ISAS - INTERNATIONAL SCHOOL FOR ADVANCED STUDIES

"Role of abnormal tau proteolysis in Alzheimer's disease neuronal degeneration"

Thesis submitted for the degree of "Doctor Philosophiae"

CANDIDATE
Gabriele Ugolini

SUPERVISORS
Prof. Michal Novak
Prof. Antonino Cattaneo

SISSA - SCUOLA INTERNAZIONALE SUPERIORE DI STUDI AVANZATI

TRIESTE
Via Beirut 2-4

TRIESTE

A mio nonno
Belisario Salani

Table of contents

Acknowledgements

I wish to express my gratitude to Prof. Michal Novak and Prof. Antonino Cattaneo for their careful supervision. I thank Dr. Andrew Bradbury, Prof. Enrico Cherubini and Prof. Andrea Nistri for helpful and stimulating discussions.

A special thank goes to Dr. Luisa Fasulo, Dr. Michela Visintin and Dr. Vittorio Verzillo for their collaboration in experimental work and their contribution to the theoretical interpretation of data.

I am grateful to to Dr. Massimo Righi, Dr. Gabriella Rossi and Dr. Marco Stebel for technical assistance and friendly discussion.

Finally, I would like to express my gratitude to Miss Barbara Stefanini, Dr. Margherita Molnar, Dr. Sonia Gasparini and Dr. Mario Barbieri for helping me whenever I needed help.

NOTE

The work described in this dissertation was carried out at the International School for Advanced Studies, Trieste, between January 1995 and August 1999. All work reported, with the exceptions listed below, arises solely from my own experiments and has not been submitted, as a whole or in part, to any other University.

1) The DNA constructs that I used in transfection experiments were prepared and sequenced by Dr. Luisa Fasulo and Dr. Michela Visintin, except for psG5-tau1-422, psG5-tau22-422 and psG5-tau22-441 (which I prepared by myself).

2) The western blot experiment shown in Fig. 4.4b (expression of tau fragments in COS cells) was performed by Dr. Michela Visintin.

3) Experiments to investigate *in vitro* cleavage of tau by caspase-3 (Fig. 4.7c and d) were performed by Dr. Vittorio Verzillo.

4) Rabbit injection and bleed (paragraph 4.9) were done by Dr. Marco Stebel.

5) Recombinant proteins to test the anti-tau421 polyclonal antibody were gently supplied by Dr. Michela Visintin (tau-421, produced by the pMAL expression and purification system, New England Biolabs) and Dr. Vittorio Verzillo (full-length tau).

Part of the data reported in the present thesis have been published or submitted in the articles listed below:

- **Ugolini G.**, Cattaneo A., Novak M.: "Co-localisation of truncated tau and DNA fragmentation in Alzheimer's Disease neurones" (NeuroReport 8, 1997, 3709-3712).

- Novak M., **Ugolini G.**, Ferencik M., Mikula I., Mitro A., Cattaneo A.: "Truncation of tau precedes fragmentation of DNA in Alzheimer's Disease" (Chemical Papers, 52, 1998, 429-431).

- Novak M., **Ugolini G.**, Fasulo L., Visintin M., Ovecka M., Cattaneo A.: "Truncation of tau and neurodegeneration" in "Alzheimer's Disease and Related Disorders" (eds. Iqbal K., Swaab D.F., Winblad B., Wisniewski H.M. – John Wiley & Sons, 1999, 281-292).

- Fasulo L.[#], **Ugolini G.**[#], Visintin M.[#], Bradbury A.M., Brancolini C., Verzillo V., Esposito G., Viglino P., Novak M., Cattaneo A.: "Tau as a substrate for caspase-3 and an effector of apoptosis". Submitted to Journal of Biological Chemistry. ([#] These authors contributed equally to this work).

Part of the data reported in the present thesis have appeared in abstract form:

- **Ugolini G.**, Cattaneo A., Novak M.
"The anti-tau monoclonal antibody MN7.51 shows a specific western blot pattern for Alzheimer's disease" - Society for Neuroscience 28th Annual Meeting, Los Angeles, CA - USA (671.4, 1714, 1998).

Main abbreviations used in the text

Aβ: amyloid-β-protein

ABC: avidin biotin complex

AcDEVD-CHO: (N-acetyl-aspartyl-glutamyl-valinyl)-3-amino-4-oxobutanoic acid

AD: Alzheimer's Disease

AP: alkaline phosphatase

ApoE: apolipoprotein E

APP: β-amyloid precursor protein

AS: antiserum

BCIP: 5-bromo-4chloro-3-indolyl-phosphate

BSA: bovine serum albumin

CBD: cortico-basal degeneration

Cdk: Cyclin-dependent kinase

CNS: central nervous system

CSF: cerebro-spinal fluid

DAB: di-amino-benzidine

DEAE-dextran: di-ethyl-amino-ethyl-dextran

DIG: digoxigenin

DMEM: Dulbecco's modified Eagle's medium

DTT: dithiothreitol

ECL: enhanced chemiluminescence

Fab: antigen-binding fragment

FAD: familial AD

FBS: fetal bovine serum

FCS: fetal calf serum

FTDP-17: fronto-temporal dementias and Parkinsonism linked to chromosome 17

GSK3: glycogen synthase kinase 3

HEPES: 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethane-sulfonic acid

IF: immunofluorescence

Ig: immunoglobulin

IHC: immunohistochemistry

ISEL: in situ end labeling (of DNA)

Kda: kilodalton

MAPK: mitogen-activated protein kinase

MARK: microtubule-affinity-regulating kinase

MP-R46: 46 kDa mannose 6-phosphate receptor

NBT: 4-nitro blue tetrazolium

NDC: non-demented control

NMR: nuclear magnetic resonance

NT2: Ntera2 (human teratocarcinoma cells)

O.D.: optical density

O/N: over night

PARP: poly-(ADP-ribose)-polymerase

PBS: phosphate buffer saline

PBST: PBS containing triton or tween

PCR: polymerase chain reaction

PHF: paired helical filament

PKA: protein kinase A

PMD: post-mortem delay

PP: phosphatase

PRE: pre-immune serum

PS: presenilin

PSP: progressive supranuclear palsy

R: repeat domain

R.T: room temperature

ScFv: single chain fragment variable

SDS: sodium dodecyl sulfate

SDS-PAGE: SDS- polyacrylamide gel electrophoresis

SF: straight filament

S/T-P: serine/threonine-proline motif

TdT: terminal deoxynucleotidyl transferase

TFE: tri-fluoro-ethanol

TMB: 3,3',5,5'-tetra-methyl-benzidene (tetra-methyl-benzidine)

TRITC: tetra-methyl-rhodamine iso-thyo-cianate (isomer R)

Abstract

Alzheimer's disease (AD) is characterized by two main histopathological hallmarks: extracellular plaques made of β-amyloid protein (Selkoe, 1999) and intraneuronal neurofibrillary lesions (Goedert et al., 1996a). Neurofibrillary lesions are accumulations of non-membrane bound bundles of paired helical filaments (PHFs), sometimes interspersed with straight filaments. The sub-unit protein constitutive of PHFs is the microtubule associated protein tau, modified in such way to form insoluble polymers (Delacourte and Buée, 1997). As it is also shown in a preliminary study introducing this work, AD-tau electrophoretic profile is characterized by a "smear" in the high molecular weight range, corresponding to pathological aggregates. In AD, tau undergoes several post-translational modifications (Mandelkow and Mandelkow, 1998), including hyperphosphorylation, oxidation, ubiquitination, glycation and abnormal proteolysis.

This thesis investigates the role of tau abnormal proteolysis in AD neuronal degeneration. The so-called "Truncation hypothesis" represents the conceptual basis of this work. Its original formulation is based on a pioneering work by M. Novak and collaborators. First, a pronase-resistant core of PHFs was shown to be made of a group of 12 Kda fragments of tau (Wischik et al., 1988a and b). These fragments were employed to raise the monoclonal antibody MN423 (Wischik et al., 1988a; Novak et al., 1989), which stains all the main neuropathological hallmarks of AD (Bondareff et al., 1991; Mena et al., 1991; Ugolini et al., 1997). Immunoelectronmicroscopy studies showed that MN423 decorates PHF isolated with or without pronase treatment (Novak et al., 1993). MN423 does not recognize full-length tau, but binds to the 12kDa fragments of core PHFs terminating at Glu-391 (Novak et al., 1993). On this basis, it was hypothesized that tau is endogenously

truncated in AD brain and Glu-391, which invariably represents the last amino acid of the 12 Kda fragments, was defined as a "truncation point" (Novak et al., 1993; Novak, 1994). MN423 immunoreactivity is also found intracellularly in AD neurons, even in the absence of neurofibrillary lesions, in the form of pre-tangle intracytoplasmic granular accumulations (Bondareff et al., 1991; Mena et al., 1991; Ugolini et al., 1997). Therefore our working hypothesis was that truncation of tau might precede its assembly into PHFs, thus representing an early intracellular event in the development of neurofibrillary pathology. In a follow-up study our group showed that, although expression of the 12 Kda fragments in a cellular context does not lead to intracellular aggregation, these truncated forms of tau fail to support microtubule assembly *in vitro* (Fasulo et al., 1996). Moreover, they are unable either to bind to microtubules or to induce their bundling *in vivo* (Fasulo et al., 1996). A truncated form of tau, terminating at Glu-391 and encompassing the two proline-rich regions, is able to induce apoptosis upon expression in COS cells, raising the possibility that tau fragments present in neuropathological conditions may affect neuron viability more profoundly than it was previously thought (Fasulo et al., 1998).

Extensive neuronal degeneration and loss is observed in many neuropathological conditions, including AD. Evidence has recently accumulated, showing that apoptotic cell death is involved in this process (Su et al., 1994; Cotman and Anderson, 1995; Lassmann et al., 1995; Anderson et al., 1996; Cotman and Su, 1996; Gervais et al., 1999). Here it is shown that, in AD cortex, truncated tau, as identified by MN423 immunohistochemistry, is co-localized with neuronal death, as revealed by DNA fragmentation (Ugolini et al., 1997).

The second part of this work was devoted to look at the question of tau truncation and cell death from the opposite angle: do truncated forms of tau have any effect on cell viability? A set of tau fragments was identified, displaying apoptotic capacity in different cellular contexts. Together with previous evidence, showing that tau is cleaved during neuronal apoptosis (Canu et al., 1998), these results suggest that tau can be, at the same time, a "substrate" and an "effector of apoptosis". Caspase-3 was identified as candidate protease responsible for the activation of this positive feed-back cycle, ultimately leading to tau-mediated cell death. The final part of this project was to attempt the derivation of an antibody specifically recognizing the cleavage site of caspase-3 on tau. This should allow a direct test of the "tau truncation" hypothesis, as modified through this work.

1. INTRODUCTION

1.1 Alzheimer's Disease: neuropathological characterization and stageing.

Alzheimer's Disease (AD) represents the most common cause of dementia in adult life (Selkoe, 1994; Price et al., 1998; Selkoe, 1999). It is associated with the selective damage of brain regions and neural circuits critical for cognition and memory. Given the complex array of factors that may start or propagate this neurodegenerative disorder, AD is more likely to be a multifactorial syndrome, rather than a disease with a single cause. Nevertheless, the final histopathological outcome of AD is the same in all cases and is identified by the two classical hallmarks of the disease: senile plaques and neurofibrillary lesions observed for the first time by Alois Alzheimer in 1906.

Senile plaques are spherical, extracellular deposits of amyloid-β-protein (Aβ) including abundant amyloid fibrils (7-10 nm) intermixed with non-fibrillary forms of the same peptide. The plaques may contain degenerating dendrites and axons within or intimately sorrounding the amyloid deposit (neuritic plaques). The plaques are often associated with activated microglia and reactive astrocytes. Aβ Deposits lacking a fibrillary organization are common in AD brain as well. These deposits are referred to as diffuse plaques. The Aβ peptides derive from the β-amyloid precursor protein (APPs). These are type-1 integral membrane glycoproteins that are subject to alternative proteolytic pathways (Selkoe, 1994 and 1999). Some APP molecules are cleaved endoproteolitically within the Aβ sequence, by a protease putatively located at level of plasma membrane and termed α-secretase. The ecto-domain of APP is released after such cleavage. Aβ peptides are generated by endoproteolytic cleavage of APP by activities termed β- and γ-secretases. About 90% of the secreted Aβ peptides are Aβ40, whereas 10% of them are Aβ42 and Aβ43, species that are highly

fibrillogenic, prone to aggregation and possibly neurotoxic (Loo et al., 1993; LaFerla et al., 1995; for a review see Yankner, 1996). The mechanism whereby Aβ peptides are harmful for neurons remains elusive. An intriguing hypothesis is that this neurotoxic action is mediated by non-neuronal cells like microglial cells (Yan et al., 1996; El Khoury et al., 1996; Giulian et al., 1996; Combs et al., 1999) that are closely associated with plaques (Probst et al., 1991). Astrocytes can play a significant role as well, since astrocytosis is associated with both AD hallmarks (Cairns et al., 1992) and astrocytes could play a central role in the inflammatory response to β-amyloid (Johnstone et al., 1999).

Neurofibrillary lesions (Goedert et al., 1996a) are intraneuronal accumulations of non-membrane-bound bundles of paired, helically wound filaments (PHFs), sometimes interspersed with straight filaments. PHF-based lesions are given different names according to the neuronal portion they occupy: neurofibrillary tangles fill up the cytoplasm of soma and proximal dendrites, neuropil threads occur in distal dendrites and dystrophic neurites indicate axons affected by the neurofibrillary pathology. The sub-unit protein constitutive of PHFs is the microtubule associated protein tau in a hyperphosphorylated state. The tau protein is modified in such a way to form insoluble aggregates, often conjugated with ubiquitin (Mori et al., 1987).

Amyloid deposits can be found in some cognitively normal individuals and their number and localization in the affected brain does not reflect the progression of AD (Arriagada et al., 1992). On the contrary, the extent and topographic distribution of neurofibrillary lesions provide a reliable pathologic correlate of the degree of dementia (Arriagada et al., 1992). The development of neurofibrillary pathology is not random but follows a stereotyped progression pattern with regard to affected cell types, cellular layers, and brain regions, with very little individual variation. This

pattern has been used to define six neuropathologic stages of AD on the basis of post-mortem brain examination (Braak and Braak, 1991). In stage I neurofibrillary lesions are developed by cells in the pre-alpha layer of the trans-entorhinal region. Stage II is characterized by a more sever involvement of this region and a mild involvement of the pre-alpha layer of the nearby entorhinal cortex. These first two stages are regarded ad clinically silent stages of AD, since patients with such pathology are cognitively unimpaired. Mild impairment of cognitive function appears in stage III and IV, when the neurofibrillary pathology becomes extensive in the trans-entorhinal and entorhinal regions and starts to affect hippocampus and a number of subcortical nuclei, including the basal forebrain magnocellular nucleus and the anterodorsal thalamic nucleus. During these intermediate stages the first ghost tangles appear (extra-cellular neurofibrillary lesions representing what is left of dead neurons). The major feature of stages V-VI is the massive diffusion of the neurofibrillary pathology in isocortical association areas. These final stages correspond to severe dementia, meeting the criteria for neuropathologic diagnosis of AD.

1.2. A possible connection between neurofibrillary pathology and Aβ plaques.

Tau has been shown to interact directly with a conformation-dependent domain of APP encompassing residues 714-723 (Smith et al., 1995). It is noteworthy that the putative tau-binding domain of APP includes residues 716 and 717, that are mutation sites associated with familial forms of AD.

Aβ fibrils can induce pathological phosphorylation (see paragraph 1.6) of tau in various systems, such as primary cultures of fetal rat hippocampal and human cortical neurons (Busciglio et al., 1995), a hybrid septal line (Le et al., 1997) and human neuroblastoma cell line (Shea et al., 1997).

A neurotoxic cascade can be imagined (Selkoe, 1999), in which progressive neuritic injury within amyloid plaques and elsewhere in the neuropil, causes disruption of neuronal metabolic homeostasis, that, in its turn, produces alteration of the kinase/phosphatase balance responsible for tau phosphorylation state (see paragraph 1.6).

Notwithstanding the above-mentioned studies, the exact relationship between neurofibrillary lesions and Aβ plaques remains an elusive question.

1.3. The genetics of Alzheimer's Disease.

It has been known for several decades that AD can occur in familial form that transmits as an autosomal dominant trait (FAD, familial AD). FAD accounts for most early onset cases but represents less than 10% of all cases, although it is likely that many of the late onset sporadic cases have some genetic predisposition determinant as well. In fact, growing evidence shows a familial susceptibility to AD onset.

At present, there are four well-confirmed genes in which mutations or polymorphisms can result in AD.

The first AD-causing gene to be identified was that encoding the precursor of Aβ, the β-amyloid precursor protein (APP) (Levy et al. 1990; Goate et al., 1991; Mullan et al., 1992). Missense mutations in APP are concentrated at level of (or near) α–, β–, γ–secretase cleavage sites. They affect the proteolytic processing of APP, favouring the production of Aβ (Levy et al., 1990; Citron et al., 1992; Suzuki et al., 1994). Yet, such mutations account for a tiny fraction of all AD cases (less than 0.1%). Moreover, although several transgenic mouse models of amyloidogenesis and Aβ-associated abnormalities have been created, nevertheless none of these models shows either a convincing AD-like phenotype (neurofibrillary tangles were never

observed) or unequivocal impairment in cognitive function (see Price et al., 1998, for a review).

Two other genes are responsible for most FAD cases (Sherrington et al., 1995; Rogaev et al., 1995; Van Broeckhoven, 1995; Price et al., 1998; Selkoe, 1999). They were termed presenilin1 (PS1) and presenilin2 (PS2), because missense mutations result in an aggressive, early-onset form of the disorder, usually beginning between the age of 40 and 60 years. PS1 and PS2 are highly homologous polytopic proteins with eight transmembrane domains. So far, they have been localized to Endoplasmic Reticulum (ER) and Golgi apparatus but their precise cellular activities are not yet known. Many mechanisms have been proposed to explain the action of mutant presenilins. It is noteworthy that FAD-linked PS1 and PS2 variants appear to influence APP processing at the γ–secretase site, leading to increase secretion of highly amyloidogenic Aβ42 and Aβ43 species (Scheuner et al., 1996). Moreover, mutant presenilins have been shown to enhance the susceptibility of various cell types to apoptosis (Wolozin et al., 1996; Keller et al., 1998).

ApoE is the first known susceptibility gene for the common late-onset form of AD (Roses, 1998). Human ApoE is a plasma and cerebrospinal fluid protein that serves as a ligand for low-density lipoprotein receptors and, through its interaction with these receptors, appears to be involved in the transport of cholesterol and other lipids among the nervous cells. ApoE is synthesized by astrocytes in brain and by macrophages in peripheral nerves during the repair response to tissue injury and regeneration. Moreover, intraneuronal synthesis has been recently shown (Xu et al., 1999).

There are three major alleles of ApoE on chromosome 19: E2, E3, E4. ApoE3 is the most prevalent allele. A disproportionate increase of the ApoE4 allele frequency in

patients with late-onset AD was first described in 1993 and subsequently confirmed by many studies (Strittmatter et al., 1993; Corder et al., 1998). The role ApoE4 plays in the etiology of AD remains an unresolved issue, although various mechanisms have been proposed such as the promotion of Aβ fibrillogenesis or the intracellular interaction with tau at an early stage of the neurofibrillary pathology (see Yankner, 1996, for a review).

The inheritance of a polymorphism in the gene encoding α2-macroglobulin, a large multifunctional protein that can act as a special kind of protease inhibitor, has been also associated with increased risk of late-onset AD (Blacker et al., 1998). Genetic epidemiological studies are under way to fully demonstrate the role of this genetic determinant in AD.

1.4. The role of the endosomal-lysosomal system in AD early neuropathology.

Recent evidence has accumulated showing that the up-regulation of the endosomal-lysosomal system is an early event in the development of AD neuropathology (Nixon and Cataldo, 1993; Cataldo et al., 1995; Nixon and Cataldo, 1995; Cataldo et al., 1996a; Cataldo et al., 1997; Troncoso et al., 1998). The neuronal endocytic pathway is a finely controlled and efficient trafficking system, designed for taking up and processing extracellular nutrients and trophic factors, for recycling and/or catabolizing receptors and other trans-membrane proteins, and for directing information to intracellular biosynthetic pathways. Thanks to endocytosis, cells are able to modify or degrade molecules inside a set of morphologically and biochemically distinct vacuolar compartments, such as early endosomes, late endosomes and lysosomes. Proteins closely linked to AD pathogenesis, such as altered APP and ApoE, are internalized and processed within the Endosomal-Lysosomal system. Lysosomal activity is

remarkably up-regulated at an early stage as part of an overall picture of metabolic "compromise" in AD neurons (Nixon and Cataldo, 1993; Cataldo et al., 1995, 1996a). The accumulation of various acid hydrolases within late endosomes and lysosomes, including proteases with potential APP secretase activities such as Cathepsin B and D (Tagawa et al., 1991; Nixon and Cataldo, 1993; Dreyer et al., 1994; Cataldo et al., 1994, 1995, 1996a) and their persistence extracellularly in association with Aβ deposits (Nixon and Cataldo, 1993; Cataldo et al., 1994, 1995, 1996b), has been observed in disorders in which Aβ is accumulated, suggesting a link between the endosomal-lysosomal system abnormalities and β–amyloidogenesis. The size of late endosomes and the extent of cathepsin trafficking to this compartment are increased in AD neurons. Moreover, in AD neocortex pyramidal neurons, trafficking pathways are likely to be activated that assist in the delivery of lysosomal hydrolases to early endosomes. For instance AD brain is characterized by increase in the levels of MP-R46 immunoreactivity in pyramidal neurons of lamina III (Cataldo et al., 1997). Since MP-R46 mediates the transport of endogenous lysosomal enzymes to the plasma membrane and early endosomes, the latter compartment is likely to be enriched in hydrolase contents as compared with the normal situation. The up-regulation of endocytosis in AD pyramidal neurons implies an abnormal stimulation of usual endocytic events that should include APP processing and normal Aβ generation. A further acceleration in APP processing is expected when trafficking of proteases to endosomes is abnormally increased as it is the case in AD. It is noteworthy that ApoE (when it is not produced by neurons) could be taken up by endocytosis and interact with APP and its cleaved derivatives inside early endosomes (Crutcher et al., 1996). In fact, the "bad" isoform of ApoE, by virtue of increased affinity for Aβ, seems to act as pathological chaperone in Aβ fibrillogenesis (Wisniewski et al., 1994).

1.5. Normal and pathological tau proteins.

Tau proteins are microtubule-associated proteins that regulate the dynamics of the microtubule network, especially involved in axonal transport and neurite outgrowth (Delacourte and Buée, 1997). They arise from alternative splicing of a single gene located on the long arm of chromosome 17 (Neve et al., 1986). It is located over 100kb at band position 17q21 (Neve et al., 1986) and contains 16 exons (Andreadis et al., 1992, 1995). Three of these exons (4a, 6, 8) are specific for peripheral tau (Goedert et al., 1992b) and are not present in Central Nervous System (CNS). In human CNS, tau proteins make up a family of six isoforms (Goedert et al., 1988,1989a and b, 1992a; Goedert and Jakes, 1990) ranging from 352 to 441 aminoacids with a molecular weight between 45 and 62 Kda (Fig. 1). The distinction between isoforms is due to the presence of two, one or no 29-aminoacid long insert (in the N-terminal portion of the molecule) and of three or four 18-aminoacid repeats (R1, R2, R3 and R4) separated by 13-14 aminoacid long less conserved interregions (in the C-terminal part). The expression of the various isoforms is developmentally regulated (Kosik et al., 1989): in adult brain all six are present whereas, in fetal brain, only the shortest isoform has been identified. The N-terminal part of tau is highly acidic and it is likely to lean out of the microtubule as a projection domain capable of interaction with other cytoskeletal proteins. Two proline-rich regions, rich in potential phosphorylation sites (Fig. 2), lie in the middle of the molecule. The highly basic C-terminus of tau is responsible for binding to microtubules and is sufficient to promote microtubule assembly *in vitro*. In particular, repeats are the main sequences involved in interaction with microtubule (Butner and Kirschner, 1991; Gustke et al., 1994) although the interregion between R1 and R2 is the most powerful sequence in promoting microtubule polymerization (Goode and Feinstein, 1994). Since the second

repeat is the one that is spliced out in 3-repeats isoforms, the R1-R2 interregion is unique to 4-repeats variants. These, in fact, are more efficient in inducing microtubule assembly (Goedert and Jakes, 1990) and show a higher binding affinity for microtubules (Goode and Feinstein, 1994; Panda et al., 1995). Yet, the repeat motifs bind to microtubule very weakly as compared to full-length tau. Our group has identified a new potential microtubule-binding domain (R') in the region immediately downstream of the last repeat (Novak, 1994; Novak et al., 1999). Finally, it has been shown that this R' domain and proline rich regions are necessary for the repeat motifs to interact productively with microtubules (Preuss et al., 1997). According to this model, R' and proline rich regions are considered as targetting domains, responsible for positioning tau on the microtubule surface, while the repeats act as catalytic domains for microtubule assembly.

Tau is a phosphoprotein and was regarded for long as an axonal protein, due to the axonal pattern of labeling obtained with the monoclonal antibody Tau-1 (Binder et al., 1985; Papasozomenos and Binder, 1987).

This antibody recognizes the dephosphorylated state of a portion of the molecule that is subject to multiple phosphorylation (Szendrei et al., 1993). However, after dephosphorylation of the tissue with alkaline phosphatase, tau proteins are abundantly detected by the Tau-1 antibody in the somatodendritic compartment as well, showing that the phosphorylation state of these molecules is different according to the cell compartments (Riederer and Binder, 1994). Phosphorylation of tau proteins is developmentally regulated. It is high in fetal brain (Brion et al., 1993; Kennessey and Yen, 1993) and decreases with age. Hyperphosphorylation in pathological conditions will be discussed in the next paragraph.

Tau proteins represent the main component of the neurofibrillary lesions characterizing a number of neurodegenerative disorders, collectively known as tauopathies (Spillantini and Goedert; 1998, Goedert et al., 1998). This family of diseases include AD, Corticobasal Degeneration (CBD), Down Syndrome, Fronto-temporal dementias and Parkinsonism linked to chromosome 17 (FTDP-17), Pick's Disease, Prion Diseases with tangles and Progressive Supranuclear Palsy (PSP). In brains affected by such disorders, abundant tau-positive filamentous regions are present. In AD neurofibrillary lesions are limited to neurons, but in most tauopathies glial cells show them as well. For frontotemporal dementias occurring in familial forms (FTDP-17), tau mutations have been shown to be a major cause of the inherited dementing disease (Spillantini et al., 1998; Hutton et al., 1998, Poorkaj et al., 1998, Goedert et al., 1998). Several exonic and intronic mutations in the tau gene have been identified in over a dozen families with FTDP-17. The known exonic mutations are missense mutations located in the microtubule binding repeats or close to them (Fig. 2), while the intronic mutations are located close to the splice-donor site of the intron following exon 10, that codes for the R1-R2 interregion and the R2 repeat. Recombinant tau proteins bearing these mutations show a reduced ability to promote microtubule assembly in vitro (Hasegawa et al., 1998; Hong et al., 1998) and to induce microtubule extension in transfected cells (Dayanandan et al., 1999). The likely primary effect of the exonic mutations is than a reduced ability of tau to interact with microtubules. Alternatively, the primary consequence may be a gain of toxic function with excess of free tau available for assembly into filaments. The net effect of the intronic mutations is increased splicing-in of exon 10 (Grover et al., 1999), resulting in an over-production of 4-repeat isoforms. Since 3- and 4-repeat tau isoforms are believed to bind different sites on microtubules (Goode and Feinstein,

1994), overproduction of the latter variants should result in an excess of tau over available binding sites on microtubules, with unbound excess tau available for self-interaction and aggregation into filamentous structures.

It is noteworthy that in AD such tau mutations as those observed in FTDP-17 have never been described, so that the relationship between hereditary and sporadic neurofibrillary pathology remains elusive.

What is the main common feature of pathological tau proteins in all sporadic and familial tauopathies? Apart from hyperphosphorylation (discussed in the next paragraph), the most obvious molecular change is the aggregation of tau proteins into insoluble polymers (Delacourte and Buée, 1997), with various filament morphologies. For instance, AD, Down syndrome, prion diseases with tangles and some forms of FTDP-17 (Seattle family A: V337M mutation) are characterized by prevalence of classical PHFs (diameter 8-20 nm and periodicity of 80 nm, see also paragraph 1.1) and a minority of straight filaments (SFs). Straight filaments of slightly different morphologies are found in Pick's disease and PSP. Ribbon-like filaments, with an irregular periodicity of 90-130 nm, are typical of FTDP-17 variants affecting 4-repeat isoforms (Multiple system tauopathy with presenile dementia, caused by the above-mentioned intronic mutation, and Dutch family 1, characterized by the P301L mutation in the R2 repeat) and CBD. The different filament morphologies displayed in different diseases correspond to heterogeneous biochemical characteristics of tau proteins and to different expression profiles of tau various isoforms (see Spillantini and Goedert, 1998 for a review).

In AD all six isoforms are present (Jakes et al., 1991) and give rise to a typical western blot profile with three major bands (and a minor band at a slightly higher molecular weight as compared with the "classical triplet"). A similar pattern can be

obtained with brain extracts of individuals affected by Down syndrome, prion disease with tangles and some form of FTDP-17 (Seattle family A).

Pick's Disease, instead, is characterized by a western blot profile with two main bands corresponding to the two lowest AD bands. Tau filaments in Pick's disease are made only of 3-repeat isoforms. CBD, PSP and FTDP-17 variants affecting 4-repeat isoforms display a western-blot pattern with 3 tau bands, lacking the AD band with the lowest molecular weight. In all these pathological conditions, filamentous aggregations have been shown to be mainly or exclusively made up of 4-repeat tau molecules.

1.6. AD PHF-tau: post-translational modifications. Is hyperphosphorylation a key-event?

A remarkable feature of "pathological" tau proteins is their hyperphosphorylation (Grundke-Iqbal et al., 1986), although tau molecules may display different phosphorylation states in the various sub-cellular compartments in normal brain as well (Riederer and Binder, 1994).

The tau protein in PHFs from the brains of AD patients is phosphorylated at more than 20 residues, many (but not all) of which are S/T-P sites (Morishima-Kawashima et al., 1995; Fig. 2). In healthy brains, tau is phosphorylated at between eight and ten of these residues (Watanabe et al., 1993).

Phosphorylation of tau proteins is developmentally regulated. It is high in fetal brain and decreases with age because of phosphatase activation (Mawal-Dewan et al., 1994; Rosner et al., 1995).

It is generally assumed that the phosphorylation state of tau is due to balance between kinase and phosphatase activities. Hyperphosphorylation could therefore be due to

either excessive phosphorylation or to reduced phosphatase activity (as suggested by Gong et al., 1993), even if both processes may be active at the same time. Hyperphosphorylation of tau has been long believed to be an early event that precedes assembly into PHFs. Yet there is no experimental evidence linking hyperphosphorylation of tau to PHF assembly (Goedert et al., 1998). In fact, synthetic PHF-like structures can be obtained *in vitro* in a phosphorylation-independent way (Goedert et al., 1996b; Perez et al., 1996; Hasegawa et al., 1997). Three-repeat isoforms tend to assemble into paired helical-like filaments, whereas four-repeat isoforms tend to assemble into straight filaments (Goedert et al., 1996b).

In a recent study the relationship between tau phosphorylation by a number of kinases (MARK, PKA, MAPK, GSK3) and tau assembly into PHFs has been investigated (Schneider et al., 1999). The proline-directed kinases MAPK and GSK3 are known to phosphorylate most S-/T-P motifs in the regions flanking the repeat motifs of tau. Schneider et al. show that this type of phosphorylation has a very weak effect on tau-microtubule interaction and PHF assembly. By contrast, MARK and PKA phosphorylate several sites within the repeats (notably the KXGS motifs including Ser262, Ser324 and Ser356). PKA phosphorylates some additional sites in the flanking regions, like Ser214, as well. According to Schneider et al., this second type of phosphorylation strongly reduces the affinity of tau for microtubules, but, at the same time, inhibits tau assembly into PHFs. Thus, phosphorylation does not prime tau for PHF assembly; on the contrary it seems to protect the protein against aggregation, suggesting that hyperphosphorylation of tau is unlikely to be directly responsible for the process of pathological aggregation.

Hyperphosphorylation is only one of a growing list of post-translational modifications undergone by tau proteins in AD (for a review see Mandelkow and Mandelkow,

1998), including ubiquitination (Mori et al., 1987), glycation (Ledesma et al., 1994), oxidation (Schweers et al., 1995) and proteolysis (discussed in the next paragraph). Ubiquitination is likely to represent a late event following PHF formation (Morishima-Kawashima et al., 1993). On the contrary, glycation of lysines in the microtubule-binding domain could result in a decreased interaction with microtubules (Ledesma et al., 1995) and oxidation of Cys322 is fundamental for the formation of di-sulfide intermolecular bridges preceding PHF assembly *in vitro* (Schweers et al., 1995).

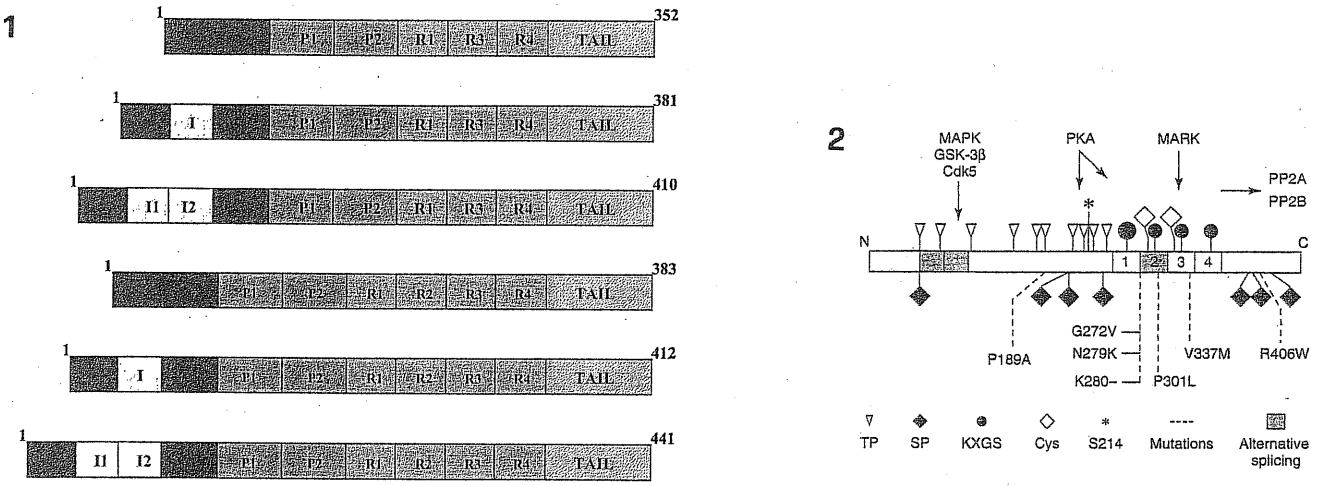


Fig. 1. *Scheme of the six tau isoforms found in human Central Nervous System. I1, I2: N-terminal Inserts; P1, P2: proline-rich domains; R1-R4: microtubule binding repeats.*

Fig. 2. *Diagram of the longest isoform, its domains, sites of phosphorylation and FTDP-17 mutations that affect tau at protein level (from: Mandelkow and Mandelkow, 1998). Phosphorylation motifs are indicated: TP (thr-Pro motif), SP (Ser-Pro motif), KXGS (non-proline motif). Abbreviations: Cdk, Cyclin-dependent kinase; Cys, Cysteine; GSK-3, Glycogen synthase kinase 3; MAPK, mitogen-activated protein kinase; MARK, microtubule-affinity-regulating kinase; PKA, protein kinase A; PP, phosphatase.*

1.7. AD PHF-tau: post-translational modifications. The role of proteolysis.

Evidence has accumulated showing that AD PHF-tau is abnormally proteolyzed or fragmented (Nieto et al., 1990; Novak et al., 1991; Goedert et al., 1992a; Novak et al., 1993; Novak, 1994; Johnson et al., 1997). It has been initially proposed that the fragments could represent the minimal unit for aggregation of PHFs (Novak, 1994; Berdnarsky and Lynch, 1996; Kenessey et al., 1997). In a recent study (Canu et al., 1998) our group has shown that tau is cleaved in cultured cerebellar granule cells when they are induced to undergo apoptosis and that calpains and caspase-3 represent candidate proteases responsible for the cleavage. In fact, tau molecule contains 3 putative caspase-3 cleavage sites and at least one is effectively cleaved by the protease *in vitro*, as it is shown in this thesis (Fasulo et al., submitted). As far as calpains are concerned, the possibility that they may be involved in the development of the neurofibrillary pathology is consistent with their roles in regulating the normal structure, dynamics, and steady-state levels of major cytoskeletal proteins, including tau (Mercken et al., 1995; Yang and Ksiezak-Reding, 1995). Phosphorylation of adult tau (but not of fetal tau) can partially protect the molecule from calpain digestion (Litersky et al. 1993; Litersky and Johnson, 1995; Mercken et al., 1995). The PHF organization strongly inhibits calpain processing of tau (Mercken et al., 1995; Yang and Ksiezak-Reding, 1995) but this does not demonstrate that calpains do not take part in a very early stage of the neurofibrillary pathology development. In fact, when tau is released from the PHFs with formic acid or guanidin, it becomes again susceptible to calpain-mediated proteolysis, regardless of tau phosphorylation state (Yang et al., 1997). On the other hand, activation of Calpain I is widespread in AD brain (Saito et al., 1993) and Calpain II is an early-appearing and pervasive component of AD neurofibrillary pathology (Grynspan et al., 1997).

Another metabolic pathway leading to proteolysis of tau is represented by the endosomal-lysosomal system, which is up-regulated in AD and could be viewed as a sort of crossroads at which both APP and tau processing converge (Nixon and Cataldo, 1995; see paragraph 1.3). For instance, Cathepsin D gene expression and cellular contents are increased in AD brain (Cataldo et al., 1995). Tau is cleaved by Cathepsin D *in vitro* (Bednarski and Lynch, 1995). In hippocampal slices, the cleavage seems to occur in an extralysosomal compartment, when Cathepsin D levels are up-regulated (Bednarski and Lynch, 1995). Cathepsin D cleavage of tau is likely to generate fragments containing the microtubule binding domain, since the most susceptible cleavage sites are located near the amino and carboxy termini of the protein (Kenessey et al., 1997). If cleavage of tau by Cathepsin D occurs *in vivo*, this implies a mis-localization at sub-cellular level of either tau or Cathepsin D, since intracellular membranes should normally separate the two proteins.

1.8. The "Truncation hypothesis".

The so-called "Truncation hypothesis" is the starting point of the projects developed by our group in the tau field. Its original formulation is based on the pioneering work carried out by M. Novak and co-workers between 1987 and 1993. First, a pronase-resistant core of PHFs was prepared and shown to be made of a group of 12 Kda fragments of tau (Wischik et al., 1988a; Wischik et al., 1988b; Jakes et al., 1991). These fragments were employed to raise the monoclonal antibody MN423 (Wischik et al., 1988a; Novak et al., 1989), which stains all the main neuropathological hallmarks of AD, including intracellular granular and neurofibrillary structures (Bondareff et al., 1991; Mena et al., 1991; this thesis). Immunoelectronmicroscopy studies showed that MN423 decorates PHF isolated with or without pronase treatment

(Novak et al., 1993). MN423 does not recognize full-length tau, but binds to the 12 Kda fragments of core PHFs terminating at Glu-391 (Novak et al., 1993). On this basis it was hypothesized that tau is endogenously truncated in AD brain and Glu-391, which invariably represents the last amino acid of the 12 Kda fragments was defined as a "truncation point" (Novak et al., 1993; Novak, 1994). MN423 immunoreactivity is also found intracellularly in AD neurones, even in the absence of Neurofibrillary tangles, in the form of pre-tangle intracytoplasmic granular accumulations (Bondareff et al., 1991; Mena et al., 1991). Therefore our working hypothesis was that truncation of tau might precede its assembly into PHFs, thus representing an early intracellular event in the development of neurofibrillary pathology. In a follow-up study our group showed that, although expression of the 12 Kda fragments in a cellular context does not lead to intracellular aggregation, these truncated forms of tau fail to support microtubule assembly *in vitro* (Fasulo et al., 1996). Moreover, they are unable either to bind to microtubules or to induce their bundling *in vivo* (Fasulo et al., 1996). A truncated form of tau, terminating at Glu-391 and encompassing the two proline-rich regions is able to induce apoptosis upon expression in COS cells, raising the possibility that tau fragments present in neuropathological conditions may affect neuron viability more profoundly than it was previously thought (Fasulo et al., 1998).

1.9. Neuronal death and neurodegeneration

Apoptosis or regulated cell death is critical for many normal physiological events and deregulation of its components can also lead to the undesired effect of uncontrolled growth of cancerous tissue (Willye et al., 1980; Bursch et al., 1992). From a morphological point of view, apoptosis is characterized by condensation of nucleus and membranes, membrane blebbing and formation of the so-called "apoptotic

bodies" (Willye et al., 1980; Bursch et al., 1992). The apoptotic process is rather fast and dead cells are usually removed within hours to days by efficient scavenger systems (often represented by specialized phagocytic cells). Apoptosis is accompanied by the activation of the proteases belonging to the caspase family (Salvesen and Dixit, 1997), which results in cleavage of a number of different substrates, including the DNA-repair enzyme PARP (Poly-(ADP-ribose)-polymerase) (Tewari et al., 1995) . Apoptosis is also identified by the degradation of nuclear DNA producing a series of oligosome-length DNA fragments that have newly generated 3'-OH ends. Conversely, normal cells have very low numbers of terminal DNA strand breaks. DNA fragmentation labeling techniques use enzymes such as terminal deoxynucleotidyl transferase (TdT) or Klenow polymerase to catalyze the addition of deoxynucleotide triphosphates to the 3'-OH termini of single or double-stranded DNA, which can be visualized immunohistochemically (Gavrieli et al., 1992). DNA fragmentation may accompany non-apoptotic cell death (Charriaut-Marlangue et al., 1995) and key morphological features of apoptotis may occur in the absence of internucleosomal DNA fragmentation (Cohen et al., 1992). Yet, DNA fragmentation is generally assumed as a hallmark of apoptosis, as long as some other morphological and/or biochemical criteria of recognition are fulfilled.

In the developing nervous system, many more neurons are generated than are retained in adult life. Neuronal attrition occurs by apoptotic cell suicide and a prominent hypothesis is that cell death is at least partly a consequence of competition for target-derived growth factors (Pettmann and Henderson, 1998; Oppenheim, 1989). This system would provide a means for matching neuronal number to target size.

Evidence is accumulating that apoptotic cell death is involved in the neuronal loss observed in many neuropathological conditions, including Alzheimer's Disease (Su et

al., 1994; Cotman and Anderson, 1995; Lassmann et al., 1995; Anderson et al., 1996; Cotman and Su, 1996; Gervais et al., 1999). Although in many of these works apoptosis was merely assessed on the basis of DNA fragmentation, nevertheless the morphology of the cells was consistent with apoptosis when it was observed (Su et al., 1994; Anderson et al., 1996). Moreover, caspase-3, the main "executioner" protease involved in apoptosis, is certainly up-regulated in AD brain (Gervais et al., 1999). Since Aβ induces apoptosis in cultured neurons (Loo et al., 1993), the concept of AD neurons dying by apoptosis has become widely accepted (Cotman and Su, 1996), without being fully demonstrated.

An obvious theoretical difficulty is represented by the slow progression of a chronic disease, like AD, which is hardly compatible with the rapidity of the apoptotic process, even if the brain environment is peculiar from the point of view of scavenger systems, due to the presence of blood-brain barrier. An intriguing possibility is that "AD-suffering" neurons have a pro-apoptotic phenotype (they would be "on the edge of death") and are more likely to die than "normal" neurons (for instance, caspase activation state may be close to the threshold beyond which apoptosis becomes an irreversible process). Within this scenario, the broad extent of DNA fragmentation in AD brain could be accounted for by the balance (at cellular level) between apoptotic stimuli and triggering of protective mechanisms (Cotman and Su, 1996), such as up-regulation of the anti-apoptotic Bcl-2 gene product (Su et al., 1996; for a review on Bcl-2 molecular family, see Reed, 1998). Alternatively, DNA fragmentation could be partially due to a very fast post-mortem phenomenon, which would be anyway far more likely for AD neurons than for "normal" ones. The latter explanation would be still consistent with the finding that Post-mortem delay does not appear to influence the number of apoptotic nuclei in AD brain (Anderson et al., 1996).

1.10. The "cell-cycle" hypothesis.

The "cell-cycle" hypothesis is somewhat complementary to the concept of AD apoptosis (for a review see Nagy et al., 1998). This hypothesis is based on a series of studies showing association of cell cycle-related proteins (like Ki-67) with neurofibrillary tangles (Smith et al., 1995; Kondratick and Vandré, 1996) and expression of cell division markers, such as Cyclin B and Cyclin E, in AD neurons (Nagy et al., 1997a; Nagy et al., 1997b).

Post-mitotic neurons are usually regarded as terminally differentiated cells incapable of undergoing further cell divisions. According to the "cell-cycle" hypothesis (and contrarily to previous belief) neurons in the adult human brain are able to re-enter the cell division cycle, but the progression of the cycle itself is normally arrested at an early stage when neurons are able to re-differentiate. However, in Alzheimer's Disease, the cell cycle would be allowed to progress into the G2 phase. At this stage, re-differentiation would be no longer permitted and neurons would enter a conflict, suffering of one of two fates: either they will die via an apoptotic pathway or they will produce AD neurofibrillary pathology (Nagy et al., 1998). This idea is supported by the observation that the Bax protein, an apoptosis inducer belonging to the same molecular family as bcl-2 (Silke and Vaux, 1998), is present in AD-affected areas of the brain but not in tangle-bearing neurons (Nagy and Esiri, 1998).

2. AIM OF THE WORK

The purpose of this thesis was to investigate the role of abnormal tau proteolysis in AD cell pathology. In particular, the following points were addressed:

i) The use of anti-tau antibodies as "structural" probes to define the nature of tau-dependent AD cellular pathology.

ii) The topographical relationship between tau abnormal proteolysis ("Truncation") in AD neurons, as assessed by immunohistochemistry, and neuronal death, as assessed by DNA fragmentation.

iii) The general impact of tau fragments on cell viability.

The theoretical context in which the experiments were thought and designed was that of the so-called "Truncation Hypothesis", as described in the previous chapter.
The original hypothesis was up-dated and modified through this work to give rise to a more "general" view of the molecular mechanisms underlying neuronal degeneration.

3. METHODS

3.1. Western blot analysis of tissue extracts.

Samples of parietal cortex from three patients with AD and three NDC age-matched controls (Tab. 4B, chapter 4) were employed for western blot experiments. Brain tissue was supplied by the Netherlands Brain Bank.

Briefly, each brain bit (1gr) was homogenized in 10 ml of sample buffer (2% SDS, 100 mm dithiotreitol, 60mM Tris, pH 6.8, 0.01% bromophenol blue), either in presence or in absence of commonly used protease inhibitors. The homogenate was spun at 3000 rpm for 10 min, at 4°C.

The supernatant was recovered and repeatedly passed through a 20-gauge needle and then through a 26-gauge needle before being centrifuged at 10,000g for 10 min at 4°C. Final supernatant was collected and its protein contents were determined.

Samples were run on Tris/glycine discontinuous SDS polyacrylamide gel after being placed in a boiling water bath for 5 min. Proteins were transferred from the gel to nitrocellulose using a semi-dry method. The blot was processed for antigen detection after blocking non-specific binding sites (blocking solution: 1xPBS containing 5% milk). MN7.51 (Tab. 4A, chapter 4), as well as other well established monoclonal antibodies in the AD field (Tau-1, PHF-1, see Tab. 4A in chapter 4), were employed at the following working dilutions: 1:20 – 1:30 for MN7.51 supernatant, 1:200 for Tau-1 (Boehringer) and 1:50 for PHF-1 (gently supplied by P. Davies). The anti-tau421 polyclonal antibody, generated according to the procedure described in paragraph 3.8, was used at 1:100

dilution. Immunoblot filter was finally reacted with 0.05% diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2.

3.2. Immunohistochemistry (IHC) and In Situ End Labeling of DNA (ISEL).

Samples of frontal and parietal cortex from 5 Alzheimer's disease (AD) cases and 3 non-demented control (NDC) cases were examined (Tab. 4C, chapter 4). Brain tissue was provided by the Netherlands Brain Bank. The age, sex, post-mortem delay (PMD) and cerebrospinal fluid (CSF) pH for each case are shown in Table 4C (chapter 4). Samples from AD and NDC brains were processed in parallel for all the staining procedures. Each block of tissue was extracted and quickly frozen in dry ice, then it was cut into smaller pieces and these were subsequently fixed in three kinds of buffer (A, B, C). Buffer A corresponds to 10% formalin in 1x Phosphate Buffer Saline (PBS), pH 7.3. It was mainly used for ISEL experiments, while buffer B (4% paraformaldehyde in 1xPBS, pH 7.3) and Buffer C (a mix of 4% paraformaldehyde and 10% formalin in 1xPBS, pH 7.3) were preferred for IHC and double labeling procedures. 25μm-thick sections from AD and NDC brains were cut on freezing microtome and collected as free-floating in 1xPBS. As washing solutions for IHC 1xPBS and/or 1xPBS containing 0.2 % Triton (PBST) were used at room temperature (R.T). Antibodies were diluted in buffered blocking solution (PBST containing 10% normal goat serum). Endogenous peroxidase was quenched by treatment with 0.5% H_2O_2 in 1xPBS for 15 minutes at R.T. After this treatment, sections were washed several times in 1xPBS. Pre-incubation in blocking solution (1 hr at R.T) was followed by several washes and incubation in primary antibody (O/N at 4° C). MN423 supernatant was used at 1:10-1:20 concentration. Several washes with 1xPBST

removed unbound antibodies. Incubation (2 hr at R.T) in secondary antibody (biotinylated anti-mouse IgG, Vector, 1:100) was followed by several washes in 1xPBST. Sections were subsequently incubated for 1hr (R.T) with the Avidin-Biotin complex (ABC kit, Vector), washed several times in 1xPBS and reacted for 2-10 min (R.T) with 0.05% DAB (Sigma chemical Co.) and 0.01% H_2O_2 in PBS. Replacement of either primary or secondary antibody with buffered blocking solution in this procedure produced no staining.

DNA fragmentation in nuclei was revealed by the ISEL technique: ApopTag peroxidase kit (Oncor) was used to detect digoxigenin (DIG) nucleotide residues added by terminal deoxynucleotodyl transferase (TdT) to the 3'-OH termini of DNA strand breaks. Staining was performed according to Oncor's ApopTag protocol. Briefly, tissue sections were incubated in TdT enzyme diluted in Reaction buffer; after blocking the TdT reaction, sections were incubated in Anti-DIG Antibody (conjugated with peroxidase) and reacted with DAB in order to visualize the DIG-dUTP-peroxidase complex.

As far as double labeling experiments are concerned, an anti-DIG antibody conjugated with alkaline phosphatase (AP) was used (AP-conjugated FAb Fragments, Boehringer; 1:500-1:1000; O/N at 4°C). The latter antibody was diluted in Tris buffer A (Tris 100mm pH 7.4; NaCl 150mm) containing Triton 0.2% and 10% fetal calf serum. After several washes (R.T) in Tris buffer A, Tris buffer B (Tris 100mm pH 8.0; NaCl 150mm) and Tris buffer C (Tris 100mm pH 9.5; NaCl 100mm), sections were incubated with 4-nitro blue tetrazolium (NBT) and 5-bromo-4chloro-3-indolyl-phosphate (BCIP) in Tris buffer C, containing 50mm $MgCl_2$ and 1mM Levamisol, for at least 15 min at R.T; TdT enzyme was replaced by distilled water in all negative controls. Pre-incubation of sections with

DNAaseI (1unit / 100 µl in DNAase I buffer: 50mM Tris pH 7.5; 50mg/ml BSA; 10mM $MnCl_2$ at 37°C for 10 min) was employed to obtain positive controls. Peroxidase reaction always followed the alkaline phosphatase reaction in all double labeling experiments. DNA fragmentation and MN423 immunoreactivity and their co-localization were quantitatively evaluated by using a 10x10 reticule and a 10x objective. The total number of MN423-positive cells and/or ISEL-positive nuclei in the gray matter was counted in at least 10 fields of frontal and parietal cortex for each case. For each series of sections 1000 (positive) cells were counted. Counts from double labeling experiments were expressed as percentages of ISEL-positive nuclei with no MN423 immunoreactivity in the cytoplasm and as percentages of MN423-positive neurons exhibiting ISEL-positive nuclei. Standard deviation was included in all cases.

3.3. Expression plasmids.

The expression vector pSG5 used for all the constructs, as well as the plasmids dGAEpSG5, tau40pSG5 and tau151-391pSG5, directing the expression of the corresponding fragments of tau, were described by Fasulo et al. (1996). The numbering of tau fragments is according to the longest human tau isoform tau40. Plasmids encoding tau fragments were generated by PCR using tau40pSG5 as a template. PCR primers contained EcoRI (5') and BamHI (3') sites for insertion into EcoRI-BamHI-cut pSG5.

The 5'primer for tau151-402, tau151-412, tau151-422, tau151-432, tau151-441, tau151-274, tau151-336 and tau151-368 was:

5'GCCCGAATTCATGATCGCCACACCGCGGGGAGCA3'.

The corresponding 3' primers were:

5'TTACAGGATCCTCAGTCCCCAGACACCACTGGCGACTTGT3',

5'TTACAGGATCCTCAGGAGACATTGCTGAGATGCCG3',

5'TTACAGGATCCTCACGAGTCTACCATGTCGATGCT3',

5'TTACAGGATCCTCACACCTCGTCAGCTAGCGTGGC3'

5'TTACAGGATCCTCACAAACCCTGCTTGGCCAGGGA3'

5'TTACAGGATCCTCACTTCCCGCCTCCCGGCTGGTG3'

5'TTACAGGATCCTCACTGGCCACCTCCTGGTTTATG3'

and 5'TTACAGGATCCTCAATTTCCTCCGCCAGGGACGTG3', respectively.

Constructs encoding for tau-head, P2dGAE and Nt-dGAE, were made using the following 5' primers: 5'GCCCGAATTCATGGCTGAGCCCCGCCAGGAG3', for tau-head and Nt-dGAE, 5'GCCCGAATTCATGAGCAGCCCCGGCTCCCCAGGC3' for P2dGAE.

The 3' primers were:

5'TTACAGGATCCTCAATTATCCTTTGAGCCACACTT3' for tau-head, 5'TTACAGGATCCTCACTCCGCCCCGTGGTCTGTCTT3' for P2 dGAE and Nt-dGAE.

Constructs encoding tau fragments tau1-422, tau22-441 and tau22-422 were made using the following 5' primers:

5'GCCCGAATTCATGGCTGAGCCCCGCCAGGAG3' (tau1-422),

5'GCCCGAATTCATGGACAGGAAAGATCAGGGGGGC3' (tau22-441 and tau22-422).

The 3' primers were:

5'TTACAGGATCCTCACGAGTCTACCATGTCGATGCT3' for tau1-422 and tau22-422, 5'TTACAGGATCCTCACAAACCCTGCTTGGCCAGGGA3' for tau22-441.

PCR was carried out with Vent DNA polymerase in a PTC100 thermal cycler (MJ Research), for 30 cycles as follows: 94°C 1 min; 60 or 65°C, 1 min; 72°C 1 min for 30 cycles, followed by 5 min at 72°C. Aliquots of each completed PCR reaction was checked on 1% agarose gels. PCR products were purified with the help of Qiaquick PCR Purification Kit (Qiagen) in accordance with the manufacturer's instructions. After digestion with EcoRI and BamHI, both inserts produced by PCR and pSG5 vector were gel-purified according to a Qiaquick Gel Extraction Kit protocol (Qiagen). Ligation reactions, transformation of competent bacteria (DH5α strain) with the ligation products, as well as Mini- and Midi- preparations of plasmid DNA were carried out by routine procedures (Sambrook et al., 1989).

All amplified inserts were sequenced by the deoxynucleotide procedure of Sanger et al. (1977).

Fragments tau151-391 and dGAE were also cloned into the scFvExpress-cyto eukaryotic expression vector (Persic et al., 1997). DNA fragments were amplified by PCR using the pSG5tau40 template. PCR primers contained Nco I (5' primers) and Sal I (3' primers) restriction sites that enabled insertion into ScFv express polylinker in frame with the myc tag (Evan et al., 1985). The 5' primers used were:

5'GGTTTGACTCATATGGCCATGGCTATCGCCACACCGCGGGGAGCA3' (for tau151-391) and GCTTTGACTCATATGGCCATGGCTATCAAACACGTCCCGGGA (for dGAE).

The 3' primer used was:

5'GAATTCACGGATCCCGTCGACCTCCGCCCCGTGGTCTGTCTTGGC3' (for both tau151-391 and dGAE).

PCR was carried out using conditions described above.

The PCR products were digested with Nco I and Sal I, gel- purified and sub-cloned into the NcoI/SalI-cut ScFv-exp-(cyto) vector (Persic et al., 1997), following the same protocol as described above. For transfection in COS cells psG5 constructs were employed, while the ScFv-exp constructs were used for transfections in NT2 cells and neurons.

3.4. Cell culture and transfection.

COS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, in 5%CO_2. The day before transfection 5.10^4 or 4.10^5 cells were plated respectively onto either 35mm dishes (containing one coverslip each), for Immunofluorescence (IF), or 90mm dishes, for cell extraction and western blot analysis. Cells were transiently transfected using DEAE-Dextran method (Fasulo et al. 1996) and analyzed 48 hours later by IF or 72 hours later by immunoblot. Transfection efficiency was around 20%.

NT2 (Ntera2/clD1) cells were maintained in DMEM high glucose, 5% FBS, 10% horse serum (Pleasure et al., 1992). Two days before transfection 5.10^4 cells were plated onto 35mm dishes (one coverslip per dish). The medium was changed 3 hours before transfection. Cells were transiently transfected using calcium phosphate method (Sambrook et al., 1989) and analyzed 72 hours later by IF. Transfection efficiency was about 10%.

Primary cell cultures were made from rat hippocampal neurons according to Malgaroli and Tsien (1992), with slight modifications. Hippocampi were dissected from 2-4 day old animals. Isolation and slicing was performed in 200 µM kinurenic acid (Sigma, St. Louis, MO) and 25 µM 2-amino-5-phosphonovalerate (Tocris Neuramin, Bristol, UK). Tissue slices were digested with trypsin in the presence of DNAse, blocked with trypsin inhibitor on ice, dissociated in medium containing DNAse. Cells were recovered and washed by two successive centrifugation steps at 500 rpm and plated on glass coverslips, coated with 50 µg/ml polyornitine and 2% Matrigel (Collaborative Research) in 35 mm NUNC Petri dishes. Cells were cultured for 5 days in 5% CO2-humidified incubator, in minimum essential medium with Earle's salts and Glutamax I (Gibco, Life Technologies) additioned with 5-10% fetal bovine serum, 7 mg/ml D-glucose, 3.6 mg/ml HEPES, 0.1 µg/ml biotin, 1.5 µg/ml vitamin B12, 30 µg/ml insulin and 100 µg/ml bovine transferrin. Proliferation of non-neural cells was prevented by the addition of 2.5-5 µM cytosine ß-D-arabinofuranoside from the second day in culture onwards. Neurons were transfected by the Calcium Phosphate method (Sambrook et al., 1989). The following main modifications were introduced in the original protocol: the medium was changed half an hour before the transfection procedure and incubation with the transfection mix lasted for 2 hours only. The latter step was followed by two washes with minimum essential medium with Earle's salts and Glutamax I (Gibco, Life Technologies). Cells were then cultured for 2 days, before being analyzed by IF.

3.5. Immunofluorescence (IF) and In Situ End-labeling of DNA (ISEL) with cell cultures.

Monoclonal antibody MN7.51 (see Tab. 4A, chapter 4) was used to detect tau expression in COS cells, at 1:10 dilution of hybridoma supernatant.

The monoclonal antibody 9E10 (see Tab. 4A, chapter 4) recognizes the myc tag (Evan et al. 1985) placed at the C-terminus of dGAE and tau151-391. It was employed to detect the expression of tau fragments in NT2 cells and neurons, at 1:2 dilution of hybridoma supernatant or as purified antibody (1:1000).

Polyclonal Antiserum 304 (generous gift of M.Goedert; see Tab4A, chapter 4) was used to detect the expression of tau-head at 1:1000 dilution.

Anti-mouse TRITC Immunoglobulins (DAKO, 1:200 dilution) or biotinylated anti-rabbit Immunoglobulins (Vector, 1:500 dilution) were chosen as secondary antibodies.

Antibodies were diluted in Blocking Solution (1xPBS containing 3% Bovine Serum Albumin, BSA, Sigma). 1xPBS and 1xPBST (see paragraph 3.2) were used for washes between incubation steps.

Apoptotic cells were identified in situ with the ISEL procedure for DNA fragmentation using the ApopTag Direct in situ Apoptosis Detection kit (Oncor), or the TdT in situ Apoptosis Detection kit (Genzyme), following the manufacturers' instructions (both procedures stain apoptotic nuclei with green fluorescein).

For double labeling, cells were fixed in 1% paraformaldehyde (10 min) on ice, then permeabilized with 70% ethanol (at –20°C) for 10 min. The ISEL procedure for detection of DNA fragmentation was always performed before indirect IF. Incubation of coverslips (1 hr at R.T.) with the anti-tau monoclonal antibody MN7.51 (or antiserum 304, to detect

the expression of tau-head) was preceded by incubation in Blocking Solution (15 min R.T.). After several washes in 1xPBS and 1xPBST, coverslips were incubated for 30 min (R.T.) with anti-mouse TRITC (or biotinylated anti-rabbit) Immunoglobulins, as secondary antibodies. When biotinylated Immunoglobulins were used, coverslips were subsequently incubated in the dark for 10 min with Avidin Rhodamine at 1:1000 dilution in 10mM Hepes and 0.15M NaCl, pH 8.2.

Coverslips were finally mounted in Vectashield mounting medium (Vector).

Apoptotic transfected cells were counted 48 hr (72 hr in the case of NT2 cells) after transfection. 200 transfected cells were examined on each coverslip. When experiments were carried out using the Genzyme reagents, the percentage of apoptotic cells out of the total number of transfected cells was multiplied by a correction factor of 1.29. Such a correction factor was worked out by comparing the apoptotic effect of tau151-391 and dGAE using the two different kits. Student's T-test was employed for the statistical analysis of counts.

3.6. Western blot analysis of cell extracts.

72 hr after transfection, cells were washed three times with PBS, extracted with ice-cold depolimeryzing Microtubules lysis buffer (50mM Tris pH 7.4, 2mM $CaCl_2$, 1%NP40 and a cocktail of protease inhibitors) for 20 min at 4°C, scraped and centrifuged 5 min at 12000 rpm. 2x SDS gel-loading buffer and dithiothreitol (stock 1 M) were added to the supernatant so that the resulting sample contained 2% SDS, 100 mm dithiotreitol, 60mM Tris (pH 6.8) and 0.01% bromophenol blue. Samples were boiled for 5 min, before being analyzed by SDS-PAGE (10% acrylamide, 20 μg of protein per lane) and blotted to

nitrocellulose using a semi-dry method. The monoclonal antibody MN7.51 (1:20 dilution of hybridoma supernatant) or the monoclonal antibody 9E10 (1:2 dilution of hybridoma supernatant) was employed as primary antibody. Peroxidase-conjugated goat anti-mouse Immunoglobulins (1:1000, DAKO) and ECL reagents (Amersham) for detection by chemiluminescence were used. Alternatively, the immunoblot filter was finally reacted with 0.05% diaminobenzidine (DAB, Sigma) and 0.01% H_2O_2.

3.7. *In vitro* cleavage of tau by caspase-3.

Recombinant human caspase-3 harboring a C-terminal His6 tag was expressed in Escherichia coli and purified on a Ni^{2+} affinity resin, as described by Canu et al. (1998). pSG5-based plasmids encoding for tau40, tau22* (D22A) and tau418* (D418A) were *in vitro* transcribed and translated with the Transcription and translation-coupled reticulocyte lysate system (Promega, Madison, WI). 2 µl of translated reticulocyte lysate were incubated for 30 min at 37°C with purified caspase-3 in 10 µl of caspase-3 buffer, containing protease inhibitors, in the presence or the absence of 150 µM Ac-DEVD-CHO. Reactions were terminated by adding 1 volume of SDS-PAGE sample buffer and boiling for 3 min, before running on SDS-PAGE.

3.8. Rabbit polyclonal antibody production.

Peptide conjugation.

Two peptides were purchased from INTERACTIVA (Germany), with the following sequences: N-V-S-S-T-G-S-I-D-M-V-D (CT+), N-V-S-S-T-G-S-I-D-M-V-D-S (CT-), corresponding respectively to 12 and 13 aminoacids of tau tail and ending respectively at

Asp-421 and Ser-422 (Fig. 3). Each peptide was conjugated to bovine serum albumin (BSA) with the following coupling procedure: the peptide was added to 1 mg/ml solution of carrier protein (BSA) in PBS pH 7.4 at a molar ratio of protein/peptide 1:30 (4°C). An equal volume of 2% glutaraldehyde solution (prepared in water just before use) was added drop-wise to the protein mixture with constant stirring (4°C). After 1 hr the reaction was stopped, by adding sodium borohydride ($NaBH_4$) to a final concentration of 10 mg/ml (1 hr at 4°C). The conjugate was dialyzed against PBS (3 times) and stored in aliquots (about 200 µg each) at -20°C. Concentration of each conjugate was determined by using the DC-protein assay kit (BIO-RAD). Each time a standard curve was prepared by using 3-5 different dilutions of a BSA standard containing from 0.2 mg/ml to 1.5 mg/ml protein.

Injection and bleed.

New Zealand female rabbit (Harlan Italy) weighting 2.5 kg was immunized with 200 µg of antigen in 1 ml of PBS plus 1 ml of incomplete Freund's adjuvant, with intra-muscular injections in the 4 legs. 21 days later the rabbit was boosted with the same amount of antigen and 11 days later blood (50 ml) was collected from the ear marginal vein. The rabbit was hyper-immunized (5 boosts). Every time next boost took place 10 days after the previous bleed and the animal was bled 11 days after the boost.

ELISA.

The Anti-sera were all tested by Enzyme-Linked Immuno-Sorbent Assay (ELISA), according to the following procedure. Plates (96-wells-immunoplate, NUNC) were coated with 100 µl per well of protein antigen (BSA-Peptide-CT+ or BSA, as control carrier protein) O/N at 4°C. The protein antigen concentration was 10 µg/ml (in 100 mM

Sodium Hydrogen Carbonate, pH 9.6 or in PBS). Wells were subsequently rinsed 3 times with PBST (PBS containing 0.1% Tween) and 3 times with PBS (the same washing procedure was employed throughout the protocol). Incubation with blocking solution BS (PBS containing 2% milk), for 1h at R.T., was followed by washes and incubation with rabbit anti-sera, at several dilutions (from 1:100 to 1:5,000) in BS for 2h at R.T. After being washed, wells were incubated in peroxidase–conjugated goat anti-rabbit Immunoglobulins (DAKO) diluted 1:2000 in BS, for at least 1h at R.T. Finally, the antibody was discarded and wells were washed out for the last time. The TMB (tetra-methyl-benzidine) reaction (100 µl of reagents per well) was used to visualize the presence of the antibody on the surface of wells. The reaction was quenched, by adding 2N Sulfuric Acid (H_2SO_4, 100 µl per well). Absorbance values (wavelength = 450 nm) were read by a spectrophotometer.

Antiserum purification.

Ammonium Sulfate (5.75 g) was slowly added to the antiserum obtained after the second boost (about 20 ml), during stirring, at 4°C. The mixture was left at 4°C (O/N) and subsequently centrifuged for 5 min (twice) at 4°C. The pellet was re-suspended in 10 ml of PBS and dialyzed against 3 changes of PBS (5 l each) at 4°C, O/N (SPECTRA/POR dialysis membrane with 8Kd cut-size and 1.7 ml/cm linear capacity, Spectrum).

Affinity cromatography column.

CnBr-activated Sepharose (0.3 g, Pharmacia) was washed and swollen with 1mM HCl. PBS buffer containing the conjugate (5 ml) was adjusted in order to finally contain 0.2M $NaHCO_3$ and 0.5M NaCl (final pH = 8.5). The swollen resin was washed in coupling buffer (0.2M $NaHCO_3$, 0.5M NaCl, pH 8.5) before the conjugate (5 ml) and the resin

(about 1 ml, after swelling) were mixed by means of an end-over-end mixer at 4°C (O/N). The resin was subsequently centrifuged (800 rpm). After removing the supernatant, the pellet was washed with 10 volumes of coupling buffer and the mixture was centrifuged again (800 rpm). The final pellet was blocked with Glycine buffer (pH 8.0) at 4°C (O/N). The two supernatants were mixed. The protein contents of the mixture and that of an aliquot of the original conjugate were measured by a spectrophotometer (λ= 280 nm). According to these measurements, the coupling efficiency was estimated to be around 50%. The resin was washed with coupling buffer and Acetate buffer (pH 4.0) for 3 times. Each time resin was centrifuged and the supernatant discarded. PBS (containing 0.02% Sodium Azide for storage of the column at 4°C) was added to the final pellet.

Immunoaffinity procedure and antibody characterization.

The resin was centrifuged and PBS was discarded. 4 ml of "purified' antiserum were added to the affinity cromatography column. The mixture was stirred at 4°C (O/N), centrifuged at 800 rpm and the supernatant was collected and named anti-tau421. In order to test the capacity of anti-tau421 to specifically recognize the BSA-CT(+), ELISA tests were performed as described above, with BSA-CT(-), BSA-CT(+) and BSA used for coating. Recombinant full-length tau and tau terminating at Asp-421 were employed in immunoblot experiments to demonstrate that anti-tau421 specifically recognizes the latter. The western blot procedure was the same as described for tissue extracts. Recombinant proteins were gently supplied by Dr. Michela Visintin (tau-421, produced by the pMAL expression and purification system, New England Biolabs) and Dr. Vittorio Verzillo (full-length tau).

3

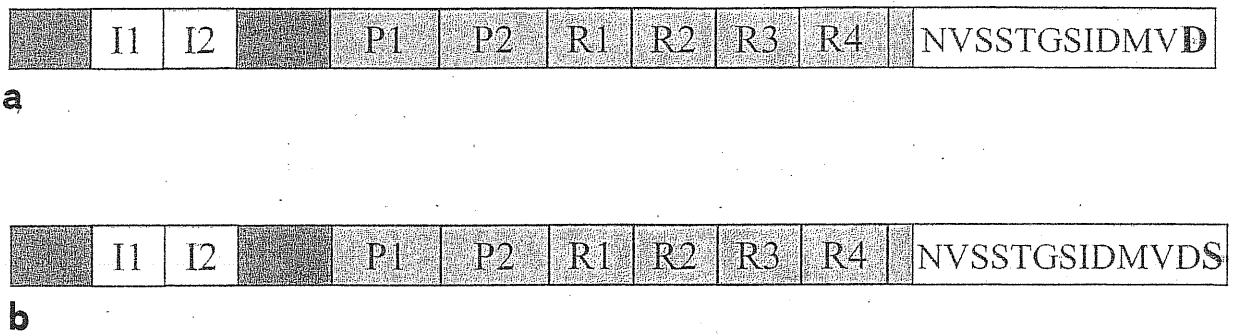


Fig. 3. *Representation of the CT(+) peptide* ***(a)*** *and CT(-) peptide* ***(b)*** *in the context of full-length tau. CT(+) peptide was used for rabbit immunization, CT(-) peptide was employed for absorption of the antiserum in order to select antibodies specifically recognizing Asp-421 as carboxy-terminus.*

4. RESULTS

4.1. MN7.51 western-blot profile distinguishes Alzheimer's disease brains from non-demented controls.

The monoclonal antibody MN7.51 recognizes all isoforms of tau in western blot, since its epitope has been localized to a short segment contained in the last two repeats, a sequence present in all isoforms (Ugolini et al., 1998; Novak et al., 1991; see Tab. 4A). The monoclonal antibody PHF-1 recognizes an epitope phosphorylated in PHF-tau and located in the carboxy-terminal portion of the molecule (Greenberg et al., 1992; Lang et al., 1992; Otvos et al., 1994; see Tab. 4A). The monoclonal antibody Tau-1 recognizes a dephosphorylated epitope inside the proline rich regions (Papasozomenos and Binder, 1987; Szendrei et al., 1993; see Tab. 4A). These three antibodies (MN7.51, PHF-1 and Tau-1) were used for a preliminary study of the tau protein patterns from post-mortal brain tissues of various AD patients and age-matched controls (see Tab. 4B). The characteristic electrophoretic profile of the SDS-soluble tau fraction revealed with MN7.51 appeared to distinguish Alzheimer's disease brains from non-demented controls. Among the samples tested, the immunoblot pattern is characterized by the appearance of a smear of polymers in the high molecular weight range (Fig. 4.1a). The same pattern could be reproduced to some extent with PHF-1 (Fig. 4.1b). Tau-1 stained extracted brain tau very well but without any discriminatory value (Fig. 4.1c). Degradation products with a molecular weight between 30 and 45 Kda were observed in all samples with Tau-1, and in AD samples with PHF-1 (which does not stain normal tau from post-mortal brain tissue), even if protease inhibitors had been added during preparation of homogenates.

Such bands were less evident with MN7.51, except that in AD2 and AD3 samples (Fig. 4.1a).

Antibody	*Protein recognized*	*Epitope*	*Use in this work*
MN7.51 (Novak et al., 1991; Ugolini et al., 1998)	*Tau (all isoforms)*	*The epitope is contained between Ser-315 and Leu-376*	*Western blot and Immunocytochemistry (Immunofluorescence)*
Tau-1 (Papasozomenos and Binder, 1987; Szendrei et al., 1993)	*Tau (all isoforms)*	*Aminoacids 189-207 when no residue is phosphorylated*	*Western blot*
PHF-1 (Greenberg et al., 1992; Lang et al., 1992; Otvos et al., 1994)	*Tau (all isoforms)*	*In the carboxy-terminal region (including phosphorylated serine residues 396 and 404)*	*Western blot*
MN10 (M. Novak, unpublished)	*Aβ peptide*	*Recognition requires the whole fragment*	*Immunohistochemistry*
AT8 (Biernat et al., 1992)	*Tau (all isoforms)*	*Around residue 200 (including phosphory lated residues 199, 202, 205)*	*Immunohistochemistry*
MN423 (Novak et al., 1989, 1991, 1993)	*All tau molecules and only those truncated at Glu-391*	*A segment of the molecule (DHGAE) terminating at Glu-391*	*Immunohistochemistry*
Antiserum 304 (Goedert et al., 1992a)	*Tau isoforms containing the N-terminal Insert I1.*	*Inside the N-terminal insert I1*	*Immunocytochemistry (Immunofluorescence)*
9E10 (Evan et al., 1985)	*Myc protein*	*C-terminal 32 Aminoacids of myc, used as peptide tag.*	*Western blot and Immunocytochemistry (Immunofluorescence)*

Tab.4A. *Summary of the antibodies employed in this work (Numbering of aminoacid residues according to the longest isoform of tau).*

Brain	CSF pH	Brain area	Sex	Age (years)	Weight (g)
AD1	6.25	SPG	F	87	1070
AD2	6.29	SPG	F	90	1104
AD3	6.99	SPG	M	58	1180
NDC1	6.38	SPG	F	73	1264
NDC2	6.75	SPG	M	74	1317
NDC3	6.94	SPG	F	81	1022

Tab.4B. *Summary of the brain samples used for western blot experiments: AD = Alzheimer's Disease brain; NDC = Non-demented control; CSF = cerebrospinal fluid; SPG = Superior Parietal Gyrus; F = female; M = male; g = grams. Post-mortem delay inferior to 4 hours in all cases.*

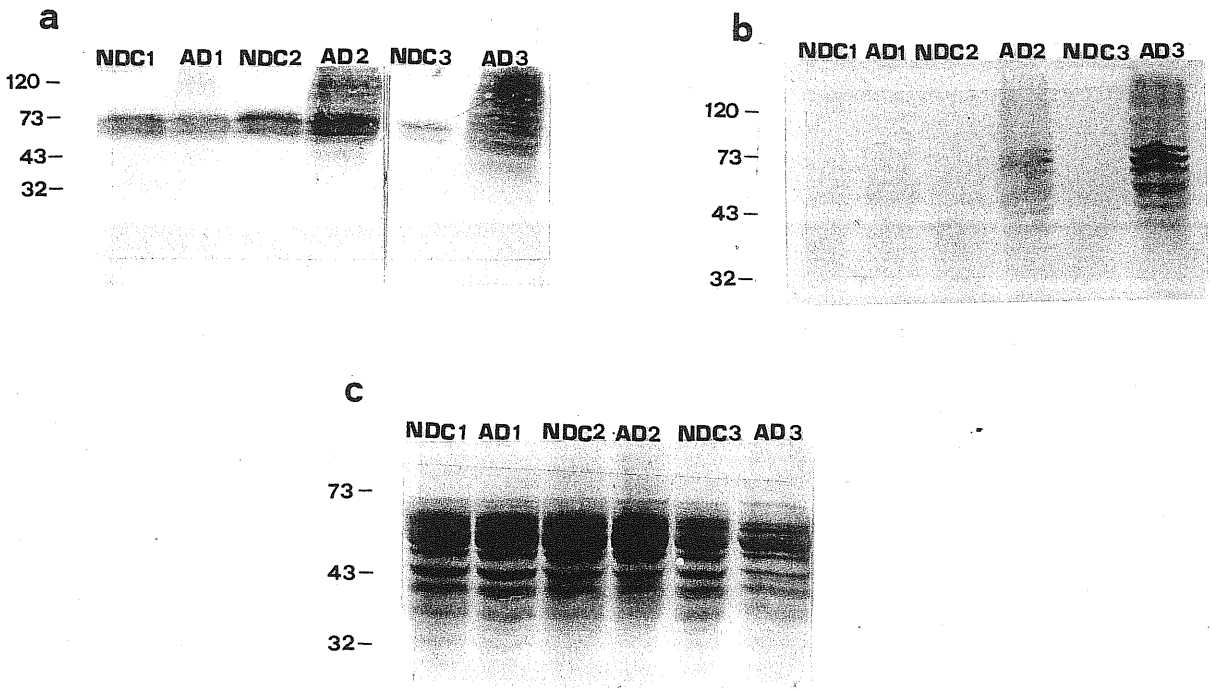


Fig. 4.1. *Western blot profile of six brain samples (NDC1, NDC2, NDC3, AD1, AD2 and AD3, see Tab. 4B) with MN7.51* ***(a)****, PHF-1* ***(b)*** *and Tau-1* ***(c)****. NDC = Non-demented control brain; AD = Alzheimer's Disease brain. Molecular weight in Kda.*

4.2. Immunohistochemical characterization of brain samples.

Brain tissue sections from AD and age-matched control brains (see Tab. 4C) were examined in order to find out AD histopathological hallmarks. Senile plaques were identified in AD brains by means of the monoclonal antibody MN10 (Michal Novak, personal communication; see Tab. 4A; Fig. 4.2a). AD neurofibrillary pathology was shown by using the monoclonal antibody AT8, that recognizes a phosphatase-sensitive epitope containing various phosphorylated residues (Ser 202, Ser199 and Thr205; Biernat et al., 1992; see Tab. 4A; Fig. 4.2b).

Brain	*Sex*	*Age (years)*	*PMD (hours)*	*CSF pH*	*ISEL+/ MN423− (%cells)*	*MN423+/ ISEL+ (%cells)*
AD4	*F*	*72*	*17.00*	*N.D*	*39±15*	*32±10*
AD5	*M*	*77*	*03.50*	*6.30*	*21±4*	*69±5*
AD6	*M*	*84*	*03.45*	*6.94*	*38±7*	*62±12*
AD7	*F*	*84*	*04.00*	*6.81*	*31±6*	*53±9*
AD8	*F*	*68*	*04.05*	*6.81*	*N.D*	*N.D*
NDC4	*M*	*80*	*04.30*	*6.22*	*Neg.*	*Neg.*
NDC5	*M*	*75*	*07.20*	*6.16*	*Neg.*	*Neg.*
NDC6	*F*	*87*	*03.00*	*7.04*	*Neg.*	*Neg.*

Tab. 4C. *Summary of the brain samples used for Immunohistochemistry. Sections were obtained from both parietal and frontal cortex in each case. The last two columns summarize results of double labeling experiments (MN423 immunohistochemistry + ISEL). PMD: post-mortem delay; CSF: cerebrospinal fluid; ISEL: in situ end labeling of DNA. AD: Alzheimer's disease brains; NDC: age-matched non-demented control brains; F: female; M: male; N.D: not determined. ISEL+/MN423−: shows % of ISEL-positive cells which were not labeled with MN423 in double labeling experiments (standard deviation is included; total number of cells counted: 1000 per series of sections). MN423+/ISEL+: shows % of MN423-positive cells exhibiting DNA fragmentation in their nuclei in double labeling experiments (standard deviation is included; total number of cells counted: 1000 per series of sections). Neg.: sections from NDC brains were only occasionally and weakly stained by MN423 and ISEL staining was virtually absent on adjacent sections.*

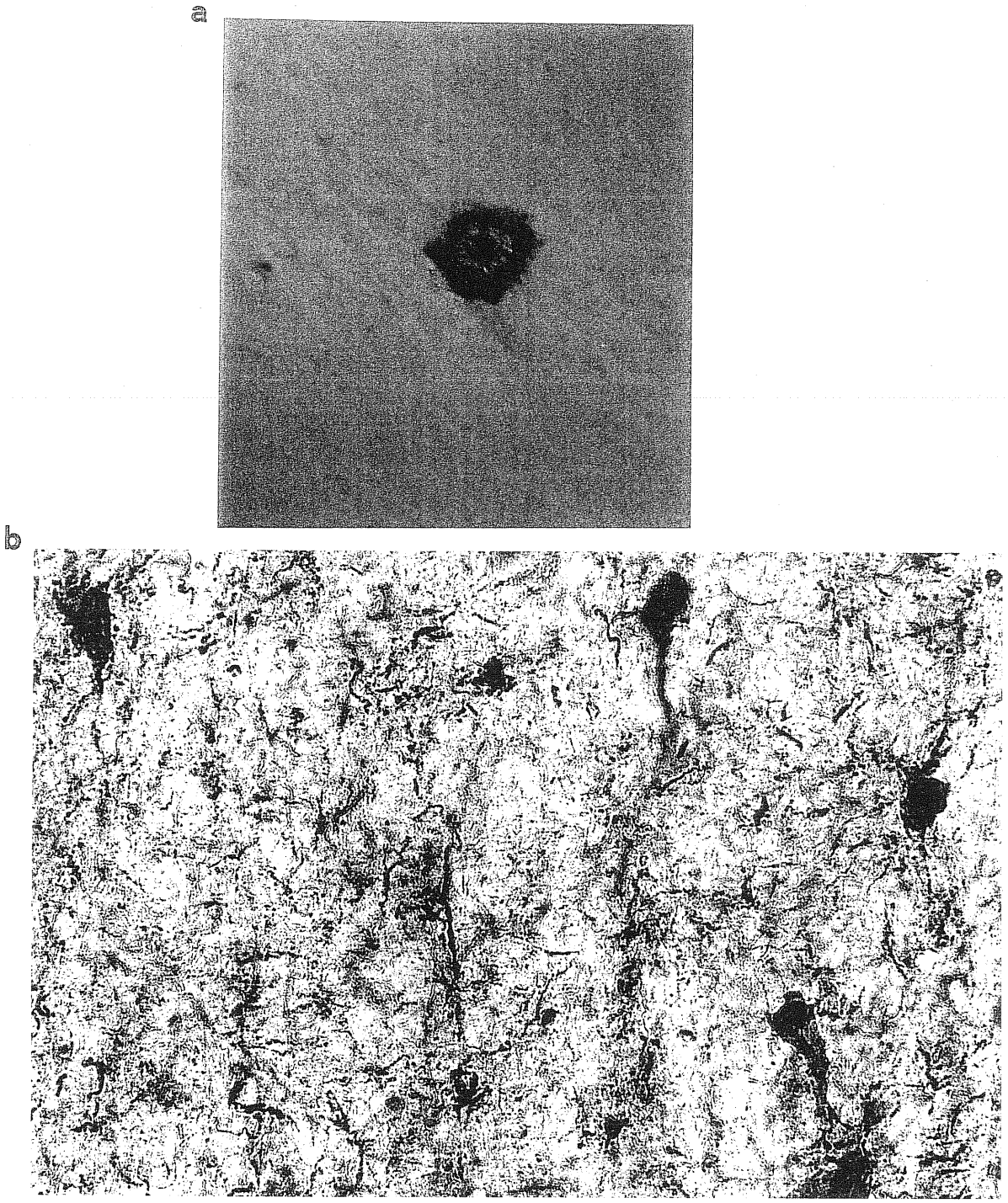


Fig. 4.2. *Histopathological hallmarks of Alzheimer's Disease (brain sample = AD5 in Tab. 4C): βamyloid plaque stained by monoclonal antibody MN10* ***(a)*** *and neurofibrillary pathology (tangles, dystrophic neurites and neuropil thread) as revealed by monoclonal antibody AT8* ***(b)****.*

4.3. Tau truncation and DNA fragmentation in AD brain.

Pre-tangle accumulation of MN423-immunoreactive material inside the cytoplasm of AD neurons.

Monoclonal antibody MN423 recognizes all and only those tau molecules truncated at Glu-391 (Novak et al., 1989, 1991, 1993; see Tab. 4A).

Tissue fixation and permeabilization, as well as staining procedure were optimized in a preliminary phase of the work.

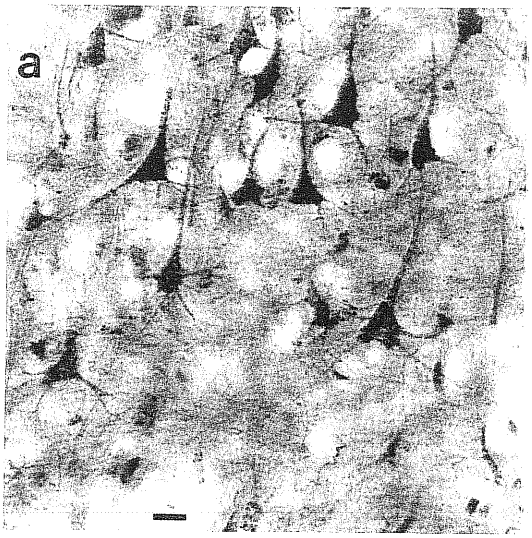
MN423 stained sections from AD brains (Fig. 4.3a) but it did not usually stain sections from age-matched NDC brains (Fig. 4.3b). Occasionally MN423 weakly labeled pyramidal neurons with no apparent cytoskeletal changes in control brains.

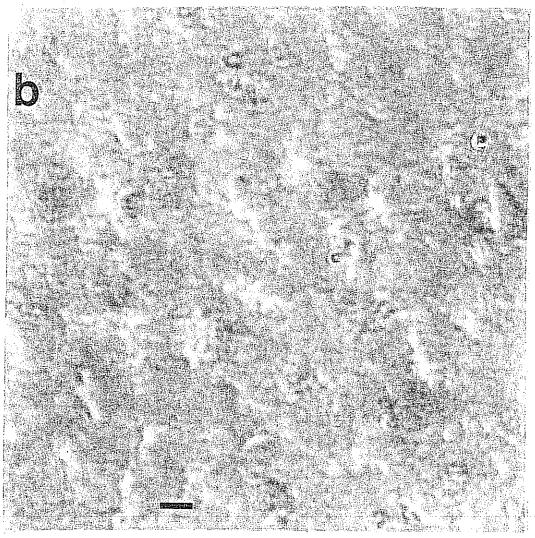
On sections from AD brains, two main patterns of staining were produced by MN423:

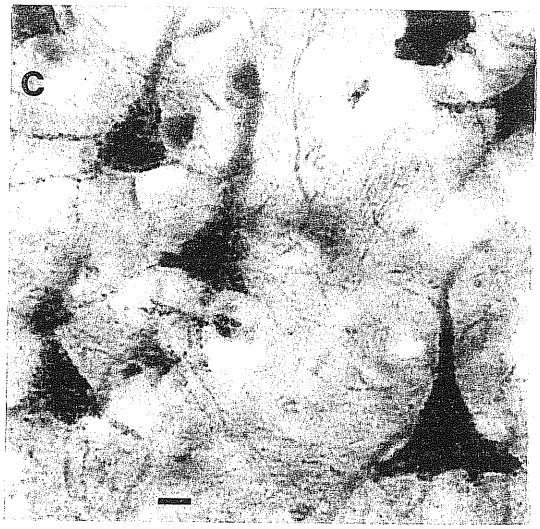
1) With paraformaldehyde-containing fixation buffers, most sections exhibited granular labeling inside of the cell bodies of cortical pyramidal neurons without showing any neurofibrillary tangles (Fig. 4.3a and 4.3c).
2) When formalin was employed as fixation buffer, classical labeling of neurofibrillary pathology hallmarks was observed: neurofibrillary tangles, dystrophic neurites and the neuritic component of senile plaques (Fig. 4.3d).

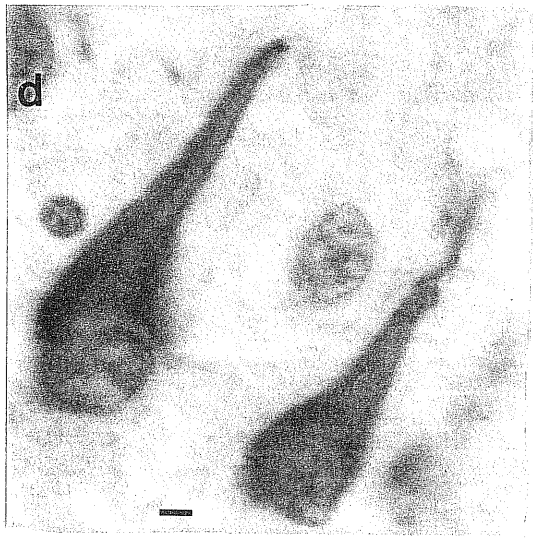
MN423-positive granular aggregations were found to be particularly abundant in the somatodentritic compartment.

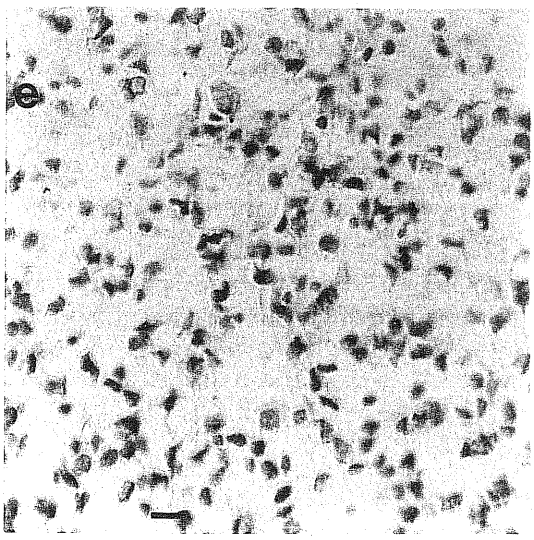
The observation of this intracellular granular material did not show any correlation with the Post-Mortem Delay (PMD) of brains (being already present with PMD as short as 3.45 h). We did not observe any PMD-related MN423 staining in control brains (Fig. 4.3b) with PMD spanning from 3.00 to 7.20 h.


a


b


c


d


e

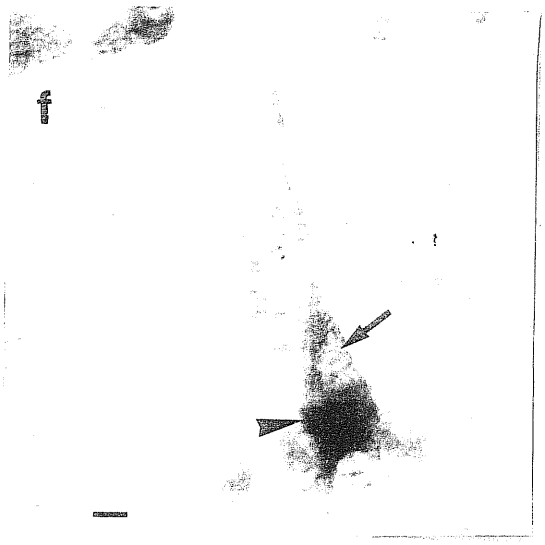

f

Fig. 4.3. *Co-localization of truncated tau and DNA fragmentation.* ***(a)*** *Pyramidal neurons of cortex, stained with MN423 (AD brain).* ***(b)*** *Cortex from NDC brain, stained with MN423: positive staining is absent.* ***(c)*** *Pre-tangle granular aggregations of MN423-positive material inside the cytoplasm of pyramidal neurons.* ***(d)*** *Typical neurofibrillary tangles stained with MN423.* ***(e)*** *ISEL-positive nuclei in AD cortex.* ***(f)*** *Double labeling showing that the tau truncation (light gray/arrow) in the cytoplasm is co-localized with DNA fragmentation in the nucleus (dark gray/arrowhead). Bar = 50μm* ***(a,b)****; 25μm* ***(c,f)****; 20μm* ***(d)****; 60μm* ***(e)****.*

DNA fragmentation in the nuclei of AD neurons.

The presence of DNA fragmentation, a commonly accepted marker of cell death, was visualized in neurons by means of ISEL technique (In-Situ End-Labeling of DNA). In sections from NDC brains, ISEL-positive nuclei were extremely rare or virtually absent. On the contrary, when sections from AD brains were processed with the ISEL technique, numerous nuclei were stained both in parietal and frontal cortex (Fig. 4.3e).

Co-localization of truncated tau and DNA fragmentation.

Examination of adjacent sections from brains AD4, AD5, AD6, AD7 stained with MN423 and ISEL respectively, showed similar pattern of labeling. Therefore experiments on co-localization of these two events were performed. Double labeling of sections from the same brain samples (Fig. 4.3f) showed that a high proportion of the nuclei of MN423-positive neurons was stained by ISEL (from a minimum of 32% to a maximum of 69%, Tab. 4C). As far as cortical gray matter is concerned, 423-positive neurons represented the majority of ISEL-positive cells (from a minimum of 61% to a maximum of 79%, Tab. 4C).

4.4. Tau fragments as effectors of apoptosis in COS cells.

Tau proteins are the main constituents of the paired helical filaments (PHFs) found in AD neurons (Wischik et al., 1988a and b). All six full length tau isoforms (Goedert et al., 1992a), as well as shorter fragments (Novak et al., 1993), are present in PHFs. The so-called 12 Kda tau fragments that encompass the microtubule binding repeat regions, are unable to bind to microtubules, but do not affect cell morphology nor viability (Fasulo et al., 1996). A previous study showed that N-terminal extension of the 12 Kda fragment,

including the two proline rich regions (fragment tau151-391), induces apoptosis in COS cells (Fasulo et al., 1998).

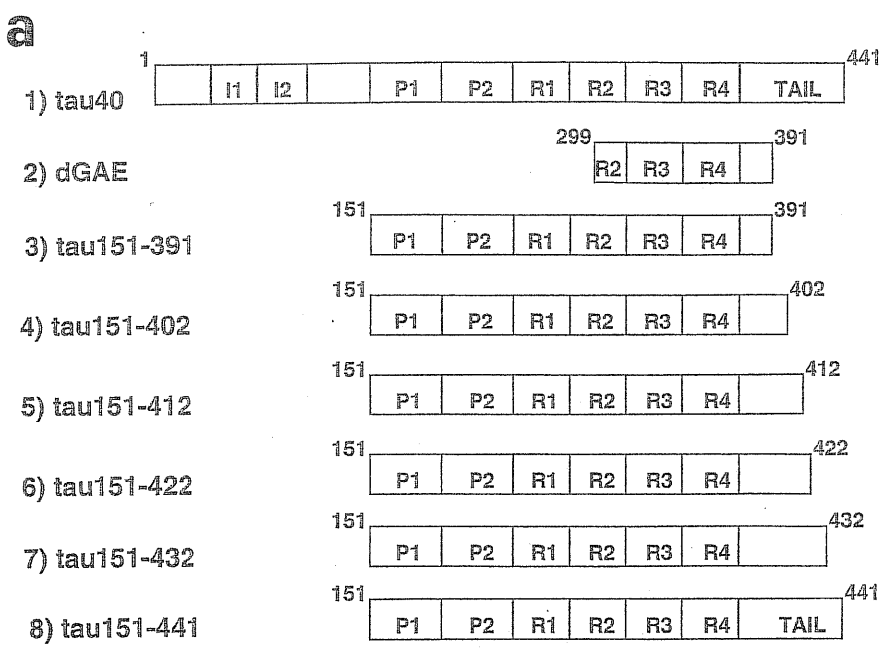
In order to systematically map the apoptotic properties of tau, a set of fragments, designed around tau151-391, and based on the longest tau isoform found in human brain (tau40), was engineered and expressed in cells.

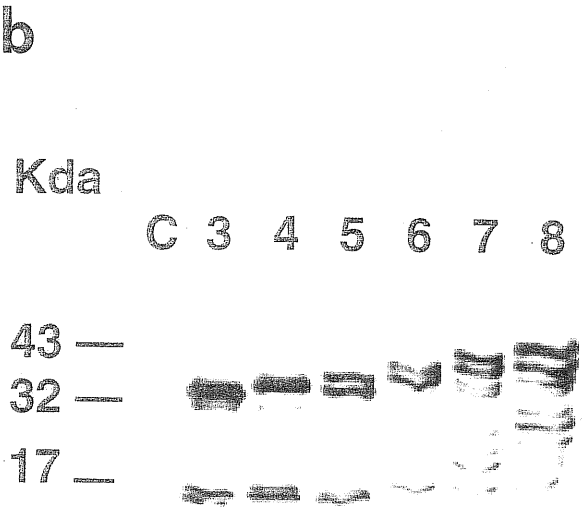
Figure 4.4a shows a first set of constructs, starting at residue 151 (start of the proline-rich region 1) and terminating at residues 391, 402, 412, 422, 432 and 441 (natural end of tau protein). Plasmids directing the expression of these fragments were transiently transfected in COS cells and the expression of the corresponding proteins was verified by Western blot (Fig. 4.4b), confirming that the fragments are expressed at comparable levels and display the predicted molecular weight. Transfected cells were immuno-stained 48 hours after the transfection with the anti-tau monoclonal antibody MN7.51 (Fig. 4.4c-h), which recognizes an epitope in the repeat region, contained in all constructs of this set (see Tab. 4A). Cells expressing fragments tau40 (Fig. 4.4c), dGAE (Fig. 4.4d), tau151-391 (Fig. 4.4e, f), tau151-422 (Fig. 4.4g) and tau 151-441 (Fig. 4.4h), were analyzed by double labeling with MN 7.51 (red) and with the ISEL procedure for DNA fragmentation (green). The percentage of transfected cells expressing fragments of tau40 and showing DNA fragmentation was determined (Fig. 4.4i). The lowest percentage of DNA fragmentation was found for cells expressing the dGAE fragment, and for this reason this fragment was used as a negative control in all experiments. On the contrary, the expression of fragments encompassing the proline rich regions, and terminating anywhere between residues 391 and 422, induced morphological changes typical of apoptosis (rounded shape, nuclear condensation and membrane blebbing) as well as DNA

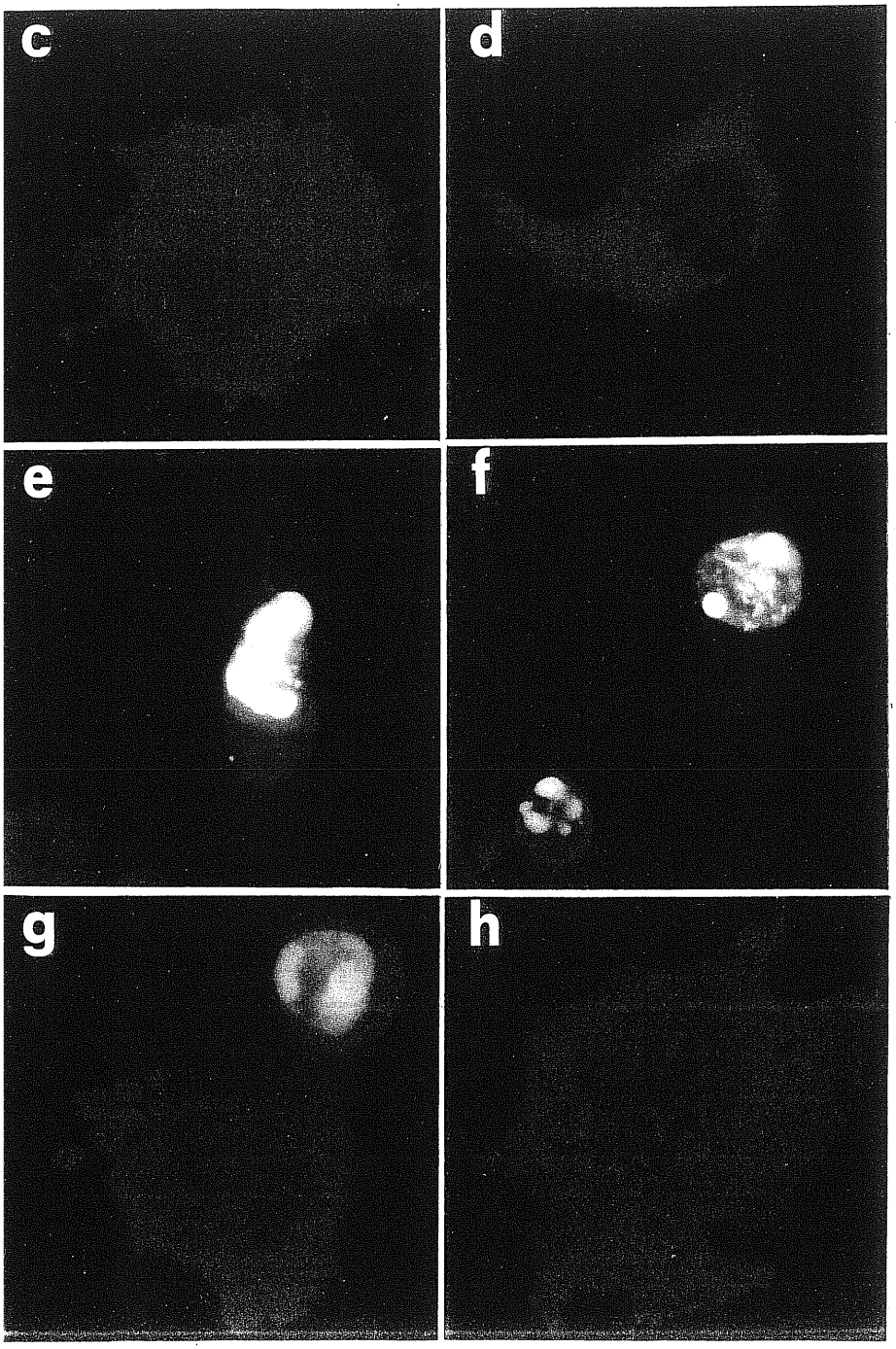
fragmentation in a large number of cells (Fig. 4.4e,f,g). Interestingly, a further C-terminal extension of the fragments, to include the natural end of the protein, resulted in the complete loss of the apoptotic properties (Fig. 4.4i). In conclusion, all fragments terminating between aminoacids 391 and 422 display the maximal ability to kill cells, with a sharp transition observed upon further extension into the 19 C-terminal residues. The full-length tau40 protein induces a slightly higher level of apoptosis than the dGAE control fragment. This is most likely due to intracellular proteolysis of the molecule, as confirmed by Western blot analysis (Fig. 4.4b), with the generation of apoptotic fragments. No such fragment can be generated from dGAE.

4.5. Fragment tau151-391 induces apoptosis in neuronal cell lines.

Fragment tau151-391 was expressed human NT-2 teratocarcinoma cells and in human SH-SY5Y neuroblastoma cells. The results for human NT-2 cells are shown in Figure 4.5. The red fluorescence signal seen with the anti tau MN 7.51 antibody is due to the transfected tau fragments, since undifferentiated NT-2 cells do not express endogenous tau, as shown (Fig. 4.5d, lane A). A comparison of NT-2 cells expressing the tau fragments dGAE (Fig. 4.5a) and tau 151-391 (Fig. 4.5b-c) showed that the latter induces apoptosis in these cells as well. The ratio between the number of apoptotic cells observed with tau151-391 and that obtained with dGAE, is comparable in NT-2 and in COS cells (Fig. 4.5e). The fragment tau151-391 induced membrane blebbing and nuclear pycnosis in human SH-SY5Y neuroblastoma cells (data not shown, personal communication by L. Fasulo).


a
1) tau40
1
441
I1
I2
P1
P2
R1
R2
R3
R4
TAIL
2) dGAE
299
391
3) tau151-391
151
4) tau151-402
402
5) tau151-412
412
6) tau151-422
422
7) tau151-432
432
8) tau151-441


b
Kda
C 3 4 5 6 7 8
43
32
17


c
d
e
f
g
h

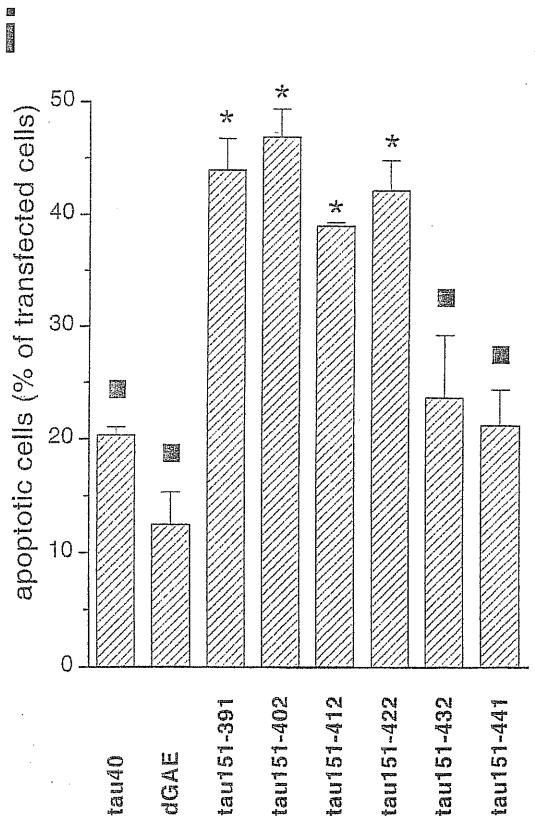

i
apoptotic cells (% of transfected cells)
50
40
30
20
10
0
*
tau40
dGAE
tau151-391
tau151-402
tau151-412
tau151-422
tau151-432
tau151-441

Fig. 4.4. *A set of fragments of tau inducing apoptosis in COS cells.* ***(a)*** *Schematic representation of Tau fragments. In this and in subsequent figures, the numbering of aminoacids corresponds to that of the longest human brain tau isoform (tau40): 1) Full length tau-40; I1, I2: N-terminal Inserts; P1, P2: proline-rich domains; R1-R4: microtubule binding repeats; 2) dGAE: one of the AD PHF core fragments; 3) tau151-391; 4) tau151-402; 5) tau151-412; 6) tau151-422; 7) tau151-432; 8) tau151-441.* ***(b)*** *Western blot of tau fragments expressed in COS cells (C: control untransfected cells). Lane numbers correspond to constructs in Fig. 4.4a.* ***(c-h)*** *Double labeling of COS cells expressing tau fragments. COS cells were transiently transfected with the tau constructs described in Fig. 1a. Cells were analyzed 48 hours after the transfection by double labeling with the anti-tau monoclonal antibody MN7.51 (red fluorescence) and with the ISEL procedure for DNA fragmentation (green fluorescence).* ***(c)*** *tau40;* ***(d)*** *dGAE;* ***(e, f)*** *tau151-391;* ***(g)*** *tau 151-422;* ***(h)*** *tau 151-441.* ***(i)*** *Percentage of cells transfected with tau fragments undergoing apoptosis. Average values from at least three independent transfection experiments are reported. At least 500 transfected cells were scored for each construct. Bars represent standard errors. Asterisks and filled squares over the bars indicate the statistical significance with respect to dGAE and tau151-391, respectively ($p < 0.001$, except for tau151-432 ($p < 0.05$)).*

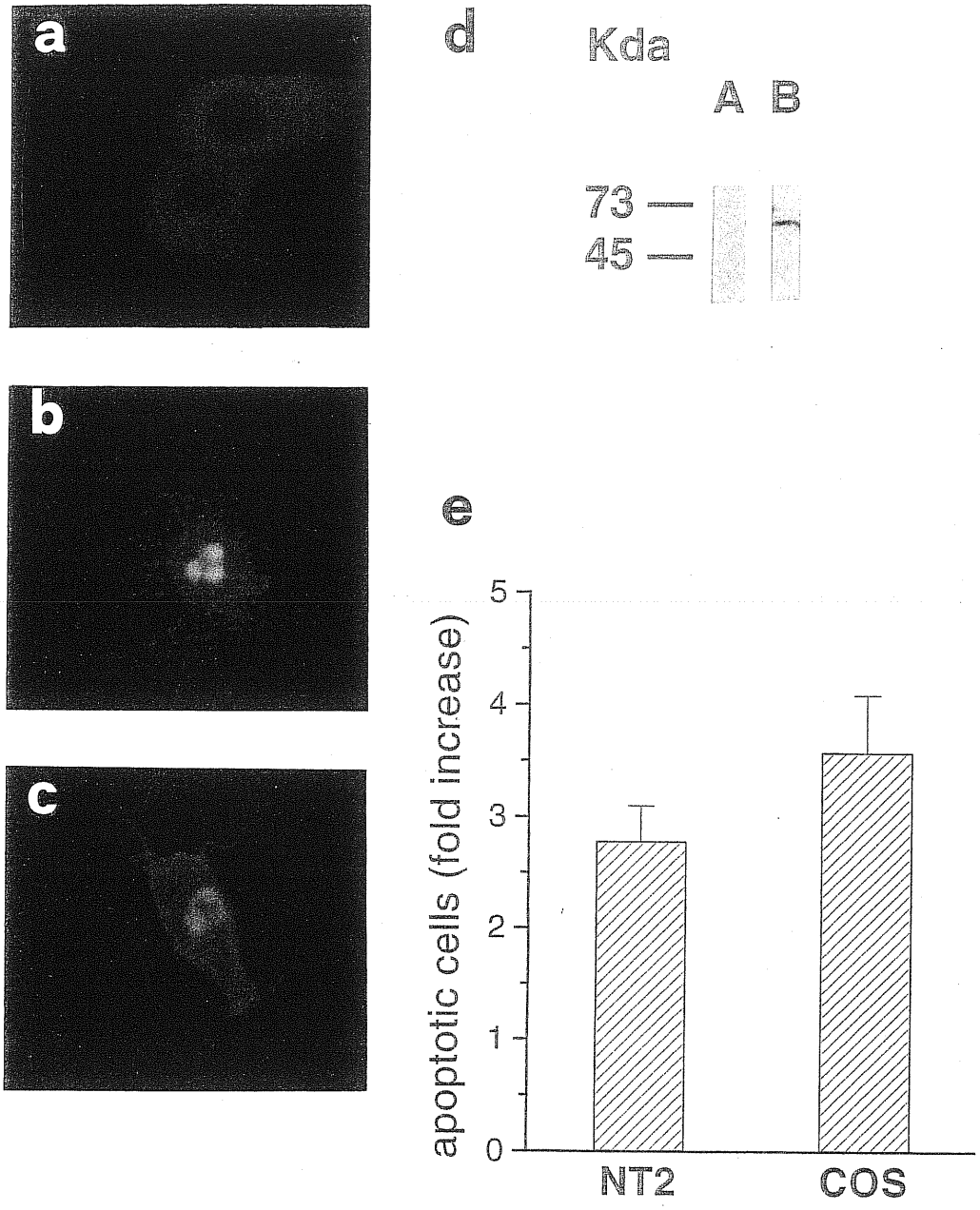


Fig. 4.5. *Fragment tau151-391 induces apoptosis in human NT-2 cells. Human NT-2 cells were transiently transfected with the tau constructs dGAE* ***(a)*** *and tau151-391* ***(b-c)****. Cells were analyzed 72 hours after the transfection by double labeling with the anti-tau monoclonal antibody MN 7.51 (red fluorescence) and with the ISEL procedure for DNA fragmentation (green fluorescence). Undifferentiated NT-2 cells do not express endogenous tau, as revealed by a Western blot comparison of cells transfected with tau40* ***(d, lane B)*** *and non-transfected NT-2 cells* ***(d, lane A).***
The number of transfected cells undergoing apoptosis was evaluated 72 hours after the transfection. The ratio between the number of apoptotic cells observed with tau151-391 and that obtained with dGAE, is expressed as fold increase of the number of apoptotic cells and is shown in ***(e)*** *for both NT-2 and COS cell transfectants. Results from at least three independent transfection experiments for each cell line are reported. At least 500 transfected cells were scored for each construct. Bars represent standard errors.*

4.6. Further mapping of the apoptotic properties of tau fragments.

A further mapping of the apoptotic properties of tau fragments showed that, unlike the C-terminal end, the N-terminal region does not appear to influence the apoptotic properties of tau fragments. Thus, fragment Nt-dGAE (tau1-391), encompassing the first 150 aminoacids of tau (Fig. 4.6a), is as toxic as tau150-391 (Fig. 4.6b). Fragment tau-head, containing the N-terminal part alone (tau1-150), does not affect cell viability, and is as harmless as the dGAE fragment (Fig. 4.6b).

In a subsequent series of experiments, the size of fragment tau151-391 was reduced at its N- and its C-termini (Fig. 4.6c). N-terminal deletion of the first proline-rich region P1 and C-terminal shortening of at least one of the microtubule repeats region reduces the apoptotic capacity of the fragment, to a level intermediate between that of tau151-391 and of dGAE (Fig. 4.6d).

4.7. A caspase-3 cleavage site divides toxic from non-toxic fragments.

A transition point in the region immediately following residue 422, divides toxic from non-toxic fragments. This transition point is close to a putative cleavage site (Asp-421) for caspase-3, the main "effector" protease of apoptosis (Salvesen and Dixit, 1997). The vicinity of the transition point to this site suggested that the use of the latter by caspase-3 *in vivo* could generate apoptotic fragments from the normal protein. Tau contains at least another potential caspase3 recognition site, starting at residue 22.

In order to verify the impact on cell viability of tau fragments generated by the cleavage at either or both sites, the corresponding tau fragments tau1-422, tau22-441 and tau22-422 were expressed (Fig. 4.7a). The deletion of the 19 C-terminal residues of tau turns it

into an effector of apoptosis, regardless of the presence of the initial 21 residues (compare fragments tau1-422 and tau22-422, Fig. 4.7b). On the contrary, the expression of fragment tau22-441 yields no substantial apoptotic effect (Fig. 4.7b).

Tau contains three caspase-3 consensus recognition sequences, located respectively at residues 22-25 (site I), 338-341(site II) and 418-421(site III).

A previous work by our group (Canu et al., 1998) showed that, when *in vitro* translated tau was treated with caspase-3, the size of tau was reduced by ~ 5 Kda. This is consistent with cleavage at either or both sites I and III, while site II was unlikely to be used.

In order to elucidate which of the two sites is used by caspase-3, tau mutants were generated, in which sites I and III were individually abolished (Fig. 4.7c). *In vitro* translated tau40 and the two mutants tau22* and tau418* were treated with caspase-3 and analyzed by SDS-PAGE.

The results showed that only site III was employed by caspase-3, while sites I and II were not used (Fig. 4.7d). Indeed, the molecular weight shift observed upon incubation with caspase-3 was only observed for tau40 (Fig. 4.7d, lanes 2 and 3) and tau22* (Fig. 4.7d, lanes 5 and 6), while tau418* was unaffected by the protease (Fig. 4.7d, lanes 8 and 9). The cleavage by caspase-3 was partially inhibited by the specific caspase-3 inhibitor AcDEVD-CHO (Fig. 4.7d, lanes 4 and 7).

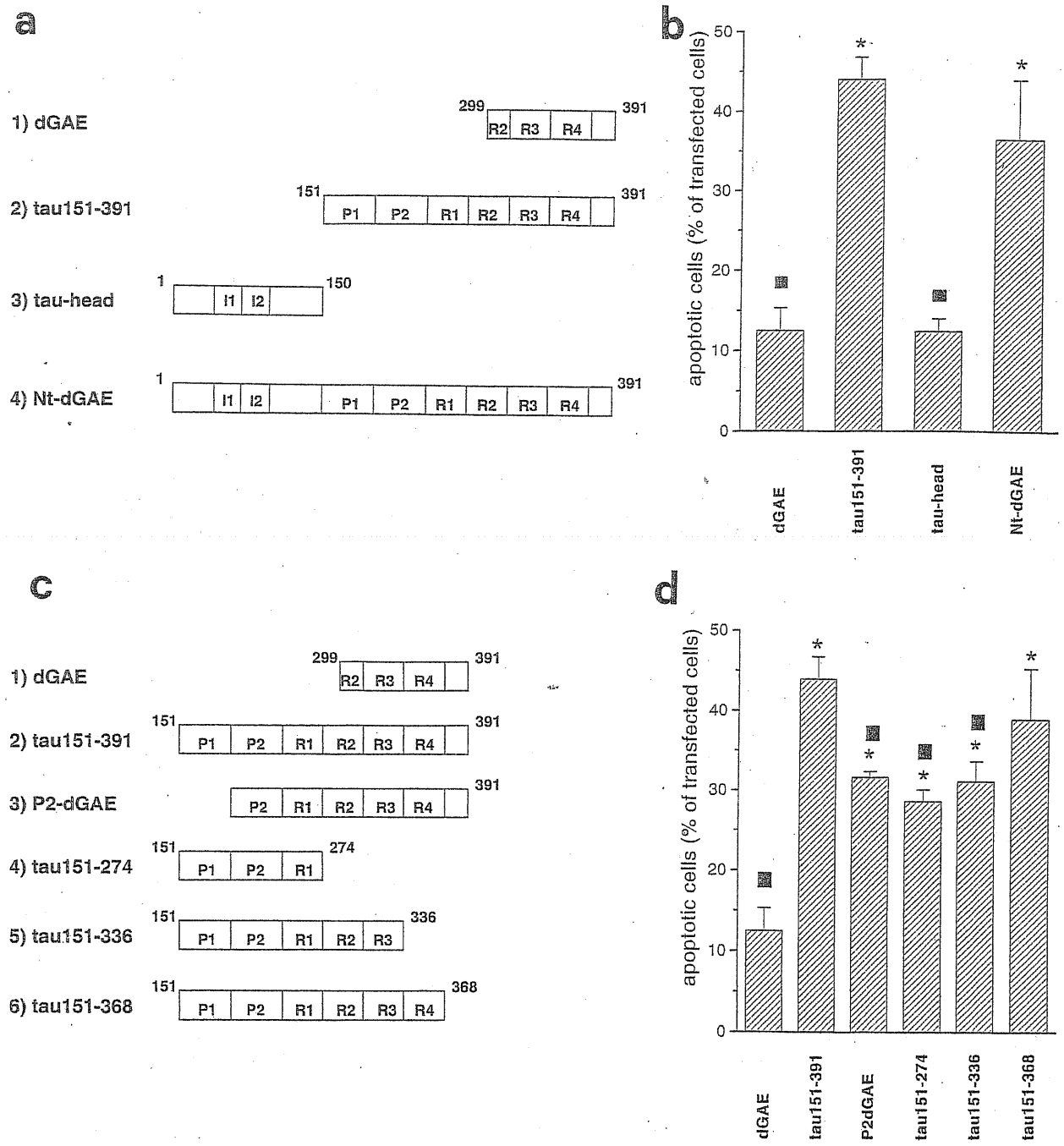
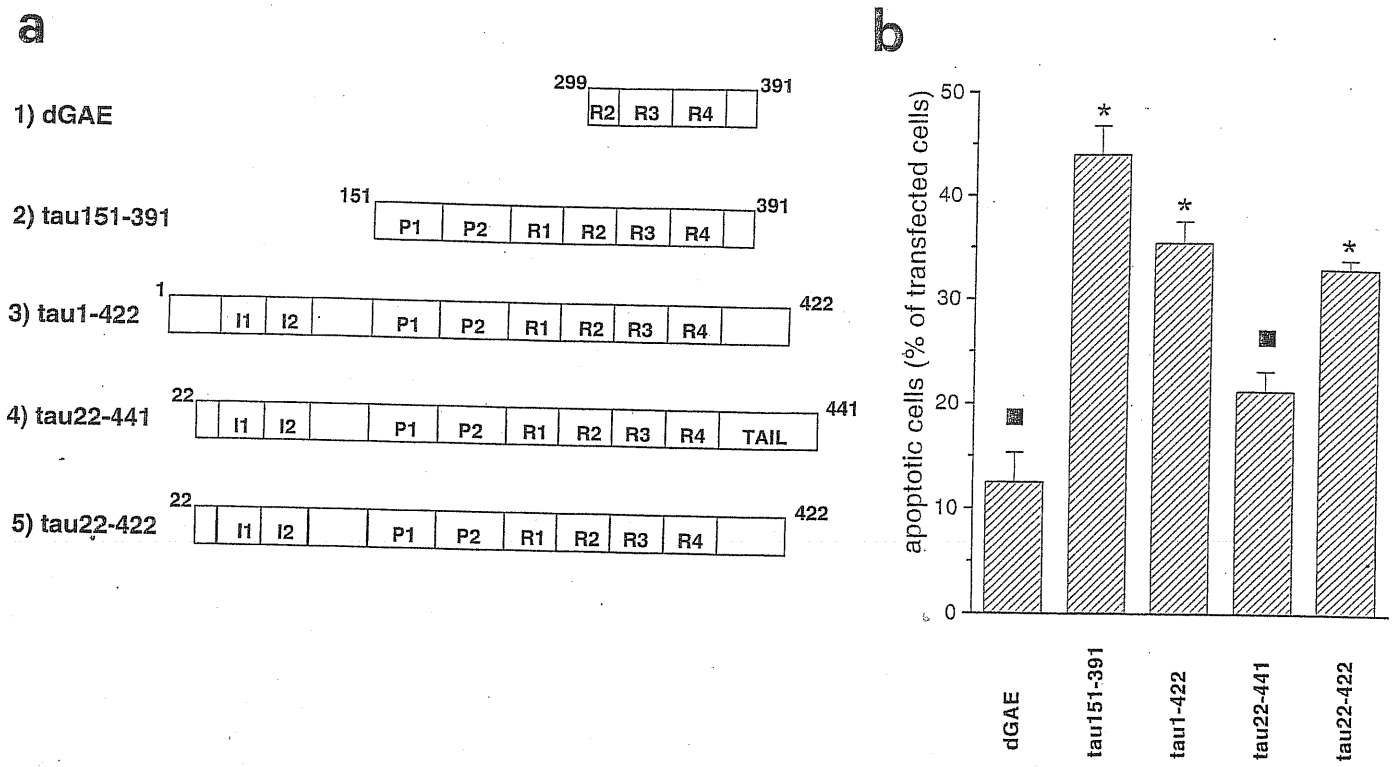
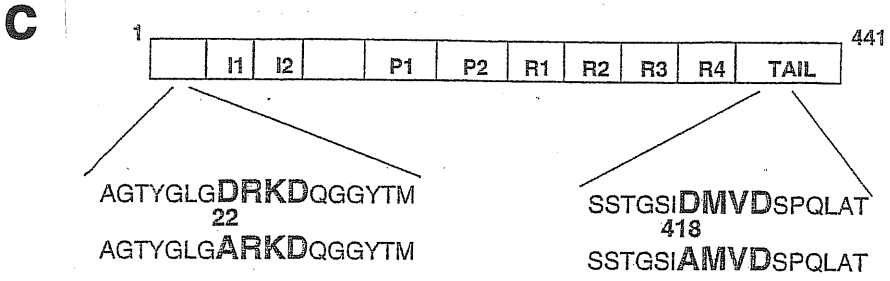


Fig. 4.6. *Further mapping of the apoptotic properties of tau fragments. The N-terminal portion of tau does not influence the apoptotic properties of tau fragments: constructs (dGAE, tau151-391, tau-head, Nt-dGAE,) and apoptosis histograms are shown in* ***(a)*** *and* ***(b)****, respectively. Microtubule repeats and proline-rich regions contribute to the apoptotic properties of tau: constructs (dGAE, tau151-391, P2-dGAE, tau151-274, tau151-336, tau151-368) and apoptosis histograms are shown in* ***(c)*** *and* ***(d)****, respectively.*
The percentage of COS cells undergoing apoptosis, upon transfection with the indicated tau constructs, was evaluated from at least three independent experiments. At least 500 transfected cells were scored for each construct. Bars represent standard errors. Asterisks indicate the statistical significance with respect to dGAE ($p < 0.01$, at least, except for tau151-368, with $p < 0.05$). Filled squares indicate the statistical significance with respect to tau151-391, with $p < 0.05$.


a
1) dGAE
299
391
R2
R3
R4
2) tau151-391
151
391
P1
P2
R1
R2
R3
R4
3) tau1-422
1
422
I1
I2
4) tau22-441
22
441
TAIL
5) tau22-422
b
apoptotic cells (% of transfected cells)
0
10
20
30
40
50
*
dGAE
tau151-391
tau1-422
tau22-441
tau22-422


c
1
441
I1
I2
P1
P2
R1
R2
R3
R4
TAIL
AGTYGLGDRKDQGGYTM
22
AGTYGLGARKDQGGYTM
SSTGSIDMVDSPQLAT
418
SSTGSIAMVDSPQLAT

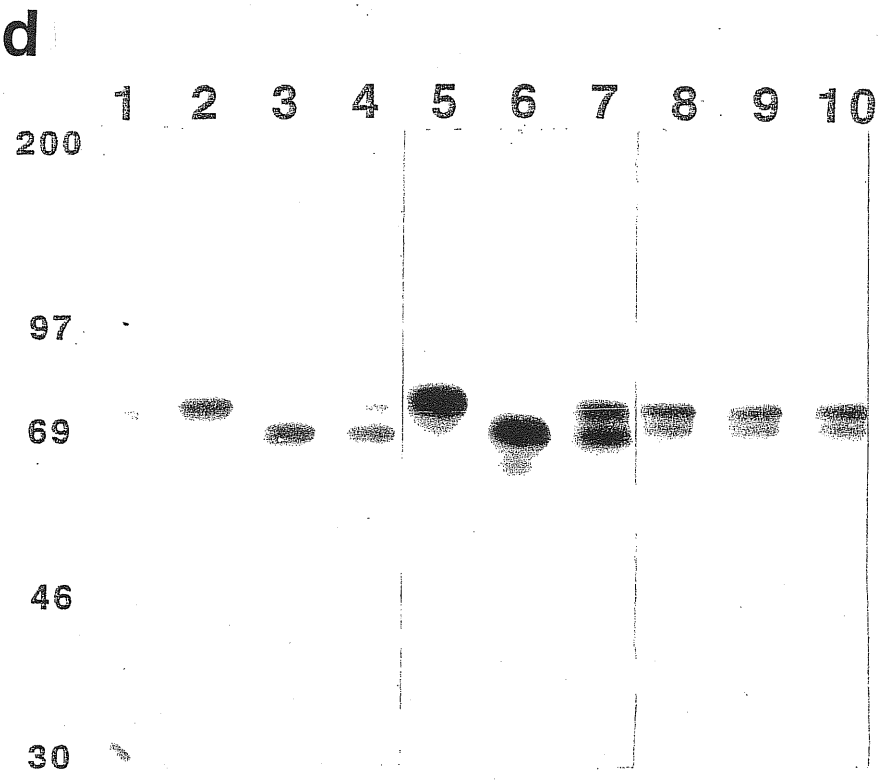

d
1
2
3
4
5
6
7
8
9
10
200
97
69
46
30

Fig. 4.7. *A putative caspase-3 cleavage site divides toxic from non-toxic tau fragments. Expression of tau fragments corresponding to cleavage at either or both the putative caspase-3 sites I and III: constructs (dGAE, tau151-391, tau1-422, tau22-441, tau22-422) and quantification of the apoptosis induced by these constructs are shown in* ***(a)*** *and* ***(b)****, respectively. The percentage of COS cells undergoing apoptosis, upon transfection with the indicated tau constructs, was evaluated from at least three independent experiments. At least 500 transfected cells were scored for each construct. Bars represent standard errors. Asterisks and filled circles over the bars indicate the statistical significance with respect to dGAE and to tau151-391, respectively, with $p < 0.001$ ($p < 0.05$ for tau151-432).*
In vitro cleavage of tau40 by caspase-3: ***(c)*** *Schematic representation of caspase-3 sites I and III on tau40. Caspase-3 consensus sequences I and III are shown (upper row), together with the mutations that were introduced to abolish them (lower row): tau22* (D22A) and tau418* (D418A).* ***(d)*** *In vitro cleavage by caspase-3 of wild-type and mutant tau40. Tau proteins were in vitro translated in the presence of 35 S-methionine, incubated with recombinant caspase-3, in the presence or absence of 150 μM AcDEVD-CHO, run on SDS-PAGE and autoradiographed. Lane 1: molecular weight marker; lanes 2-3-4: wild type tau40 incubated with proteolysis buffer (lane 2), caspase-3 (lane 3) and caspase-3 with AcDEVD-CHO (lane 4); lanes 5-6-7: tau22* incubated with proteolysis buffer (lane 5), caspase-3 (lane 6) and caspase-3 with AcDEVD-CHO (lane 7); lanes 8-9-10: tau418* incubated with proteolysis buffer (lane 8), caspase-3 (lane 9) and caspase-3 with AcDEVD-CHO (lane 10).*

4.8. Neuronal transfection.

Primary cultures of rat hippocampal neurons were transfected with the constructs tau151-391 and dGAE, sub-cloned into the ScFv-cyto eukaryotic expression vector. A variant of the Calcium Phosphate method, designed in the lab, had to be set up for neuronal transfections. Since neurons normally express tau, the presence of the two fragments could not be assessed by an anti-tau antibody. Thus, it was detected by means of the monoclonal antibody 9E10, recognizing a myc epitope located downstream of the cloned sequence in the ScFv-cyto expression vector (Evan et al., 1985; see Tab. 4A). At least 10% of neurons were stained by 9E10 72 hours after transfection (Fig. 4.8a and b). No apparent "apoptotic change" could be observed in neuronal cultures transfected with both constructs. When DNA fragmentation was examined, few nuclei were stained by the ISEL technique both in neurons expressing tau151-391 and in neurons expressing dGAE. Yet, in both cases, many cells seemed to detach from the substrate, after transfection .

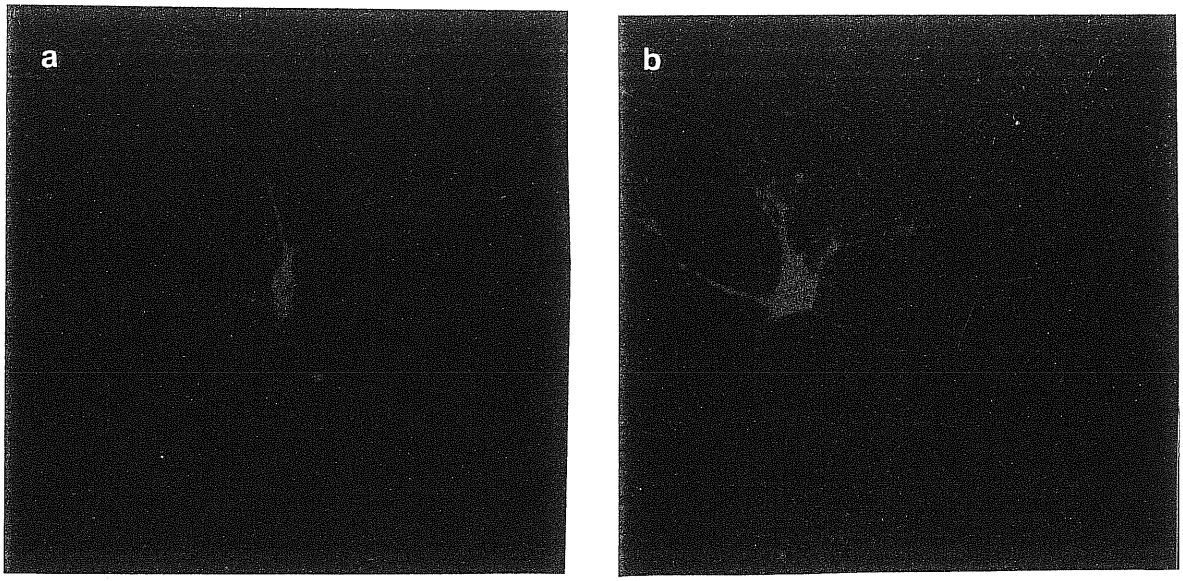


Fig. 4.8. *Neuronal transfections. Rat hippocampal neurons expressing constructs tau 151-391* ***(a)*** *and dGAE* ***(b)****, respectively. Red fluorescence corresponds to 9E10 staining.*

4.9. Production of anti-tau421 polyclonal antibody.

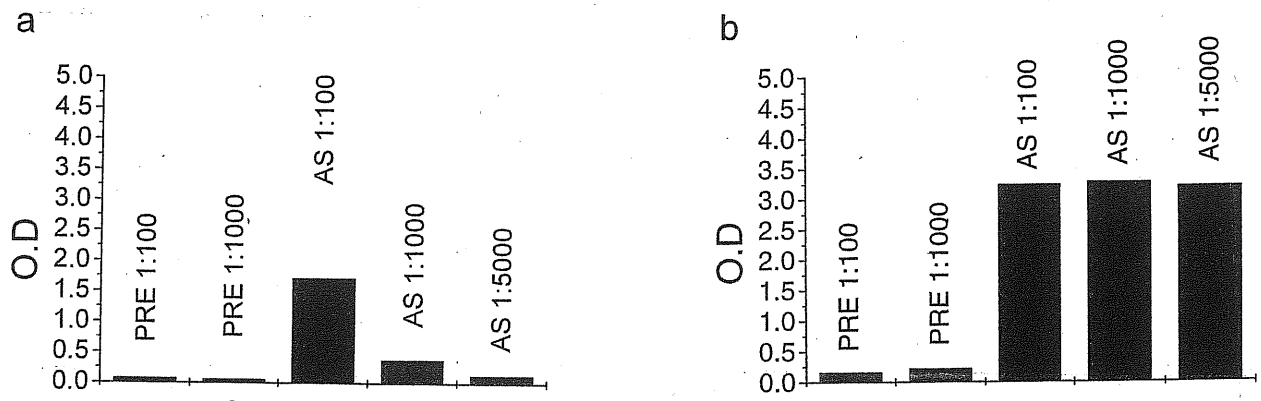
On the basis of results shown in the previous paragraph, tau is both a substrate and an effector of apoptosis. Caspase-3 is able to cleave tau in vitro and is likely to cleave tau in a neuronal culture model of apoptosis (Canu et al., 1998). A working hypothesis has been consequently drawn, according to which cleavage of tau by the apoptotic protease caspase-3 is enough to turn tau into an effector of apoptosis. In order to test directly this hypothesis, an antibody "probe" was produced, specifically reacting with Tau molecule ending at Asp-421, the residue at which caspase-3 actually cleaves tau in vitro. Two peptides (CT(+) and CT(-), see Fig. 3 in the previous chapter) were conjugated to bovine serum albumin (BSA), corresponding respectively to 12 and 13 amino-acids of tau tail and ending respectively at Asp-421 and Ser-422. CT(+) was used for rabbit immunization. Enzyme-Linked Immuno-Sorbent Assay (ELISA) was performed with several antisera (first bleed corresponding to primary response plus four subsequent boosts; pre-immune serum was employed as negative control). The primary response already contains a high titer of antibodies specifically recognizing the BSA-CT(+) conjugate (Fig. 4.9b). After the third boost (Fig. 4.9g-h), the response against BSA-CT(+) seems to decrease at the highest dilutions (1:1000, 1:5000), as compared with the primary response. On the other hand, an undesired response against the carrier protein BSA progressively increases from the first to the fourth boost (Fig. 4.9c, e, g, i), at least at the lowest dilution tested (1:100). The antiserum obtained after the second boost was partially purified as it is described in Methods (3.8), by absorbing it with the BSA-CT(-) peptide, with the purpose of creating a probe that specifically recognizes tau truncated at Asp-421. At the end of the procedure, the polyclonal antibody (anti-tau421) was tested in

ELISA for recognition of the BSA-CT+ conjugate as compared with the BSA-CT(-) one. Results are summarized in Tab. 4D. For 3 different dilutions of the antibody and 2 different coating concentrations of the conjugates, recognition is appreciably biased towards BSA-CT(+).

Western blot experiments showed that anti-tau421 antibody was not able to react with recombinant full-length tau, while it could recognize recombinant tau terminating at Asp-421 (Fig. 4.9k, lanes B and A respectively). MN7.51 was used as a control anti-tau antibody.

4.10. Preliminary results with the anti-tau421 antibody.

The new immunochemical reagent was used in a western blot experiment performed with two samples (one AD brain extract and one control brain extract, see Table 4B). This preliminary result is shown in Fig. 4.10. At least three bands were visualized by the antibody in the lanes (A and B), corresponding to either sample. The intermediate band seems to correspond to tau protein, as it is revealed by MN7.51 on a control brain extract (Fig. 4.10, lane C).



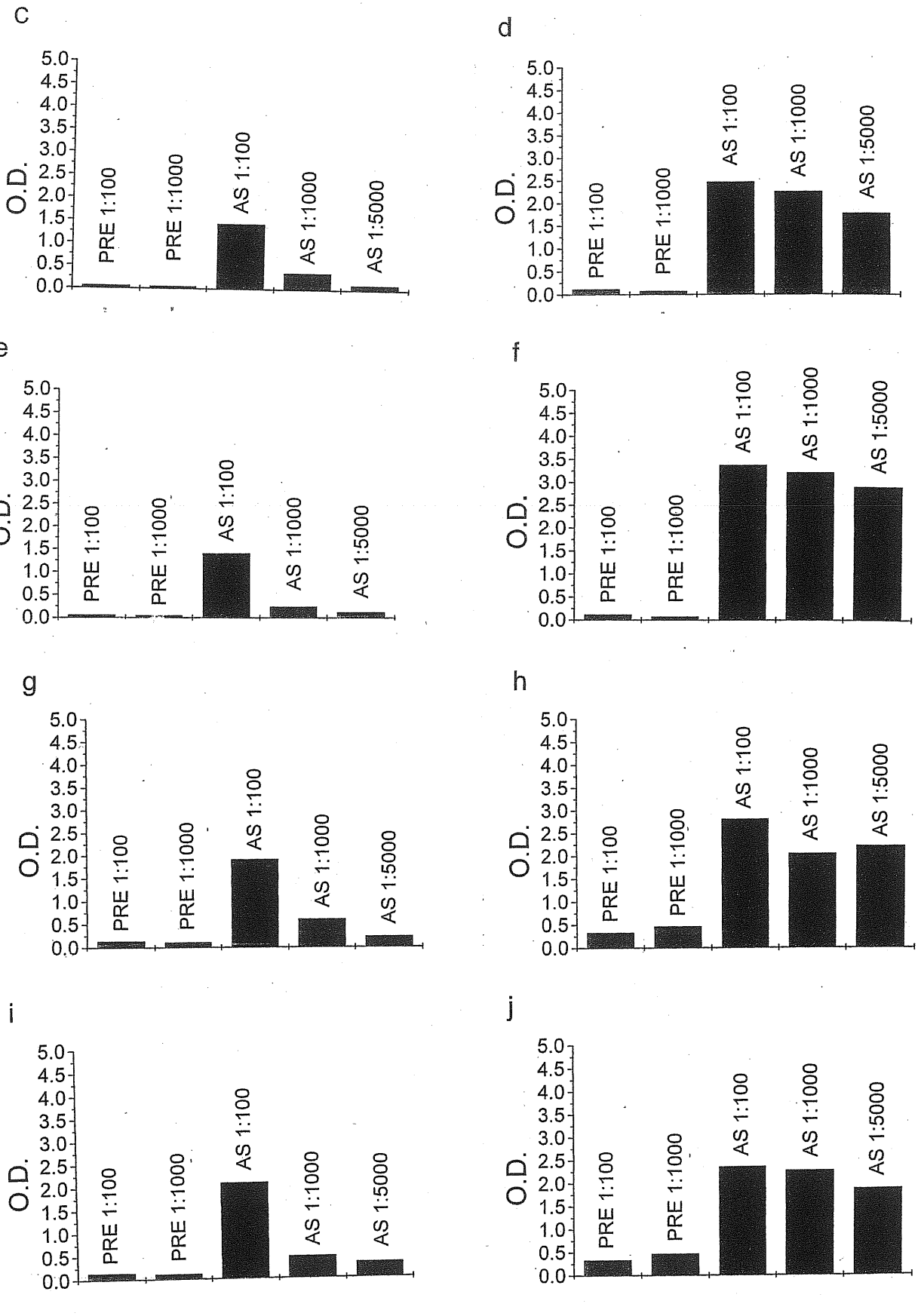

c
d
e
f
g
h
i
j
O.D.
PRE 1:100
PRE 1:1000
AS 1:100
AS 1:1000
AS 1:5000
k
A
B
C
D
73
43
32

Fig. 4.9. *Enzyme-Linked Immuno-Sorbent Assay (ELISA) was performed on several antisera. Histograms show O.D (optical density) measures (Y axis) plotted against different dilutions of antiserum (AS) and pre-immune (PRE) control serum (X axis).*

(a) *First bleed corresponding to primary response (Coating with BSA)*

(b) *First bleed corresponding to primary response (Coating with BSA-CT(+) conjugate).*

(c) *First boost. Response to BSA.*

(d) *First boost. Response to BSA-CT(+) conjugate.*

(e) *Second boost. Response to BSA.*

(f) *Second boost. Response to BSA-CT(+).*

(g) *Third boost. Response to BSA.*

(h) *Third boost. Response to BSA-CT(+).*

(i) *Fourth boost. Response to*

(j) *Fourth boost. Response to BSA-CT(+).*

(k) *Western blot experiment showing that, after the immunoaffinity procedure the anti-tau421 polyclonal antibody does not react with recombinant full-length tau (lane B), while it can recognize recombinant tau terminating at Asp-421 (lane A). MN7.51 is able to recognize both recombinant proteins (lane C and D).*

	Antibody dilution 1:500	*Antibody dilution 1:1000*	*Antibody dilution 1:5000*
BSA-CT(-) 10 μg/ml	*0.121*	*0.082*	*0.046*
BSA-CT(+) 10 μg/ml	*0.199*	*0.142*	*0.053*
BSA-CT(-) 1 μg/ml	*0.112*	*0.077*	*0.047*
BSA-CT(+) 1 μg/ml	*0.187*	*0.118*	*0.063*

Tab. 4D. *Summary of the absorbance measures obtained with three different dilutions of the antibody and two different coating concentrations of the two conjugates (BSA-CT(-) and BSA-CT(+)), once the antibody was pre-adsorbed on the BSA-CT(-) conjugate. Values are reported in Optical Density (O.D.) units. Background response minor to 0.05 O.D. units.*

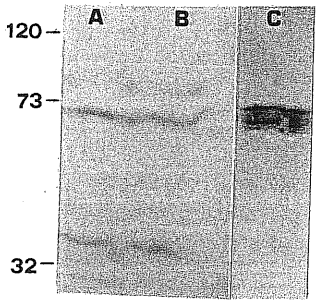


Fig. 4.10. *Immunoblot experiment with anti-tau421 on brain extracts AD2 and NDC3 (see Tab. 3B). Lane A: AD2; lane B: NDC3.*
Lane C: NDC3 western blot profile with MN7.51.
Molecular weight in Kda.

5. DISCUSSION

5.1. Tau polymers in Alzheimer's disease.

The main common feature of pathological tau proteins in AD and all sporadic and familial tauopathies is their aggregation into insoluble polymers with various filament morphologies. The presence of these polymers results in a typically "smeared" western blot profile. The smears seem to consist largely of the carboxy-terminal portion of tau and ubiquitin (Delacourte and Buée, 1997).

I used three anti-tau antibodies (MN7.51, PHF-1 and Tau-1, see Tab. 4A in the previous chapter) to verify if the corresponding immunoblot pattern could be diagnostic of AD pathology.

MN7.51 is a pan-tau monoclonal antibody recognizing all six tau isoforms (Novak et al., 1991; Jakes et al., 1991). Here it is shown that MN7.51 western blot profile allows distinguishing AD brains from control ones, thanks to the presence of tau polymers in the high molecular weight range. A similar western blot pattern is obtained with antibodies that recognize hyperphosphorylated PHF-tau, like PHF-1 (Greenberg et al., 1992), but not with tau-1, recognizing a dephosphorylated epitope in the proline-rich region of tau (Papasozomenos and Binder, 1987).

The simplest and most direct explanation for this difference between MN7.51 and tau-1 western blot patterns is that AD tau making up polymers is in a hyperphosphorylated state and can be visualized only by antibodies recognizing either phosphorylated or phosphorylation-independent epitopes. These high molecular weight MN7.51 immunoreactive bands are not abolished by SDS, DTT and boiling, and must therefore reflect the presence of tightly bond aggregates of tau full-length molecules or fragments (Delacourte and Buée, 1997).

The nature of these AD-specific aggregates revealed by MN7.51 is not completely clear. They are likely to correspond to the SDS-soluble fraction of PHFs (the so-called "Dispersed PHFs": Greenberg and Davies, 1990; Lee et al., 1991; Goedert et al., 1996a). The presence of ubiquitinated tau is another possibility that can be considered, although not necessarily alternative to the previous one, since tau ubiquitination seems to be preceded by tau aggregation (Morishima-Kawashima et al., 1993; Delacourte and Buée, 1997).

These experiments do not identify AD-specific low molecular weight tau fragments. In fact bands between 30 and 45 Kda are seen by Tau-1 in every sample and are likely to reflect degradation products that are generated during PMD or sample preparation. Similar bands are less evident with MN.7.51, (with the partial exception of 2 AD cases). This difference between tau-1 and MN7.51 western-blot profiles could be due to partial occlusion of MN7.51 epitope. Total occlusion of the MN7.51 epitope is known to occur in the PHF-bound tau protein (Harrington et al., 1991), in which the epitope sequence is concealed and inaccessible to binding of antibodies. This result might suggest that tau fragments containing the MN7.51 epitope are prone to aggregation and are therefore rather rare as soluble monomers. Of course, this finding should be confirmed by a broader study, using a reasonably higher number of brain extract samples, with more sensitive detection techniques (ECL, for instance).

5.2. Tau truncation as an early event in the development of neurofibrillary pathology.

Evidence has accumulated showing that AD tau is abnormally proteolysed or fragmented (Nieto et al., 1990; Novak et al., 1991; Goedert et al., 1992a; Novak et al., 1993; Novak, 1994; Johnson et al., 1997).

The pronase-resistant core of PHFs was shown to be made of a group of 12 Kda fragments of tau (Wischik et al., 1988a and b; Jakes et al., 1991) and was employed to raise the monoclonal antibody MN423 (Wischik et al., 1988a; Novak et al., 1989). This antibody stains all the main neuropathological hallmarks of AD, including intracellular granular and neurofibrillary structures (Bondareff et al., 1991; Mena et al., 1991). Immunoelectronmicroscopy studies showed that MN423 decorates PHF isolated with or without pronase treatment (Novak et al., 1993). MN423 does not recognize full-length tau, but binds to the 12 Kda fragments of core PHFs as well as to all tau fragments terminating at Glu-391 (Novak et al., 1993). On this basis it was hypothesized that tau is endogenously truncated in AD brain at Glu-391 (Novak et al., 1993; Novak, 1994). This residue, that invariably represents the last amino acid of the 12 Kda fragments, was defined as a "truncation point". The truncated forms of tau fail to support microtubule assembly *in vitro* (Fasulo et al., 1996). Moreover, they are unable either to bind to microtubules or to induce their bundling, when expressed *in vivo* (Fasulo et al., 1996). One of the aims of the present study was to further investigate the distribution of MN423-immunoreactivity in AD neurons. Results support the idea that truncation of tau is an early event in AD cellular neuropathology. In fact, by MN423 immunohistochemistry, intense granular staining was shown inside of neurons that did not exhibit any overt neurofibrillary changes, suggesting that truncation of tau precedes the formation of tangles. In other words cytoplasmic accumulation of MN423-immunoreactivity is likely to represent an early pre-tangle stage in the development of neuropathology at single cell level. Of course, the regional distribution of such kind of cellular staining should depend on the disease stage (early stage at single cell level does not necessarily mean early stage in disease progression). This study took into consideration cortical brain tissue from AD patients

severely affected by the disease. Yet, in principle, this type of labeling should be reproduced with brain tissue from patients suffering from "pre-symptomatic" AD, as long as brain regions are examined that are affected by the disease before isocortical areas (see paragraph 1.1).

In order to rule out the possibility that tau gets truncated due to long PMD, brains with different PMD were employed for this study (Tab. 4C in the previous chapter). The span of delay was from 3hr 45min up to 17 hr (Tab. 4C in the previous chapter). The granular staining with MN 423 was already present in the AD brain with PMD as short as 3hr 45 min. However, NDC brains with PMD spanning from 3.00 to 7.20hrs did not show any PMD-dependent staining with 423. On the basis of these data it can be concluded that tau truncation, as identified by MN423, is unlikely to be produced by a postmortem proteolytic event and shows no correlation with PMD of the brains studied. Thus, occasional MN423-staining of neurons, observed in NDC brains without any neurofibrillary pathology, could be considered as pre-AD stage or as a sign of "pathological" aging.

5.3. Tau truncation and neuronal death in AD.

It is well documented that neurons die in AD (Su et al., 1994; Cotman and Anderson, 1995; Lassmann et al., 1995; Anderson et al., 1996; Cotman and Su, 1996). To assess the impact of tau truncation on neuronal degeneration in AD, the ISEL technique was employed in order to identify nuclei with fragmented DNA, as DNA fragmentation is a commonly accepted marker of cell death (Gavrieli et al., 1992). In accordance with previous results (Su et al., 1994; Cotman and Anderson, 1995; Lassmann et al., 1995) few or none ISEL-positive nuclei were found out in sections from NDC brains, while numerous nuclei were labeled in sections from AD brains (Fig. 4.3e in the previous

chapter). Adjacent sections from AD brain tissues showed that the regional patterns of 423 and ISEL staining were similar. MN423/ISEL double labeling studies demonstrated that truncation of tau and cell degeneration are correlated.

The scenario in which cell death precedes truncation becomes rather unlikely, since a significant subpopulation of MN423-positive neurons does not display any sign of DNA fragmentation in their nuclei (Tab. 4C in the previous chapter). Nevertheless, a small number of ISEL-positive nuclei that does not show MN423 immunoreactivity in their nuclei could be found as well. This result can be accounted for by the presence of dying glial cells (Su et al., 1994; Cotman and Anderson, 1995; Lassmann et al., 1995) in which truncation of tau has never been shown so far. A parallel explanation is supplied by the "Intermediate Truncation" hypothesis, discussed in the next paragraph. In this view, fragments ending at Glu-391 might be the final outcome of a cleavage process with many possible intermediate products. During C-terminal proteolysis, Glu-391 might constitute an accumulation point that proteases are not able to overcome. According to the "Intermediate Truncation" hypothesis, all tau fragments with a carboxy-terminus between Glu-391 and Asp-421 are potentially "toxic". Of course, the presence of these fragments inside neurons is not detectable with MN423, unless the carboxy-terminal residue is Glu-391.

This work on Tau truncation and DNA fragmentation, published by NeuroReport in 1997, represents one of the earliest studies in which markers for pathological forms of tau are shown to colocalize with neuronal death.

5.4. Tau as "a substrate and an effector of apoptosis".

Tau fragments and neuronal death.

Is tau truncation a mere by-product of cellular degeneration before neuron dies, or is it an early fundamental event in the cascade of molecular events ultimately leading to neuronal death? To answer this question, our group has recently investigated the impact of truncated tau on cell viability. The main outcome of that preliminary study was that a fragment of tau ending at Glu-391 and encompassing the two proline rich regions is able *per se* to induce apoptosis upon expression in COS cells (Fasulo et al., 1998). This fragment (tau 151-391) is longer than the 12 Kda fragments described by Novak and co-workers, having the same carboxy-terminus (Wischik et al., 1988a and b; Novak, 1994).

In order to systematically map the apoptotic properties of tau, a set of fragments, designed around tau151-391, and based on the longest tau isoform found in human brain (tau40), was engineered and expressed in cells. A class of tau fragments was identified, displaying apoptotic capacity when expressed in different cell contexts. The cell death process was defined as apoptosis according to the following criteria: i) membrane blebbing plus nuclear condensation and breakdown, ii) DNA fragmentation, iii) processing of PARP (for the latest point, see Fasulo et al., 1998).

Most of the experiments were performed in COS cells. Yet, the apoptotic capacity of Tau151-391 was clearly shown in NT2 cells as well. This is a human cell line, which displays characteristics expected of a human committed neuronal progenitor cell (Pleasure et al., 1992; Pleasure and Lee, 1993). Furthermore, the fragment tau151-391 induces membrane blebbing and nuclear pycnosis in human SH-SY5Y neuroblastoma cells (Luisa Fasulo, personal communication). These data allow thinking that the results described in the previous sections and here commented, may be grossly

independent of the cell line context, revealing a general mechanism involving tau. As tau is naturally expressed in neurons, this is the main cell type in which such mechanism should take place. Surprisingly, expression of the apoptotic fragment tau151-391 in primary cultures of rat hippocampal neurons was not followed by any overt DNA fragmentation in the nuclei of adherent cells. Three main explanations can account for this result:

1) Endogenous tau exerts a "protective" role.
2) There is an experimental bias against apoptotic cells, detaching from the substrate.
3) Rat hippocampal neurons represent a remarkable exception as far as the apoptotic properties of tau fragments are concerned.

Experimental modulation of the levels of endogenous tau either with antisense oligonucleotides (Caceres and Kosik, 1990) or using cultures derived from tau K.O mice (Harada et al., 1994) would allow testing hypothesis 1.

Determination of DNA fragmentation by agarose gel electrophoresis after collection of cells detached from the substrate and/or careful cell density counts (Mettling et al., 1995) after transfection would allow testing hypothesis 2.

Combination of tau fragments expression with sub-threshold pro-apoptotic stimuli could also be instructive.

Mapping of the apoptotic properties of tau fragments in COS cells. Tau as a substrate for caspase-3. Protective role of the last 19 amino acids of the protein.

Deletion mapping experiments in COS cells showed that the shortest fragment with full apoptotic capacity is tau151-368, which comprises the proline rich regions P1 and P2, as well as the microtubule binding domains R1, R2, R3 and R4. The N-terminal portion of the molecule does not appear to have any relevant effect in determining the apoptotic capacity of tau fragments. The longest fragment with full apoptotic capacity

is tau1-422, which only lacks the 19 C-terminal residues. On the contrary, the presence of the latter aminoacids seems to exert a "protective" effect. In fact, a "transition" point was observed around residue 422, with C-terminal extension yielding a non-toxic protein, like full-length tau. This transition point overlaps with one of the three putative caspase-3 cleavage sites present in tau protein (Fig. 4.7c). According to the experimental data obtained by Dr. V. Verzillo in our lab (Fig. 4.7d), this site is the only one that can be effectively used by caspase-3 *in vitro*. On the other hand, tau can be cleaved by caspases in a culture model of neuronal apoptosis (Canu et al., 1998) and fragments of tau ending at Glu-391 (as revealed by MN423 immunoreactivity) co-localize with DNA fragmentation in AD neurons (Ugolini et al., 1997; see previous paragraph).

Thus, the absence of the 19 C-terminal residues of tau causes a dramatic change in the properties of the protein, turning it into an apoptotic effector. Various mechanisms can underlie this change: i) cleavage of the C-terminal peptide may cause a structural rearrangement of the remaining part of the molecule, possibly by preventing an intra-molecular interaction; ii) the C-terminal part of tau might be involved in interactions with other cellular proteins, including tau itself. In this case, cleavage of this peptide would destroy these interactions.

As a crucial step towards a mechanistic understanding of the function of the C-terminal peptide, the NMR solution structure of tau423-441 was determined by Paolo Viglino and co-workers, collaborating with our group (Fasulo et al., submitted). These data represent the first piece of experimental structural information on any part of tau. The two main features of the solved structure are: i) the peptide displays a well defined right-handed α-helix structure (in TFE); ii) the helical conformation is stabilized by a C-terminal capping motif, involving the glycine residue at the

penultimate position (Fig. 5a and b). Hydrophobicity is very unevenly distributed along tau sequence, being substantially concentrated at the tubulin-binding repeats and at the C-terminal fragment (Ruben et al., 1991). Therefore, the sharp conformational propensity of tau423-441 for a C-capping-stabilized helical geometry suggests that this part of tau is potentially involved in intra- or inter-molecular protein-protein interactions (Yanagawa et al., 1998). The structure exhibited by the tau423-441 may play a role in the proteolysis of tau in the cell. Thus, an exposed frayed helix C-end would hardly escape the processing by cellular carboxy-peptidases, leading to progressive unwinding of the helix. By contrast, a blocked helix should exhibit a greater resistance against cleavage by carboxy-peptidases. Cleavage of tau at residue 421 by caspase-3 could make the protein more prone to further proteolysis, generating apoptotic fragments. In this view, fragments ending at Glu-391, identified in AD brain by Novak and coworkers (Wischik et al., 1988a; Novak et al., 1989; Bondareff et al., 1991; Mena et al., 1991; Novak et al., 1993; Ugolini et al., 1997) might be the final outcome of a cleavage process with many possible intermediate products ("Intermediate Truncation" hypothesis). During C-terminal proteolysis, Glu-391 might constitute an accumulation point that proteases are not able to overcome. Accumulation of fragments ending at this residue might be due to an intrinsic resistance to proteolysis of the upstream region, which has a predicted α-helical structure (Ruben et al., 1991), or to a concomitant pathological aggregation process, involving the microtubule binding domains (Schweers et al., 1995).

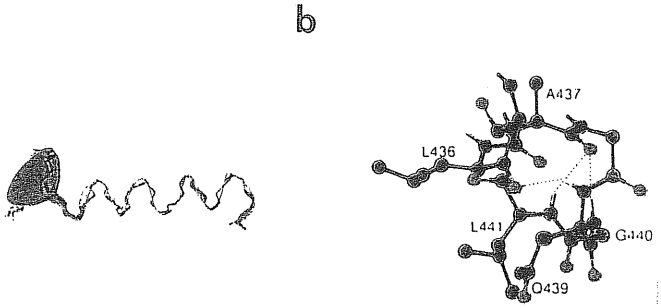


Fig. 5. *Solution structure of the hybdrophobic C-terminal fragment 423-441 of tau. The solution structure of the hydrophobic C-terminal fragment 423-441 of tau was determined by ^{1}H NMR and restrained modeling in TFE/water mixture.* ***(a)*** *Under these conditions tau423-441 folds into a regular right-handed α-helix geometry, extending from Ala-426 up to Gln-439.* ***(b)*** *Helix C-capping motif of tau423-441 as obtained from NMR-restrained calculations. Motif involves Gly-440 and Leu-441 (Fasulo et al., submitted).*

A vicious feed-forward mechanism involving tau.

In a complementary set of experiments our group has shown that, in a culture model of neuronal apoptosis, the production of tau fragments is an early and reversible event, with caspase-3 and calpain identified as proteases contributing to tau cleavage *in vivo* (Canu et al., 1998).

Altogether, these data allow speculating about the duplex role of tau as a substrate and an effector of apoptosis. Many neurodegeneration-associated gene products have been shown to be substrate for apoptotic proteases of the caspase family (Salvesen and Dixit, 1997). These include huntingtin and other polyglutamine expansion disease-associated proteins (Nasir et al., 1996; Goldberg et al., 1996; Wellington et al., 1998) as well as the AD-associated proteins presenilin-1, presenilin-2 and APP (Kim et al., 1997; Loetscher et al.,1997; Van de Craen et al.,1999; Barnes et al., 1998; Gervais et al., 1999; Weidemann et al., 1999). On the other hand, many of the neurodegeneration-associated gene products induce a pro-apoptotic phenotype in cultured cells. These include polyglutamine expansion disease-associated proteins (Igarashi et al., 1998; Sanchez et al., 1999), mutant APP (Yamatsuji et al., 1996),

presenilins (Mattson and Guo, 1997), and amyloidogenic Aβ-peptides (Yankner et al., 1989; Loo et al., 1993, LaFerla et al., 1995).

On this basis, tau may be part of a vicious circle, in which not only it is a substrate for proteases activated by sub-threshold pro-apoptotic stimuli, but also induces apoptosis by virtue of the fragments generated by those proteases.

In this way, a positive-feedback intracellular loop involving tau would be generated, similar to the extracellular cycle that has been described for the neurotoxic fragments of APP generated by caspase-3 cleavage of APP itself (Gervais et al., 1999; Yuan and Yankner, 1999). According to this model, caspase-3 contributes to the formation of APP neurotoxic fragments and, upon release in the extracellular space, these induce the degeneration of neighbouring cells. This form of self-propagating process has been proposed to apply to other proteins involved in neurodegenerative diseases, including Huntington's disease (Ona et al., 1999; Yuan and Yankner, 1999). It may be speculated that cleavage of tau and APP respectively represent two main intracellular and extracellular steps whereby a neurodegeneration "wave" slowly propagates inside AD brain. Apoptosis is generally considered to be an all-or-none phenomenon. As previously discussed in paragraph 1.9, it is difficult to think that caspase-mediated protein cleavage can underlie a chronic, delayed neurodegenerative process. One would think that caspase-activated phenomena would be rather acute than prolonged and chronic. One intriguing possibility is that sporadic apoptosis, perhaps as a result of stress or even the daily wear-and-tear of a normal ageing process may act as the seed of future degeneration by producing neurotoxic products in a very local fashion. Activation of the apoptotic machinery could be focal and might not reach the threshold beyond which the cell death process becomes irreversible, perhaps due to up-regulation of anti-apoptotic gene products (Su et al., 1996). It is therefore possible

to imagine a scenario in which a state of cellular "suffering" may spread slowly starting from local pro-apoptotic stimuli of undefined and possibly various nature. Dying neurons would start to appear only when a broad population of cells had been brought to a "threshold" state.

5.5. Tau fragments, aggregation into PHFs and cell viability: a growingly complicated picture.

The mechanisms whereby tau protein is modified to take part in AD neurofibrillary pathology and aggregation *in vivo* are fundamentally unknown. Tau shows distinct post-translational changes in AD brain, including hyperphosphorylation, oxidation, ubiquitination and proteolysis (for a review, see Mandelkow and Mandelkow, 1998). For many years it has been thought that tau phosphorylation is the main event leading to neuronal degeneration with formation of neurofibrillary tangles and eventually to cell death (Mandelkow and Mandelkow, 1993; Goedert et al., 1996a). Yet, there is no experimental evidence linking hyperphosphorylation of tau to PHF assembly (Goedert et al., 1998). In fact, synthetic PHF-like structures can be obtained *in vitro* in a phosphorylation-independent way (Goedert et al., 1996b; Perez et al., 1996; Hasegawa et al., 1997). In a recent study the relationship between tau phosphorylation by a number of kinases (MARK, PKA, MAPK, GSK3) and tau assembly into PHFs has been investigated (Schneider et al., 1999). The authors conclude that hyperphosphorylation does not prime tau for PHF assembly. On the contrary, it protects the protein against aggregation, suggesting that hyperphosphorylation of tau is unlikely to be directly responsible for the process of pathological aggregation.

Abnormal proteolysis of tau is seen in AD brain (Nieto et al., 1990; Novak et al., 1991; Goedert et al., 1992a; Novak et al., 1993; Novak, 1994).

It has also been proposed that tau fragments could represent the minimal unit for aggregation of PHFs (Novak, 1994; Bednarsky and Lynch, 1996; Kenessey et al., 1997). In fact, truncated forms of tau are unable either to bind to microtubules or to induce their bundling *in vivo* (Fasulo et al., 1996). Yet, insoluble polymers of truncated tau have not been described, upon over-expression of the 12 Kda PHF-core tau in COS cells (Fasulo et al., 1996).

Is there a relationship between tau aggregation and neuronal death in AD? The question is still unclear. The cell-cycle-associated prolyl-isomerase Pin1 binds to PHF-tau, resulting in depletion of soluble Pin1 and, possibly, in mitotic arrest and apoptotic cell death (Lu et al., 1999). Thus, sequestration of Pin1 into PHFs could contribute to neuronal death. Topographic association has been described between DNA fragmentation and neurofibrillary pathology, as revealed by antibodies recognizing PHF-phosphorylated tau (Sugaya et al., 1997). In contrast, previous reports have underlined that a large subset of nuclei displaying DNA fragmentation is not associated with either intracellular neurofibrillary tangles or pre-tangle neurons (Su et al., 1994). This observation is consistent with the finding that neuronal loss in AD brain can be remarkable in areas, like visual cortex, where neurofibrillary pathology is almost absent (Leuba and Kraftsik, 1994), raising the possibility that AD neuronal death may occur without development of the neurofibrillary pathology. On the other hand, two neuron populations have been identified in AD brain: one dying via a Bax-dependent pathway, the other primarily affected by neurofibrillary pathology (Nagy and Esiri, 1998; Nagy et al., 1998). Moreover in tangle-bearing neurons the anti-apoptotic bcl-2 gene product is down-regulated while it is up-regulated in neurons displaying DNA fragmentation (Su et al., 1996).

This work shows colocalization of nuclear DNA fragmentation and cytoplasmic tau molecules truncated at Glu-391, suggesting that tau truncation is an early process in AD neuronal degeneration. The identification of a set of tau fragments inducing apoptosis in various cellular contexts highlights the possibility that abnormal proteolysis of tau affects cell viability in a more general and profound fashion than it was previously thought. Tau cleavage by caspase-3, as described in the previous paragraph, could produce a double effect: induction of apoptosis in cells that are unable to undergo neurofibrillary change and promotion of PHF formation in tangle-bearing cells.

A growingly complicated picture is emerging in which the relationship between presence of abnormal forms of tau protein (hyperphosphorylated, truncated, proteolysed etc.), formation of PHFs and neuronal loss, is not so simple as it was previously imagined.

5.6. Derivation of a polyclonal anti-tau421 antibody. Future perspectives.

If cleavage of tau by caspase-3 at Asp-421 is an early event occurring in AD cellular pathology, fragments terminating at this residue should be found in AD neurons.

As a conclusion of this Ph.D. work, the derivation of a polyclonal antibody was undertaken, in order to obtain a probe selectively recognizing all and only those tau molecules cleaved at Asp-421 (caspase-3 site III). This probe should allow direct testing of the hypothesis described in paragraph 5.4 ("tau as a substrate and an effector of apoptosis"). The polyclonal antibody (anti-tau421) produced by the procedure described in the previous sections ("Methods" and "Results") is able to discriminate between recombinant tau molecules ending at Asp-421 and their full-length counterparts. This antibody was used as immunochemical reagent on two

samples (AD brain extract and age-matched control brain extract). According to this very preliminary result, tau is cleaved at Asp-421 in both cases. Of course, it is necessary to examine many more samples before being able to comment this finding and draw conclusions. However, if this result were confirmed in future, it could be explained in two ways:

i) Cleavage of tau at Asp-421 is a very early process occurring both in AD and "normal" aging (but does a really "normal" aging exist?).

ii) Cleavage of tau at that site occurs during PMD or sample preparation.

Extraction of rat brain tissue performed with varying PMD, in the presence of caspase inhibitors, followed by Immunoblot with the anti-tau421 antibody, should allow testing the latter "trivial" possibility. The anti-tau421 antibody is going to be used in immunohistochemistry (on AD and normal brain sections) and in further immunoblot experiments with human (AD and control) brain extracts. Moreover, immunoblot experiments will be performed, with cerebellar granule cells, after apoptosis induction. It is known that tau is cleaved in this system and its cleavage is prevented by caspase inhibitors (Canu et al., 1998). Tau molecules cleaved at Asp-421 are therefore likely to appear when cells start to undergo apoptosis, contributing to reinforce the apoptotic cascade. The generation of a wider panel of antibodies against C-terminal epitopes of tau fragments, along the lines described in this work, will allow an in-depth study of the kinetics and spatio-temporal distribution of tau proteolysis in AD.

5.7. Conclusion.

The main achievements of this work are:

1) *The anti-tau monoclonal antibody MN7.51 yields a western blot profile that allows distinguishing between AD brains and non-demented controls.*

2) *MN423 immunoreactivity, diagnostic of tau truncated at residue Glu-391, is associated with DNA fragmentation in AD brain.*

3) *A set of tau fragments encompassing the two proline rich regions and the repeat motifs displays apoptotic capacity in different cells (including cell lines of "neuronal type").*

4) *A caspase-3 cleavage site divides toxic from non-toxic fragments.*

On the basis of these results, a new working hypothesis on the pathological consequences of abnormal tau proteolysis has been formulated.

5) *As part of the endeavors to test this hypothesis, an antibody specifically recognizing tau cleaved by caspase-3 at Asp-421 has been generated.*

References

Anderson AJ, Su JH, Cotman CW: DNA damage and apoptosis in Alzheimer's disease: colocalization with c-Jun immunoreactivity, relationship to brain area, and effect of postmortem delay. J Neurosci 16, 1710-1719 (1996).

Andreadis A, Brown WM, Kosik KS: Structure and novel exons of the human tau gene. Biochemistry 31, 10626-10633 (1992).

Andreadis A, Broderick JA, Kosik KS: Relative exon affinities and suboptimal splice site signals lead to non-equivalence of two cassette exons. Nucleic Acids Res 23, 3585-93 (1995).

Arriagada PV, Growdon JH, Hedley-White ET, Hyman BT: Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's Disease. Neurology 42, 631-639 (1992).

Barnes NY, Li L, Yoshikawa K, Schwartz LM, Oppenheim RW, Milligan CE: Increased production of amyloid precursor protein provides a substrate for caspase-3 in dying motoneurons. J Neurosci 18, 5869-5880 (1998).

Bednarski E, Lynch G: Cytosolic proteolysis of tau by cathepsin D in hippocampus following suppression of cathepsins B and L. J Neurochem 67, 1846-1855 (1996).

Biernat J, Mandelkow EM, Schroter C, Lichtenberg-Kraag B, Steiner B, Berling B, Meyer H, Mercken M, Vandermeeren A, Goedert M, et al: The switch of tau protein to an Alzheimer-like state includes the phosphorylation of two serine-proline motifs upstream of the microtubule binding region. EMBO J 11, 1593-1597 (1992).

Binder LI, Frankfurter A, Rebhun LI: The distribution of tau in the mammalian central nervous system. J Cell Biol 101, 1371-1378 (1985).

Blacker D, Wilcox MA, Laird NM, Rodes L, Horvath SM, Go RC, Perry R, Watson B Jr, Bassett SS, McInnis MG, Albert MS, Hyman BT, Tanzi RE: Alpha-2 macroglobulin is genetically associated with Alzheimer disease. Nat Genet 19, 357-360 (1998).

Bondareff W, Wischik CM, Novak M, Roth M: Sequestration of tau by granulovacuolar degeneration in Alzheimer's disease. Am J Pathol 139, 641-647 (1991).

Braak H, Braak E: Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 82, 239-259 (1991).

Brion JP, Smith C, Couck AM, Gallo JM, Anderton BH: Developmental changes in tau phosphorylation: fetal tau is transiently phosphorylated in a manner similar to paired helical filament-tau characteristic of Alzheimer's disease. J Neurochem 61, 2071-2080 (1993).

Bursch W, Oberhammer F, Schulte-Hermann R: Cell death by apoptosis and its protective role against disease. Trends Pharmacol Sci 13, 245-251 (1992).

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

Butner KA, Kirschner MW: Tau protein binds to microtubules through a flexible array of distributed weak sites. J Cell Biol 115, 717-730 (1991).

Caceres A, Kosik KS: Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons. Nature 343, 461-463 (1990).

Cairns NJ, Chadwick A, Luthert PJ, Lantos PL: Astrocytosis, beta A4-protein deposition and paired helical filament formation in Alzheimer's disease. J Neurol Sci 112, 68-75 (1992).

Canu N, Dus L, Barbato C, Ciotti MT, Brancolini C, Rinaldi AM, Novak M, Cattaneo A, Bradbury A, Calissano P: Tau cleavage and dephosphorylation in cerebellar granule neurons undergoing apoptosis. J Neurosci 18, 7061-7074 (1998).

Cataldo AM, Hamilton DJ, Nixon RA: Lysosomal abnormalities in degenerating neurons link neuronal compromise to senile plaque development in Alzheimer disease. Brain Res 640, 68-80 (1994).

Cataldo AM, Barnett JL, Berman SA, Li J, Quarless S, Bursztajn S, Lippa C, Nixon RA: Gene expression and cellular content of cathepsin D in Alzheimer's disease brain: evidence for early up-regulation of the endosomal-lysosomal system. Neuron 14, 671-680 (1995).

Cataldo AM, Hamilton DJ, Barnett JL, Paskevich PA, Nixon RA: Properties of the endosomal-lysosomal system in the human central nervous system: disturbances mark most neurons in populations at risk to degenerate in Alzheimer's disease. J Neurosci 16, 186-199 (1996a).

Cataldo AM, Barnett JL, Mann DM, Nixon RA: Colocalization of lysosomal hydrolase and β-amyloid in diffuse plaques of the cerebellum and striatum in Alzheimer's disease and Down's syndrome. J Neuropathol Exp Neurol 55, 704-715 (1996b).

Cataldo AM, Barnett JL, Pieroni C, Nixon RA: Increased neuronal endocytosis and protease delivery to early endosomes in sporadic Alzheimer's disease: neuropathologic evidence for a mechanism of increased beta-amyloidogenesis. J Neurosci 17, 6142-6151 (1997).

Charriaut-Marlangue C, Ben-Ari Y: A cautionary note on the use of the TUNEL stain to determine apoptosis. Neuroreport 7, 61-64 (1995).

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

Cohen GM, Sun XM, Snowden RT, Dinsdale D, Skilleter DN: Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. Biochem J 286, 331-334 (1992).

Combs CK, Johnson DE, Cannady SB, Lehman TM, Landreth GE: Identification of microglial signal transduction pathways mediating a neurotoxic response to amyloidogenic fragments of beta-amyloid and prion proteins. J Neurosci 19, 928-939 (1999).

Corder EH, Lannfelt L, Bogdanovic N, Fratiglioni L, Mori H: The role of APO-E polymorphisms in late-onset dementias. Cell Mol Life Sci 54, 928-934 (1998).

Cotman CW, Anderson AJ: A potential role for apoptosis in neurodegeneration and Alzheimer's disease. Mol Neurobiol 10, 19-45 (1995).

Cotman CW, Su JH: Mechanisms of neuronal death in Alzheimer's disease. Brain Pathol 6, 493-506 (1996).

Crutcher KA, Tolar M, Harmony JAK, Marques M: Apolipoprotein E-derived 22 kDa fragment neurotoxicity: a new pathophysilogical mechanism in Alzheimer's disease. Neurobiol Aging 17, 5155 (1996).

Dayanandan R, Van Slegtenhorst M, Mack TG, Ko L, Yen SH, Leroy K, Brion JP, Anderton BH, Hutton M, Lovestone S: Mutations in tau reduce its microtubule binding properties in intact cells and affect its phosphorylation. FEBS Lett 446, 228-232 (1999).

Delacourte A, Buée L: Normal and pathological Tau proteins as factors for microtubule assembly. Int Rev Cytol 171:167-224 (1997)

Dreyer RN, Bausch KM, Fracasso P, Hammond LJ, Wunderlich D, Wirak DO, Davis G, Brini CM, Buckholz TM, Konig G: Processing of the pre-beta-amyloid protein by cathepsin D is enhanced by a familial Alzheimer's disease mutation. Eur J Biochem 224, 265-271 (1994).

El Khoury J, Hickman SE, Thomas CA, Cao L, Silverstein SC, Loike JD: Scavenger receptor-mediated adhesion of microglia to beta-amyloid fibrils. Nature 382, 716-719 (1996).

Evan GI, Lewis GK, Ramsay G, Bishop JM: Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol Cell Biol 5, 3610-6 (1985).

Fasulo L, Ovecka M, Kabat J, Bradbury A, Novak M, Cattaneo A: Overexpression of Alzheimer's PHF core fragments: implications for the tau truncation hypothesis. Alzheimer's Research 2, 195-200 (1996).

Fasulo L, Visintin M, Novak M, Cattaneo A: Tau truncation in Alzheimer's disease: expression of a fragment encompassing PHF core tau induces apoptosis in COS cells. Alzheimer's Reports 1, 25-32 (1998).

Fasulo L, Ugolini G, Visintin M, Bradbury A, Brancolini C, Verzillo V, Esposito G, Viglino P, Novak M, Cattaneo A: Tau as a Substrate for Caspase-3 and an Effector of Apoptosis. Submitted to J Biol Chem.

Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119, 493-501 (1992).

Gervais FG, Xu D, Robertson GS, Vaillancourt JP, Zhu Y, Huang J, LeBlanc A, Smith D, Rigby M, Shearman MS, Clarke EE, Zheng H, Van Der Ploeg LH, Ruffolo SC, Thornberry NA, Xanthoudakis S, Zamboni RJ, Roy S, Nicholson DW: Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic Aβ peptide formation. Cell 97, 395-406 (1999).

Giulian D, Haverkamp LJ, Yu JH, Karshin W, Tom D, Li J, Kirkpatrick J, Kuo LM, Roher AE: Specific domains of β-amyloid from Alzheimer plaque elicit neuron killing in human microglia. J Neurosci 16, 6021-6037 (1996).

Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, Giuffra L, Haynes A, Irving N, James L, Mant R, , Newton P, Rooke K, Roques P, Talbot C, Pericak-Vance M, Roses A, Williamson R, Rossor M, Owen M, Hardy J: Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349, 704-706 (1991).

Goedert M, Wischik CM, Crowther RA, Walker JE, Klug A: Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated protein tau. Proc Natl Acad Sci U S A 85, 4051-4055 (1988).

Goedert M, Spillantini MG, Potier MC, Ulrich J, Crowther RA: Cloning and sequencing of the cDNA encoding an isoform of microtubule-associated protein tau containing four tandem repeats: differential expression of tau protein mRNAs in human brain. EMBO J 8, 393-399 (1989a).

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

Goedert M, Jakes R: Expression of separate isoforms of human tau protein: correlation with the tau pattern in brain and effects on tubulin polymerization. EMBO J 9, 4225-4230 (1990).

Goedert M, Spillantini MG, Cairns NJ, Crowther RA: Tau proteins of Alzheimer paired helical filaments: abnormal phosphorylation of all six brain isoforms. Neuron 8, 159-168 (1992a).

Goedert M, Spillantini MG, Crowther RA: Cloning of a big tau microtubule-associated protein characteristic of the peripheral nervous system. Proc Natl Acad Sci U. S. A. 89, 1983-1987 (1992b).

Goedert M, Trojanowsky JQ, Lee VMY: The neurofibrillary pathology of Alzheimer's Disease. In "The molecular and genetic basis of neurological disease", 2nd edn (eds Rosenberg RN, Prusiner SB, Di Mauro S, Barchi RL, Butterworth-Heinemann, Boston, 1996a).

Goedert M, Jakes R, Spillantini MG, Hasegawa M, Smith MJ, Crowther RA: Assembly of microtubule-associated protein tau into Alzheimer-like filaments induced by sulphated glycosaminoglycans. Nature 383, 550-553 (1996b).

Goedert M, Crowther RA, Spillantini MG: Tau mutations cause frontotemporal dementias. Neuron 21, 955-958 (1998).

Goldberg Y.P., Nicholson D.W., Rasper D.M., Kalchman M.A., Koide H.B., Graham R.K., Bromm M., Kazemi-Esfarjani P., Thornberry N.A., Vaillancourt J.P. & Hayden M.R. Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. Nat Genet 13, 442-449 (1996).

Gong CX, Singh TJ, Grundke-Iqbal I, Iqbal K: Phosphoprotein phosphatase activities in Alzheimer disease brain. J Neurochem 61, 921-927 (1993).

Goode BL, Feinstein SC: Identification of a novel microtubule binding and assembly domain in the developmentally regulated inter-repeat region of tau. J Cell Biol 124, 769-782 (1994).

Greenberg SG, Davies P: A preparation of Alzheimer paired helical filaments that displays distinct tau proteins by poly-acrylamide gel electrophoresis. Proc Natl Acad Sci U S A, 87, 5827-5831 (1990).

Greenberg SG, Davies P, Schein JD, Binder LI: Hydrofluoric acid-treated tau-PHF proteins display the same biochemical properties as normal tau. J Biol Chem 267, 564-569 (1992).

Grover A, Houlden H, Baker M, Adamson J, Lewis J, Prihar G, Pickering-Brown S, Duff K, Hutton M: 5' splice site mutations in tau associated with the inherited dementia FTDP-17 affect a stem-loop structure that regulates alternative splicing of exon 10. J Biol Chem 274, 15134-15143 (1999).

Grundke-Iqbal I, Iqbal K, Tung YC, Quinlan M, Wisniewski HM, Binder LI: Abnormal phosphorylation of the microtubule-associated protein tau in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci U. S. A. 83, 4913-4927 (1986).

Grynspan F, Griffin WR, Cataldo A, Katayama S, Nixon RA: Active site-directed antibodies identify calpain II as an early-appearing and pervasive component of neurofibrillary pathology in Alzheimer's disease. Brain Res 763, 145-58 (1997).

Gustke N, Trinczek B, Biernat J, Mandelkow EM, Mandelkow E: Domains of tau protein and interactions with microtubules. Biochemistry 33, 9511-9522 (1994).

Harada A, Oguchi K, Okabe S, Kuno J, Terada S, Ohshima T, Sato-Yoshitake R, Takei Y, Noda T, Hirokawa N: Altered microtubule organization in small-calibre axons of mice lacking tau protein. Nature 369, 488-91 (1994).

Harrington CR, Mukaetova-Ladinska EB, Hills R, Edwards PC, Montejo de Garcini E, Novak M, Wischik CM: Measurement of distinct immunochemical presentations of tau protein in Alzheimer disease. Proc Natl Acad Sci U S A 88, 5842-5846 (1991).

Hasegawa M, Crowther RA, Jakes R, Goedert M: Alzheimer-like changes in microtubule-associated protein Tau induced by sulfated glycosaminoglycans. Inhibition of microtubule binding, stimulation of phosphorylation, and filament assembly depend on the degree of sulfation. J Biol Chem 272, 33118-33124 (1997).

Hasegawa M, Smith MJ, Goedert M: Tau proteins with FTDP-17 mutations have a reduced ability to promote microtubule assembly. FEBS Lett 437, 207-210 (1998).

Hong M, Zhukareva V, Vogelsberg-Ragaglia V, Wszolek Z, Reed L, Miller BI, Geschwind DH, Bird TD, McKeel D, Goate A, Morris JC, Wilhelmsen KC, Schellenberg GD, Trojanowski JQ, Lee VM: Mutation-specific functional impairments in distinct tau isoforms of hereditary FTDP-17. Science 282, 1914-1917 (1998).

Hutton M, Lendon CL, Rizzu P, Baker M, Froelich S, Houlden H, Pickering-Brown S, Chakraverty S, Isaacs A, Grover A, Hackett J, Adamson J, Lincoln S, Dickson D, Davies P, Petersen RC, Stevens M, de Graaff E, Wauters E, van Baren J, Hillebrand M, Joosse M, Kwon JM, Nowotny P, Kuei Che L, Norton J, Morris JC, Reed LA, Trojanowsky J, Basun H, Lannfelt L, Neystat M, Fahn S, Dark F, Tannenberg T, Dodd PR, Hayward N, Kwok JBJ, Schofield PR, Andreadis A, Snowden J, Craufurd D, Neary D, Owen F, Oostra BA, Hardy J, Goate A, Van swieten J, Mann D, Lynch T, Heutink P: Association of missense and 5'-splice-site mutations in tau with the inherited dementia FTDP-17. Nature 393, 702-705 (1998).

Igarashi S, Koide R, Shimohata T, Yamada M, Hayashi Y, Takano H, Date H, Oyake M, Sato T, Sato A, Egawa S, Ikeuchi T, Tanaka H, Nakano R, Tanaka K, Hozumi I, Inuzuka T, Takahashi H, Tsuji S: Suppression of aggregate formation and apoptosis by transglutaminase inhibitors in cells expressing truncated DRPLA protein with an expanded polyglutamine stretch. Nat. Genet. 18, 111-117 (1998).

Jakes R, Novak M, Davison M, Wischik CM: Identification of 3- and 4-repeat tau isoforms within the PHF in Alzheimer's disease. EMBO J 10, 2725-2729 (1991).

Johnson GV, Seubert P, Cox TM, Motter R, Brown JP, Galasko D: The tau protein in human cerebrospinal fluid in Alzheimer's disease consists of proteolytically derived fragments. J Neurochem 68, 430-433 (1997).

Johnstone M, Gearing AJ, Miller KM: A central role for astrocytes in the inflammatory response to beta-amyloid; chemokines, cytokines and reactive oxygen species are produced. J Neuroimmunol 93, 182-193 (1999).

Keller JN, Guo Q, Holtsberg FW, Bruce-Keller AJ, Mattson MP: Increased sensitivity to mitochondrial toxin-induced apoptosis in neural cells expressing mutant presenilin-1 is linked to perturbed calcium homeostasis and enhanced oxyradical production. J Neurosci 18, 4439-4450 (1998).

Kenessey A, Yen SH: The extent of phosphorylation of fetal tau is comparable to that of PHF-tau from Alzheimer paired helical filaments. Brain Res 629, 40-46 (1993).

Kenessey A, Nacharaju P, Ko LW, Yen SH: Degradation of tau by lysosomal enzyme cathepsin D: implication for Alzheimer neurofibrillary degeneration. J Neurochem 69, 2026-2038 (1997).

Kim TW, Pettingell WH, Jung YK, Kovacs DM, Tanzi RE: Alternative cleavage of Alzheimer-associated presenilins during apoptosis by a caspase-3 family protease. Science 277, 373-376 (1997).

Kondratick CM, Vandré DD: Alzheimer's disease neurofibrillary tangles contain mitosis-specific phosphoepitopes. J Neurochem 67, 2405-2416 (1996).

Kosik KS, Orecchio LD, Bakalis S, Neve RL: Developmentally regulated expression of specific tau sequences. Neuron 2, 1389-1397 (1989).

LaFerla FM, Tinkle BT, Bieberich CJ, Haudenschild CC, Jay G: The Alzheimer's Aβ peptide induces neurodegeneration and apoptotic cell death in transgenic mice. Nat Genet 9, 21-30 (1995).

Lang E, Szendrei GI, Lee VM, Otvos L Jr: Immunological and conformation characterization of a phosphorylated immunodominant epitope on the paired helical filaments found in Alzheimer's disease. Biochem Biophys Res Commun 187, 783-790 (1992)

Lassmann H, Bancher C, Breitschopf H, Wegiel J, Bobinski M, Jellinger K, Wisniewski HM: Cell death in Alzheimer's disease evaluated by DNA fragmentation in situ. Acta Neuropathol 89, 35-41 (1995).

Le WD, Xie WJ, Kong R, Appel SH: Beta-amyloid-induced neurotoxicity of a hybrid septal cell line associated with increased tau phosphorylation and expression of beta-amyloid precursor protein. J Neurochem 69, 978-985 (1997).

Ledesma MD, Bonay P, Colaco C, Avila J: Analysis of microtubule-associated protein tau glycation in paired helical filaments. J Biol Chem 269, 21614-21619 (1994).

Ledesma MD, Bonay P, Avila J: Tau protein from Alzheimer's disease patients is glycated at its tubulin-binding domain. J Neurochem 65, 1658-1664 (1995).

Lee VM, Balin BJ, Otvos L Jr, Trojanowski JQ: A68: a major subunit of paired helical filaments and derivatized forms of normal Tau. Science 251, 675-8 (1991).

Leuba G, Kraftsik R: Visual cortex in Alzheimer's disease: occurrence of neuronal death and glial proliferation, and correlation with pathological hallmarks. Neurobiol Aging 15, 29-43 (1994).

Levy E, Carman MD, Fernandez-Madrid IJ, Power MD, Lieberburg I, van Duinen SG, Bots GT, Luyendijk W, Frangione B: Mutation of the Alzheimer's disease amyloid gene in hereditary cerebral hemorrhage, Dutch type. Science 248, 1124-1126 (1990).

Litersky JM, Scott CW, Johnson GV: Phosphorylation, calpain proteolysis and tubulin binding of recombinant human tau isoforms. Brain Res 604, 32-40 (1993).

Litersky JM, Johnson GV: Phosphorylation of tau in situ: inhibition of calcium-dependent proteolysis. J Neurochem 65, 903-911 (1995).

Loetscher H, Deuschle U, Brockhaus M, Reinhardt D, Nelboeck P, Mous J, Grunberg J, Haass C, Jacobsen H: Presenilins are processed by caspase-type proteases. J. Biol. Chem. 272, 20655-20659 (1997).

Loo DT, Copani A, Pike CJ, Whittemore ER, Walencewicz AJ, Cotman CW: Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc. Natl. Acad. Sci. USA 90, 7951-7955 (1993).

Lu PJ, Wulf G, Zhou XZ, Davies P, Lu KP: The prolyl isomerase Pin1 restores the function of Alzheimer-associated phosphorylated tau protein. Nature 399, 784-788 (1999).

Malgaroli A, Tsien RW: Glutamate-induced long-term potentiation of the frequency of miniature synaptic currents in cultured hippocampal neurons. Nature 357, 134-139 (1992).

Mandelkow EM, Mandelkow E: Tau as a marker for Alzheimer's disease. Trends Biochem Sci 18, 480-483 (1993).

Mandelkow EM, Mandelkow E: Tau in Alzheimer's disease. Trends Cell Biol 8, 425-427 (1998).

Mattson MP, Guo Q. Cell and molecular neurobiology of presenilins: a role for the endoplasmic reticulum in the pathogenesis of Alzheimer's disease? J Neurosci Res 50, 505-513 (1997).

Mawal-Dewan M, Henley J, Van de Voorde A, Trojanowski JQ, Lee VM: The phosphorylation state of tau in the developing rat brain is regulated by phosphoprotein phosphatases. J Biol Chem 269, 30981-30987 (1994)

Mena R, Wischik CM, Novak M, Milstein C, Cuello AC: A progressive deposition of paired helical filaments (PHF) in the brain characterizes the evolution of dementia in Alzheimer's disease. An immunocytochemical study with a monoclonal antibody against the PHF core. J Neuropathol Exp Neurol 50, 474-490 (1991).

Mercken M, Grynspan F, Nixon RA: Differential sensitivity to proteolysis by brain calpain of adult human tau, fetal human tau and PHF-tau. FEBS Lett 368, 10-14 (1995).

Mettling C, Gouin A, Robinson M, el M'Hamdi H, Camu W, Bloch-Gallego E, Buisson B, Tanaka H, Davies AM, Henderson CE: Survival of newly postmitotic motoneurons is transiently independent of exogenous trophic support. J Neurosci 15, 3128-3137 (1995).

Mori H, Kondo J, Ihara Y: Ubiquitin is a component of paired helical filaments in Alzheimer's disease.Science 235, 1641-1644 (1987).

Morishima-Kawashima M, Hasegawa M, Takio K, Suzuki M, Titani K, Ihara Y: Ubiquitin is conjugated with amino-terminally processed tau in paired helical filaments. Neuron 10, 1151-1160 (1993).

Morishima-Kawashima M, Hasegawa M, Takio K, Suzuki M, Yoshida H, Titani K, Ihara Y: Proline-directed and non-proline-directed phosphorylation of PHF-tau. J Biol Chem 270, 823-829 (1995).

Mullan M, Crawford F, Axelman K, Houlden H, Lilius L, Winblad B, Lannfelt L: A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nat Genet 1, 345-347 (1992).

Nagy Z, Esiri MM, Smith AD: Expression of cell division markers in the hippocampus in Alzheimer's disease and other neurodegenerative conditions. Acta Neuropathol 93, 294-300 (1997a).

Nagy Z, Esiri MM, Cato AM, Smith AD: Cell cycle markers in the hippocampus in Alzheimer's disease. Acta Neuropathol 94, 6-15 (1997b).

Nagy Z, Esiri MM: apoptosis-related protein expression in the hippocampus in Alzheimer's Disease. Neurobiol Aging 18, 565-571 (1998)

Nagy Z, Esiri MM, Smith AD: The cell division cycle and the pathophysiology of Alzheimer's disease. Neuroscience 87, 731-739 (1998).

Nasir J, Goldberg YP, Hayden MR: Huntington disease: new insights into the relationship between CAG expansion and disease. Hum Mol Genet 5, 1431-1435 (1996).

Neve RL, Harris P, Kosik KS, Kurnit DM, Donlon TA: Identification of cDNA clones for the human microtubule-associated protein tau and chromosomal localization of the genes for tau and microtubule-associated protein 2. Brain Res 387, 271-280 (1986).

Nieto A, Montejo de Garcini E, Correas I, Avila J: Characterization of tau protein present in microtubules and paired helical filaments of Alzheimer's disease patient's brain. Neuroscience 37, 163-170 (1990).

Nixon RA, Cataldo AM: The lysosomal system in neuronal cell death: a review. Ann N Y Acad Sci 679, 87-109 (1993).

Nixon RA, Cataldo AM: The endosomal-lysosomal system of neurons: new roles. Trends Neurosci 18, 489-496 (1995).

Novak M, Wischik CM, Edwards P, Pannell R, Milstein C: Characterization of the first monoclonal antibody against the pronase resistant core of the Alzheimer PHF. Prog Clin Biol Res, 317, 755-761 (1989).

Novak M, Jakes R, Edwards PC, Milstein C, Wischik CM: Difference between the tau protein of Alzheimer paired helical filament core and normal tau revealed by epitope analysis of monoclonal antibodies 423 and 7.51. Proc. Natl. Acad. Sci. USA, 88, 5837-5841 (1991).

Novak M, Kabat J, Wischik CM: Molecular characterization of the minimal protease resistant tau unit of the Alzheimer's disease paired helical filament. EMBO J 12, 365-370 (1993).

Novak M: Truncated tau protein as a new marker for Alzheimer's disease. Acta Virol 38, 173-189 (1994).

Novak M, Ugolini G, Fasulo L, Visintin M, Ovecka M, Cattaneo A: Truncation of tau and neurodegeneration. In "Alzheimer's Disease and Related Disorders" (eds. Iqbal K., Swaab D.F., Winblad B., Wisniewski H.M. – John Wiley & Sons, 281-292, 1999).

Ona VO, Li M, Vonsattel JP, Andrews LJ, Khan SQ, Chung WM, Frey AS, Menon AS, Li XJ, Stieg PE, Yuan J, Penney JB, Young AB, Cha JH, Friedlander RM: Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. Nature 399, 263-267 (1999).

Oppenheim RW: The neurotrophic theory and naturally occurring motoneuron death. Trends Neurosci 12, 252-255 (1989).

Otvos L Jr, Feiner L, Lang E, Szendrei GI, Goedert M, Lee VM: Monoclonal antibody PHF-1 recognizes tau protein phosphorylated at serine residues 396 and 404. J Neurosci Res 39, 669-673 (1994).

Panda D, Goode BL, Feinstein SC, Wilson L: Kinetic stabilization of microtubule dynamics at steady state by tau and microtubule-binding domains of tau. Biochemistry 34, 11117-11127 (1995).

Papasozomenos SC, Binder LI: Phosphorylation determines two distinct species of Tau in the central nervous system. Cell Motil Cytoskeleton 8, 210-26 (1987).

Perez M, Valpuesta JM, Medina M, Montejo de Garcini E, Avila J: Polymerization of tau into filaments in the presence of heparin: the minimal sequence required for tau-tau interaction. J Neurochem 67, 1183-1190 (1996).

Persic L, Righi M, Roberts A, Hoogenboom HR, Cattaneo A, Bradbury A: Targeting vectors for intracellular immunization. Gene 187, 1-8 (1997).

Pettmann B, Henderson CE: Neuronal Cell Death. Neuron 20, 633-647 (1998).

Pleasure SJ, Page CD, Lee VMJ: Pure, postmitotic polarized human neurons derived from Ntera2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. J Neurosci 12, 1802-1815 (1992).

Pleasure SJ, Lee VMY: Ntera 2 cells: A human cell line which displays characteristics expected of a human committed neuronal progenitor cell. J Neurosci Res 35, 585-602, 1993.

Poorkaj P, Bird TD, Wijsman E, Nemens E, Garruto RM, Anderson L, Andreadis A, Wiederholt WC, Raskind M, Schellenberg GD: Tau is a candidate gene for chromosome 17 frontotemporal dementia. Ann Neurol 43, 815-825 (1998).

Preuss U, Biernat J, Mandelkow EM, Mandelkow E: The 'jaws' model of tau-microtubule interaction examined in CHO cells. J Cell Sci 110, 789-800 (1997).

Price DL, Sisodia SS, Borchelt DR: Genetic neurodegenerative diseases: the human illness and transgenic models. Science 282, 1079-1083 (1998).

Probst A, Langui D, Ulrich J: Alzheimer's disease: a description of the structural lesions. Brain Pathol 1, 229-239 (1991).

Reed JC: Bcl-2 family proteins. Oncogene 17, 3225-3236 (1998).

Riederer BM, Binder LI: Differential distribution of tau proteins in developing cat cerebellum. Brain Res Bull 33, 155-161 (1994).

Rogaev EI, Sherrington R, Rogaeva EA, Levesque G, Ikeda M, Liang Y, Chi H, Lin C, Holman K, Tsuda T, et al: Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. Nature 376, 775-778 (1995).

Roses AD: Apolipoprotein E and Alzheimer's disease. The tip of the susceptibility iceberg. Ann N Y Acad Sci 855, 738-743 (1998).

Rosner H, Rebhan M, Vacun G, Vanmechelen E: Developmental expression of tau proteins in the chicken and rat brain: rapid down-regulation of a paired helical filament epitope in the rat cerebral cortex coincides with the transition from immature to adult tau isoforms. Int J Dev Neurosci 13, 607-617 (1995).

Ruben G.C., Iqbal K., Grundke-Iqbal, I., Wisniewski H.M., Ciardelli T.L. & Johnson J.E.jr. The microtubule-associated protein tau forms a triple stranded left-hand helical polymer. J. Biol. Chem. 266, 22019-22027 (1991).

Saito K, Elce JS, Hamos JE, Nixon RA: Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. Proc Natl Acad Sci U S A 90, 2628-2632 (1993).

Salvesen GS, Dixit VM: Caspases: intracellular signalling by proteolysis. Cell 91, 443-446 (1997).

Sambrook J, Fritsch EF, Maniatis T: Molecular cloning, a laboratory manual (Second Edition). Cold Spring Harbor Laboratory Press (1989).

Sanchez I., Xu C.J., Juo P., Kakizaka A., Blenis J. & Yuan J. Caspase-8 is required for cell death induced by expanded polyglutamine repeats. Neuron 22, 623-633 (1999).

Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A 74, 5463-5467 (1977).

Schneider A, Biernat J, von Bergen M, Mandelkow E, Mandelkow EM: Phosphorylation that detaches tau protein from microtubules (Ser262, Ser214) also protects it against aggregation into Alzheimer paired helical filaments. Biochemistry 38, 3549-3558 (1999).

Scheuner D, Eckman C, Jensen M, Song X, Citron M, Suzuki N, Bird TD, Hardy J, Hutton M, Kukull W, Larson E, Levy-Lahad E, Viitanen M, Peskind E, Poorkaj P, Schellenberg G, Tanzi R, Wasco W, Lannfelt L, Selkoe D, Younkin S: Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. Nat Med 2, 864-870 (1996).

Schweers O, Mandelkow EM, Biernat J, Mandelkow E: Oxidation of cysteine-322 in the repeat domain of microtubule-associated protein tau controls the in vitro assembly of paired helical filaments.Proc Natl Acad Sci U S A. 92, 8463-8467 (1995).

Selkoe DJ: Cell biology of the amyloid beta-protein precursor and the mechanism of Alzheimer's disease. Annu Rev Cell Biol 10, 373-403 (1994).

Selkoe DJ: Translating cell biology into therapeutic advances in Alzheimer's Disease. Nature 399 (Supp), A23-A29 (1999).

Shea TB, Prabhakar S, Ekinci FJ: Beta-amyloid and ionophore A23187 evoke tau hyperphosphorylation by distinct intracellular pathways: differential involvement of the calpain/protein kinase C system. J Neurosci Res 49, 759-768 (1997).

Sherrington R, Rogaev EI, Liang Y, Rogaeva EA, Levesque G, Ikeda M, Chi H, Lin C, Li G, Holman K, et al: Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature 375, 754-60 (1995).

Silke J, Vaux DL: Cell death: shadow baxing. Curr Biol 8, R528-531 (1998).

Smith MA, Siedlak SL, Richey PL, Mulvihill P, Ghiso J, Frangione B, Tagliavini F, Giaccone G, Bugiani O, Praprotnik D, Kalaria RN, Perry G: Tau protein directly interacts with the amyloid beta-protein precursor: implications for Alzheimer's disease. Nat Med 1, 365-369 (1995).

Smith TW, Lippa CF: Ki-67 immunoreactivity in Alzheimer's disease and other neurodegenerative disorders. J Neuropathol Exp Neurol 54, 297-303 (1995).

Spillantini MG and Goedert M: Tau protein pathology in neurodegenerative diseases. Trends Neurosci 21, 428-433 (1998).

Spillantini MG, Murrell JR, Goedert M, Farlow MR, Klug A, Ghetti B: Mutation in the tau gene in familial multiple system tauopathy with presenile dementia. Proc Natl Acad Sci U S A 95, 7737-7741 (1998).

Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, Roses AD: Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. Proc Natl Acad Sci USA 90, 1977-1981 (1993).

Su JH, Anderson AJ, Cummings BJ, Cotman CW: Immunohistochemical evidence for apoptosis in Alzheimer's disease. Neuroreport 5, 2529-2533 (1994).

Su JH, Satou T, Anderson AJ, Cotman CW: Up-regulation of Bcl-2 is associated with neuronal DNA damage in Alzheimer's disease. Neuroreport 7, 437-440 (1996).

Sugaya K, Reeves M, McKinney M: Topographic associations between DNA fragmentation and Alzheimer's disease neuropathology in the hippocampus. Neurochem Int 31, 275-281 (1997).

Suzuki N, Cheung TT, Cai XD, Odaka A, Otvos L Jr, Eckman C, Golde TE, Younkin SG: An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. Science 264, 1336-40 (1994).

Szendrei GI, Lee VM, Otvos L Jr: Recognition of the minimal epitope of monoclonal antibody Tau-1 depends upon the presence of a phosphate group but not its location. J Neurosci Res 34, 243-249 (1993).

Tagawa K, Kunishita T, Maruyama K, Yoshikawa K, Kominami E, Tsuchiya T, Suzuki K, Tabira T, Sugita H, Ishiura S: Alzheimer's disease amyloid beta-clipping enzyme (APP secretase): identification, purification, and characterization of the enzyme. Biochem Biophys Res Commun 177, 377-387 (1991).

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM: Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81, 801-809 (1995).

Troncoso JC, Cataldo AM, Nixon RA, Barnett JL, Lee MK, Checler F, Fowler DR, Smialek JE, Crain B, Martin LJ, Kawas CH: Neuropathology of preclinical and clinical late-onset Alzheimer's disease. Ann Neurol 43, 673-676 (1998).

Ugolini G, Cattaneo A, Novak M: Co-localization of truncated tau and DNA fragmentation in Alzheimer's Disease neurons. NeuroReport 8, 3709-3712 (1997).

Ugolini G, Cattaneo A, Novak M: The anti-tau monoclonal antibody MN7.51 shows a specific western blot pattern for Alzheimer's disease. Society for Neuroscience 28th Annual Meeting, Los Angeles, CA - USA (671.4, 1714, 1998).

Van Broeckhoven C: Presenilins and Alzheimer's disease. Nat Genet 11, 230-232 (1995).

Van de Craen M, De Jonghe C, Van den Brande I, Declercq W, Van Gassen G, Van Criekinge W, Vanderhoeven I, Fiers W, Van Broeckhoven C, Hendriks L, Vandenabeele P: Identification of caspases that cleave presenilin-1 and presenilin-2. Five presenilin-1 (PS1) mutations do not alter the sensitivity of PS1 to caspases. FEBS Lett. 445, 149-154 (1999).

Watanabe A, Hasegawa M, Suzuki M, Takio K, Morishima-Kawashima M, Titani K, Arai T, Kosik KS, Ihara Y: In vivo phosphorylation sites in fetal and adult rat tau. J Biol Chem 268, 25712-25717 (1993).

Weidemann A., Paliga K., D rrwang U., Reinhard F.B, Schuckert O., Evin G. & Masters C.L. Proteolytic processing of the Alzheimer's disease amyloid precursor protein within its cytoplasmic domain by caspase-like proteases. J Biol Chem 274, 5823-5829 (1999).

Wellington CL, Ellerby LM, Hackam AS, Margolis RL, Trifiro MA, Singaraja R, McCutcheon K, Salvesen GS, Propp SS, Bromm M, Rowland KJ, Zhang T, Rasper D, Roy S, Thornberry N, Pinsky L, Kakizuka A, Ross CA, Nicholson DW, Bredesen DE,

Hayden MR: Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. J Biol Chem 273, 9158-9167 (1998).

Wischik CM, Novak M, Thogersen HC, Edwards PC, Runswick MJ, Jakes R, Walker JE, Milstein C, Roth M, Klug A: Isolation of a fragment of tau derived from the core of the paired helical filament of Alzheimer disease. Proc Natl Acad Sci USA 85, 4506-4510 (1988a).

Wischik CM, Novak M, Edwards PC, Klug A, Tichelaar W, Crowt: Structural characterization of the core of the paired helical filament of Alzheimer disease. Proc Natl Acad Sci USA 85, 4884-4888 (1988b).

Wisniewski T, Castano EM, Golabek A, Vogel T, Frangione B: Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro. Am J Pathol 145, 1030-1035 (1994).

Wolozin B, Iwasaki K, Vito P, Ganjei JK, Lacana E, Sunderland T, Zhao B, Kusiak JW, Wasco W, D'Adamio L: Participation of presenilin 2 in apoptosis: enhanced basal activity conferred by an Alzheimer mutation. Science, 274: 1710-1713 (1996).

Wyllie AH, Kerr JF, Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol 68, 251-306 (1980).

Xu PT, Gilbert JR, Qiu HL, Ervin J, Rothrock-Christian TR, Hulette C, Schmechel DE: Specific regional transcription of apolipoprotein E in human brain neurons. Am J Pathol 154, 601-611 (1999).

Yamatsuji T., Okamoto T., Takeda S., Murayama Y., Tanaka N. & Nishimoto I. Expression of V642 APP mutant causes cellular apoptosis as Alzheimer trait-linked phenotype. EMBO J. 15, 498-509 (1996).

Yan SD, Chen X, Fu J, Chen M, Zhu H, Roher A, Slattery T, Zhao L, Nagashima M, Morser J, Migheli A, Nawroth P, Stern D, Schmidt AM: RAGE and amyloid-beta peptide neurotoxicity in Alzheimer's disease. Nature 382, 685-691 (1996).

Yanagawa H., Chung S.H., Ogawa Y., Sato K., Shibata-Seki T., Masai J. & Ishiguro K. Protein anatomy: C-tail region of human tau protein as a crucial structural element in Alzheimer's paired helical filament formation in vitro. Biochemistry 37, 1979-1988 (1998).

Yang LS, Ksiezak-Reding H: Calpain-induced proteolysis of normal human tau and tau associated with paired helical filaments. Eur J Biochem 233, 9-17 (1995).