nordic, Stockholm; G, Innogenetics, Zwujndrecht.

2. CULTURES OF CELLS AND BRAIN SLICES

In **Papers I, III** and **IV,** SH-SY5Y cells were grown to 70-80% confluence in 100-mm-diameter dishes or 6-well culture plates in an atmosphere of 5% CO_2 /95% air at 37°C employing DMEM/F12 medium (1:1) supplemented with 5% fetal bovine serum (FBS), 100 units/ml penicillin, and 0.1mg/ml streptomycin. In order to minimize

stress responses induced by serum deprivation, the cells were then cultured in 0.5% FBS media for two days. Prior to treating the cells with zinc, the cultures were kept in fresh serum free media for 2h. For primary neuronal culture in **Paper I**, tissues of cortical cortice from brains of SD pups at prenatal 16-18 days were cultured in 6-well plates or 100-mm dishes in Neurobasal media supplemented with 1% B-27 (Invitrogen, Carlsbad, CA) with or without drug treatments.

CD rats (**Paper II)** and Wistar rats (**Paper III)** (Male, 150-200 g) were decapitated when deeply anesthetized, and brains were rapidly removed. 350 μm-thick coronal slices were sectioned with a vibroslicer (Campden Instruments LTD, London, UK), cultured in oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 3.5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 2.0 mM $CaCl_2$, 11 mM D (+)-glucose, 25 mM $NaHCO_3$, pH 7.4, with or without treatment, and homogenized in buffer containing 50 mM Tris-HCl, pH 7.0, 1.0 mM PMSF, 1.0 mM EDTA, 2.0 mg/L Aprotinin, 10 mM β-mercaptoethanol, 20 mM β-glycerophosphate, 2.0 mM Na_3VO_4 and 100 mM NaF.

3. IMMUNOSTAINING

3.1. Immunohistochemistry

The severity of neurofibrillary degeneration in human brain was classified into control, transentorhinal, limbic, isocortical stages according to the criteria established by Braak and Braak (Braak and Braak, 1991). Autopsy tissue blocks of the medial temporal cortex from 16 individuals (4 cases each stage), ages 50 to 93 years, were from Professor Heiko Braak, Dept of Anatomy, J.W. Goethe University, Frankfurt, Germany. 50-100 μM formalin-fixed frozen sections were stained by the free-floating method with antibodies to tau, p70S6K, ERK1/2, and MEK1/2 by the avidin-biotin-peroxidase complex kit (Vector, Burlingame, CA) (**Papers I and II)**.

The 400 μM rat brain slices were cultured, fixed in periodate / lysine / paraformaldehyde solution, embedded in paraffin, and cut into 6 μM sections. Rat brain slices from treated and untreated groups were stained with different antibodies to tau, p70S6K, ERK1/2, and MEK1/2 by the avidin-biotin-peroxidase complex kit (Vector, Burlingame, CA) (**Paper II)**.

3.2. Immunocytochemistry and immuno-electron microscopy

SH-SY5Y cells were grown on FALCON® culture slides (8-well), treated with or without zinc, fixed in 4% paraformadehyde, incubated with antibodies to p70S6K (T421/S424), ERK1/2 (T202/Y204), and S6 (235/236), and visualized by the avidin-

biotin-peroxidase complex kit (Vector, Burlingame, CA) (**Paper III**). For electron microscopy, the zinc-treated cells were fixed in buffered 3% paraformaldehyde plus 0.1% glutaraldehyde. The ultra-thin sections of the collected cell pellets were incubated with antibody against p70S6K (T421/S424) or PHF-1, and detected with protein A coated with 10 nm gold particles (**Paper III**).

3.3. Double immunofluorescent staining and confocal microscopy

In **Paper I**, 50-100 μM floating or 30 μM formalin-fixed frozen sections were incubated by anti-rabbit secondary antibodies conjugated with CY3 (red) or CY5 (far red) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) to stain bound antibodies to p-p70S6K or ubiquitin, and CY2 (green) -conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) to stain bound mAb AT8. Fluorescent stainings were visualized by a BioRad Laser Scanning Confocal Imaging System (Radiance Plus) equipped with a Nikon Eclipse inverted microscope (TE300), in which an argon ion laser exciting at 488 nm with a diachronic beamsplitter 560DCLP and a bandpass filter HQ515/30 was used to detect CY2, and a HeNe laser exciting at 543 nm with E570LP emission filter to CY3.

In **Paper IV**, zinc-treated SH-SY5Y cells grown on FALCON® culture slides (8-well) were incubated with rabbit primary antibodies to p-p70S6K (T421/S424) and mAb to tyrosinated -tubulin, and visualized with CY5 conjugated anti-rabbit IgG, and CY2 conjugated anti-mouse IgG.

4. PREPARATION OF HOMOGENATES AND CELL EXTRACTS

Homogenates of frozen tissue blocks of the medial temporal cortex of 13 control and 22 AD brains from Dr. Irina Alafuzoff, Kuopio Finland were prepared in **Papers I and IV**, and protein concentration was measured by the Bradford method (Bradford, 1976). Extracts were made in primary cultured cortical neurons from embryonic SD at 16-18 days old, SH-SY5Y cells (**Papers I, III, and IV**), SH-SY5Y cells transfected with htau40 and mock transfected groups (**Paper IV**), as well as homogenates from cultured rat brain slices (**Papers II and III)**, protein concentrations were determined with the BCA kit (Pierce Chemical, Rockford, IL).

5. INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY, WESTERN/DOT BLOTTING

Levels of p70S6K (total, T389 or T421/S424), tau, S6 (total, S235/236 and S240/244), and markers for proteolytic system (Rab5, Lamp-1 and ubiquitin) were measured by indirect enzyme-linked immunosorbent assay (ELISA) in homogenates from AD and control cases (**Paper I)**.

For Western blotting used (**Papers I, II, III**, and **IV)**, boiled samples were electrophoresed on 10-12% % SDS-polyacrylamide gels, and the separated proteins transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies (**Table 2**), and the bound IgGs recognized by anti-rabbit secondary antibody conjugated to horseradish peroxidase and visualized with ECL™ Western blotting detection reagents (Amersham Pharmacia Biotech, England). For dot blotting (**Paper IV)**, membranes with dotted samples (3 μg/dot in triplicate) were incubated with phospho-site-specific antibodies to S262, S214, T212, T212/S214, followed by secondary antibody linked with horseradish peroxidase (Amersham Biosciences AB, Uppsala, Sweden) at room temperature for 1h. Immunoreactive proteins were detected according to the enhanced chemiluminescence protocol (Amersham Biosciences AB, Uppsala, Sweden). Intensities of blots and dots were quantified with Quantity One 4.3.0 software (Bio-Rad Laboratories Inc., Hercules, CA).

6. CELL VIABILITY ASSAY

SH-SY5Y cells seeded in 96-well plates were cultured and treated with zinc sulfate or other reagents for certain time periods. At the desired time points, the culture medium was changed to phenol red-free medium containing 0.3mg/ml MTT. The plates were then kept in a humidified chamber for 3h at 37°C. The formed formazan crystals were dissolved by adding 100μl DMSO /well. Absorbances at 570nm were recorded with a spectrophotometer (**Papers III and IV**).

7. OTHER METHODS

For the methods used only in **Paper IV** such as *in vitro* tau phosphorylation by p70S6K, tau assembly assay, immunoprecipitation, and measurements of protein content in each cell, please see details in **Paper IV**. For measuring the activities of PP-1, PP-2B, and PP-2A in the extracts of rat brain slices only used in **Paper II**, please refer to **Paper II** for details.

8. STATISTICS

Levels of p70S6K, tau, S6 and proteolytic system markers in brain homogenates between AD and control groups were compared with Student's independent *t*-test (**Paper I**). The Pearson correlation and stepwise regression between p70S6K and tau levels were also analyzed. For regression analysis, the variable entered/removed criteria were probability-of-F-to-enter ≤0.05 and probability-of-F-to-remove ≥0.1 (**Paper I**). Statistical comparisons between different experimental groups were done by one-way analysis of variance (ANOVA) followed by least significant

difference (LSD) post-hoc test. The accepted level of significance was set at a p value ≤ 0.05 (**Papers I, II, III, and IV**).

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