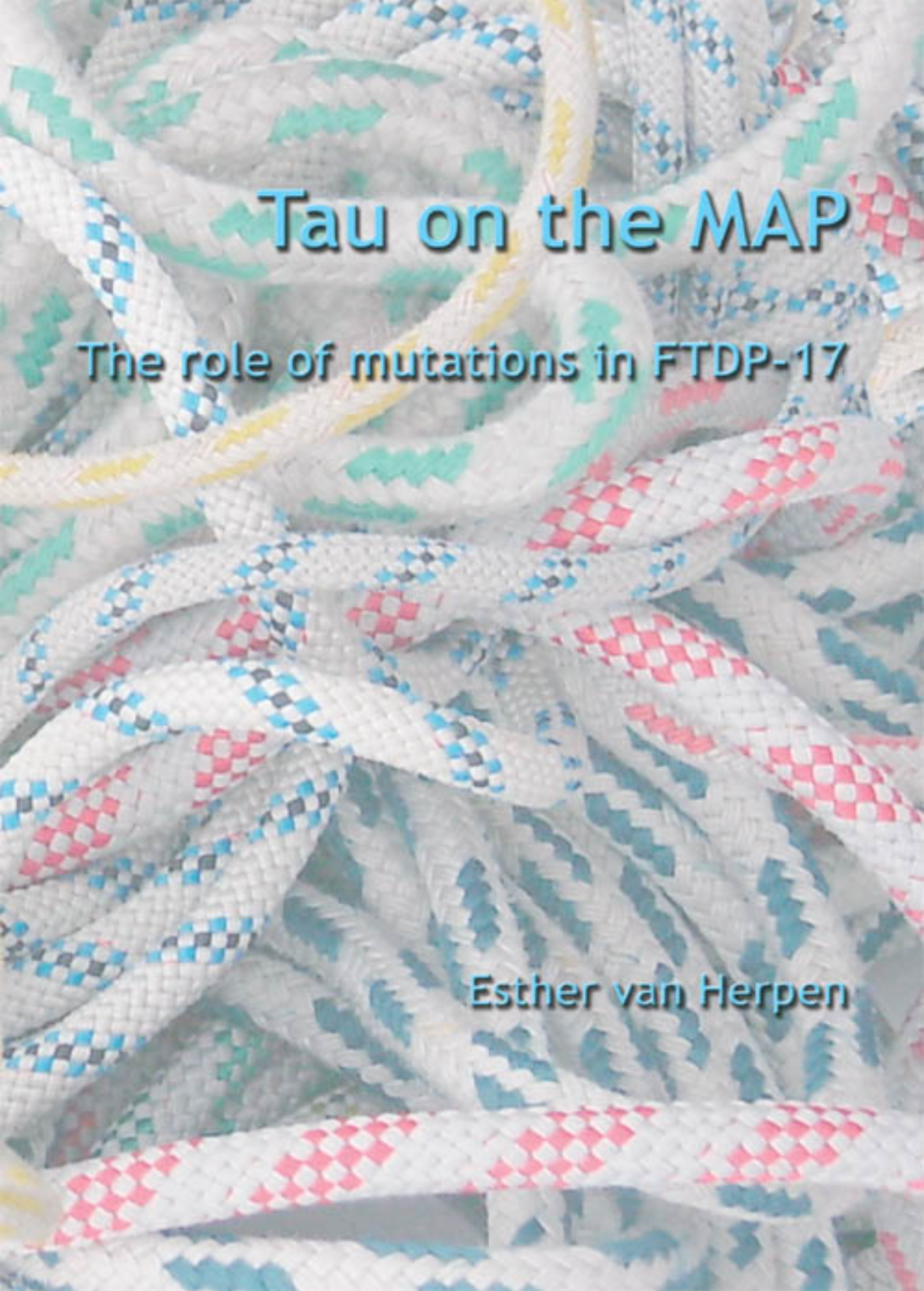
Tau on the MAP
The role of mutations in FTDP-17
Esther van Herpen

Tau on the MAP

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De rol van mutaties in FTDP-17

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The role of mutations in FTDP-17

De rol van mutaties in FTDP-17

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
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en volgens het besluit van het College voor Promoties

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Esther van Herpen

geboren te Alkmaar

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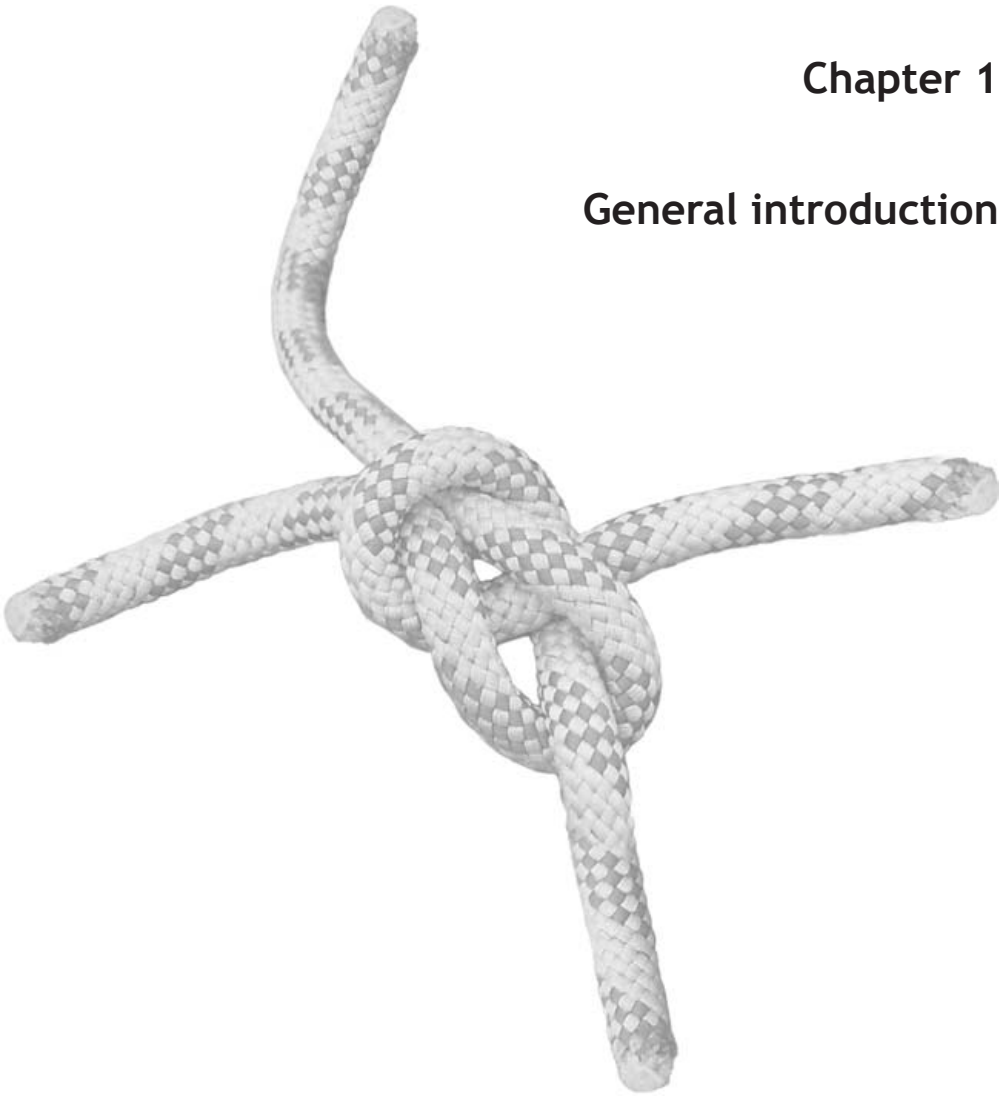
Tao Te Ching



Chapter 1

General introduction

General introduction

1.1 Neurodegeneration and dementia

In our aging population the prevalence of neurodegenerative disorders will increase rapidly over time. Prevalence values for dementia rise from 5% to more than 30% between the ages of 65 and 85 years. This serious threat and burden for patients, families and society questions the desirability of continuous increasing life expectancies. Currently, dementing disorders affect more than 20 million people worldwide, a number expected to double in the next 30 years (Hofman *et al.*, 1991). Neurodegenerative disorders are characterized by the degeneration of specific populations of neurons. They comprise a diverse set of disorders, of which the most common is Alzheimer's disease (AD). AD affects approximately 10% of the population over 65 years of age (Evans *et al.*, 1989; Hebert *et al.*, 1995). Up until now, therapeutic options in preventing or delaying onset of AD are limited, resulting in both an increased number of people with Alzheimer's disease and an increased proportion of the total population affected (Hebert *et al.*, 2001; Hofman *et al.*, 1995). Genetic studies using both families and (isolated) populations have resulted in the identification of new genes, playing a role in the pathogenesis of neurodegeneration (Rocchi *et al.*, 2003). Although mutations in these genes explain only a small proportion of cases, the findings have been of great importance in understanding the disease process and in opening new research opportunities.

1.2 Frontotemporal dementia

Historically, frontotemporal dementia (FTD) has been known as Pick's disease (PiD), named after Arnold Pick, a German professor in neurology (Pick, 1892). In 1892, he described the first patients with clinical symptoms resembling what is now called FTD. He emphasized the progressive behavioral disturbances and the bilateral focal cerebral atrophy. In 1911, Alois Alzheimer described the neuropathological changes, including the typical neuronal inclusion bodies (Alzheimer, 1911), and in 1926 the term Pick's disease was introduced (Figure 1) (Onari & Spatz, 1926). Other important milestones were the biochemical isolation of a protein able to induce microtubule formation (the tau protein) by Weingarten in 1975 (Weingarten *et al.*, 1975) and the observation that hyperphosphorylated tau was the predominant protein component of the inclusion bodies described by Grundke-Iqbal in 1986 (Grundke-Iqbal *et al.*, 1986a; Grundke-Iqbal *et al.*, 1986b). This research mainly focused on AD, but showed its importance in relation with other neurodegenerative disorders. In the 1980's, several clinical studies were published, describing a form of dementia affecting predominantly the frontal and temporal lobes of the brain (Brun, 1987; Neary *et al.*, 1988). The underlying pathology showed to be distinct from Alzheimer's disease. In 1994, a consensus statement was published by groups from Lund, Sweden and Manchester, UK, in which the name FTD was chosen to cover this clinical and pathological entity (Lund and Manchester Groups, 1994).

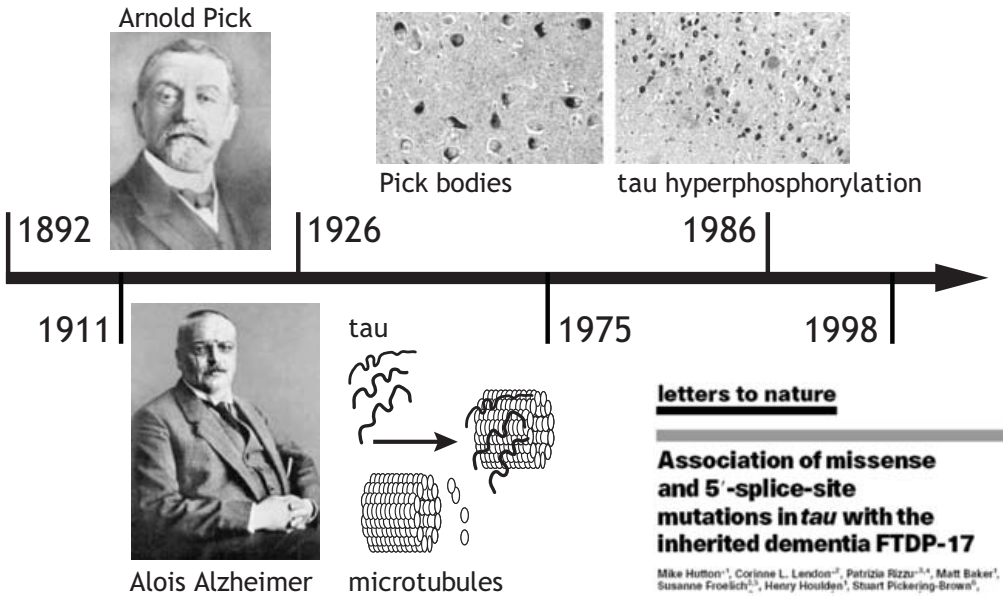


Figure 1: Milestones in FTD research
Timetable of the research to frontotemporal dementia, starting in 1892 with the first clinical description of the disease by Arnold Pick. In 1911, Alois Alzheimer described the neuropathology of FTD. In 1926, the characteristic inclusion bodies were named Pick bodies. In 1975 the tau protein, involved in binding to microtubules was biochemically isolated and a detailed characterization started, leading to the observation that hyperphosphorylated tau protein was the main component of the inclusion bodies in 1986. Two roads connected when in 1998 mutations in the *tau* gene were detected in patients with FTDP-17.

FTD is, together with AD and vascular dementia, the most common form of presenile dementia (Varma *et al.*, 2002). The first disease symptoms on average start at 57.6 ± 9.0 years, with a broad range from 30 to 80 years (Rosso *et al.*, 2003a). The disease duration varies between 5 to 15 years (Neary *et al.*, 1998). The prevalence of FTD is estimated to be between 10 to 20% of all dementias. After AD and Lewy body disease it is the third most common cortical dementia (Barker *et al.*, 2002). FTD has been and continues to be under-diagnosed or misdiagnosed, and increased understanding of the disorder will lead to a better diagnosis. A recent study of the prevalence of FTD in the Netherlands showed at maximum a prevalence of 9.4 per 100.000 inhabitants at age 60-69 years, although that might be overestimated due to the small geographical area (Rosso *et al.*, 2003a). Other prevalence values range between 2 and 15 per 100.000 habitants (Ratnavalli *et al.*, 2002; Stevens *et al.*, 1998). FTD is mostly sporadic, but a familial form with an autosomal dominant pattern of inheritance is seen in about 20% of patients. Over 40% of FTD patients have a familiar history of dementia (Neary *et al.*, 1990; Stevens *et al.*, 1998).

Clinically, FTD is characterized by progressive behavioral changes and disturbances of language. Disinhibition, loss of initiative and diminished attention for surroundings are often the initial symptoms, sometimes misdiagnosed as depression. Furthermore, stereotypic and

ritual behavior or apathy and withdrawal are characteristic of the disorder (Heutink *et al.*, 1997; van Swieten *et al.*, 1999). In contrast to AD, memory problems are not prominent at the initial stage of the disease (Lund and Manchester Groups, 1994; Neary *et al.*, 1998). Later in the disease process, cognitive decline, economy of speech or even mutism and rigidity can occur (Heutink *et al.*, 1997). Focal atrophy of the frontal and temporal lobes is characteristic for this disease, sometimes asymmetric and usually most severe frontally (Neary *et al.*, 1998). Neuropathologically, neuronal loss, gliosis and spongiosis are characteristics of FTD. Approximately 35% of the patients with FTD have cellular inclusions that stain positive for the microtubule associated protein tau (MAPT). These depositions of abnormally hyperphosphorylated tau can vary considerably in their characteristics, ranging from pretangles to Pick bodies, location (neuronal and/or glial inclusions) and in quantity (Mann *et al.*, 2000; Spillantini *et al.*, 1998a).

However, the majority of patients with FTD (65%) show frontotemporal neuronal loss but no disease-specific lesions and no tau pathology. Several other names are used for this neuropathological entity, such as frontotemporal lobar degeneration (FTLD) or dementia lacking distinctive histology (DLDH).

1.3 Tauopathies

Interestingly, tau pathology is also a characteristic observation in several other neurodegenerative disorders, such as Alzheimer's disease (Braak & Braak, 1991), Pick's disease (Hof *et al.*, 1994), progressive supranuclear palsy (PSP) (Hauw *et al.*, 1990; Hof *et al.*, 1994), corticobasal neurodegeneration (CBD) (Paulus & Selim, 1990) and amyotrophic lateral sclerosis/parkinsonism-dementia complex of Guam (Hirano *et al.*, 1966). This group of disorders is covered by the name tauopathies and is overviewed in table 1 (Spillantini *et al.*, 1998a).

Table 1. Overview of tauopathies

Disease	References
Alzheimer's disease	(Braak & Braak, 1991)
Amyotrophic lateral sclerosis/parkinsonism-dementia complex of Guam*	(Hirano *et al.*, 1966)
Argyrophilic grain disease*	(Braak & Braak, 1989; Tolnay *et al.*, 1997)
Corticobasal degeneration*	(Paulus & Selim, 1990)
Creutzfeldt-Jacob disease	(Shin *et al.*, 1989)
Dementia pugilistica*	(Hof *et al.*, 1992a)
Down's syndrome	(Mann *et al.*, 1989)
Frontotemporal dementia with parkinsonism linked to chromosome 17*	(Foster *et al.*, 1997; Spillantini *et al.*, 1998a)
Gerstmann-Sträussler-Scheinker disease	(Ghetti *et al.*, 1989)
Myotonic dystrophy	(Kiuchi *et al.*, 1991)
Niemann-Pick disease, type C	(Love *et al.*, 1995)
Pick's disease*	(Hof *et al.*, 1994)
Postencephalitic parkinsonism	(Geddes *et al.*, 1993)
Prion protein cerebral amyloid angiopathy	(Ghetti *et al.*, 1996)
Progressive subcortical gliosis*	(Mann, 1998)
Progressive supranuclear palsy*	(Hauw *et al.*, 1990; Hof *et al.*, 1992b)
Tangle only dementia*	(Bancher *et al.*, 1987)

*diseases where tau pathology is the most prominent neuropathological feature

1.4 Frontotemporal dementia and parkinsonism linked to chromosome 17

In 1994 linkage to chromosome 17q21-22 was found for a single family with an unusual syndrome called the disinhibition-dementia-parkinson-amyotrophic complex (DDPAC) (Wilhelmsen *et al.*, 1994). Other families, with a variety of clinical and pathological characteristics, were reported to be linked to the same region (Bird *et al.*, 1997; Heutink *et al.*, 1997; Petersen *et al.*, 1995; Wijker *et al.*, 1996). The term frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) was proposed and chosen at a consensus meeting in Ann Harbor in 1996 to cover the spectrum of 13 large families with significant linkage to chromosome 17q21-22 (Foster *et al.*, 1997). The most common clinical features were abnormal behavior

with disturbed executive function combined with relatively preserved memory, orientation and visuo-spatial function. Parkinsonian features of rigidity and postural instability were seen early in the course of the disease in some of the patients (Foster *et al.*, 1997). Essentially, the same description as the Lund and Manchester groups gave for FTD (Lund and Manchester Groups, 1994). The neuropathology of FTDP-17 is characterized by marked neuronal loss in affected brain regions, with extensive neuronal or neuronal and glial fibrillary pathology composed of hyperphosphorylated tau protein, but without other disease-specific brain lesions or deposits (eg. β-amyloid or α-synuclein) in the majority of the cases (Figure 1) (Foster *et al.*, 1997; Spillantini *et al.*, 1998a).

Since tau pathology is the hallmark of the tauopathies, including FTDP-17, and the gene encoding the tau protein being located at chromosome 17q21-22, within or near the FTDP-17 locus, it was an obvious candidate from the beginning. In 1998 genetic and molecular biological analysis from several groups showed that tau is indeed the responsible gene. Several exonic and intronic mutations were found in most of the FTDP-17 families (Dumanchin *et al.*, 1998; Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998b). This was a major discovery in the field of dementia research, showing for the first time that tau dysfunction can cause neurodegeneration itself. Convincing evidence was provided that tau plays a central and not just a secondary role in the disease process. Many novel *tau* mutations have been identified in predominantly smaller families over recent years, widening the clinical spectrum (Rosso & van Swieten, 2002). These findings provided valuable information and options for research into the pathophysiological mechanisms of tau dysfunction and the relationship to neurodegeneration in general.

1.5 Microtubule associated protein tau

The microtubule associated protein tau (MAPT) is a member of the family of microtubule associated proteins (MAP). Tau and other MAP homologues are found in many animal species, ranging from *Caenorhabditis elegans* to chicken, bovine and human (Goedert *et al.*, 1996a; Himmler, 1989; McDermott *et al.*, 1996). Although one or more MAP proteins are found in virtually every eukaryotic cell type, expression of tau is primarily neuronal. The tau protein is abundant in both the central (CNS) and peripheral nervous systems (PNS), predominantly in axons (Binder *et al.*, 1985; Tashiro *et al.*, 1997). The human *tau* gene encompasses over 100 kb of genomic sequence on the long arm of chromosome 17 (17q21) (Neve *et al.*, 1986). The *tau* gene contains 16 exons, but exons 4A, 6 and 8 are only found in the transcripts in the PNS. Exon 4A is found in human, bovine and rodents, with a high degree of homology. Exon -1 and exon 14 are transcribed, but not translated (Andreadis *et al.*, 1992; Neve *et al.*, 1986). Exon 13 does not splice to exon 14, but instead, the intron between exons 13 and 14 is retained in the transcript (Andreadis *et al.*, 1992). Exons 2, 3 and 10 are alternatively spliced, giving rise to six different tau isoforms in the adult human brain, ranging from 352 to 441 amino acids. The molecular weights of these isoforms vary between 50 and 70 kDa.
In the carboxy-terminal part of tau, three (3R) or four (4R) imperfect repeats are present, containing domains important for binding to microtubules. The repeat domains are encoded by exons 9-13, where exon 10 encodes the additional fourth repeat (Andreadis *et al.*, 1992;

Goedert *et al.*, 1989a; Goedert *et al.*, 1989b). The repeat motifs consist of 31 or 32 amino acids; each of them contains the characteristic and highly conserved Pro-Gly-Gly-Gly motif (Lee *et al.*, 1989). The alternative splicing of exons 2 and 3 results in the absence (0N) or presence of 29 (1N) or 58 (2N) inserted amino acids located in the amino terminus of the protein (Figure 2). The expression of the tau gene is regulated developmentally. In fetal human brain, only the shortest isoform (0N3R) is expressed. In adult human brain, all six isoforms are expressed (Goedert *et al.*, 1989b). The ratio between 3R and 4R isoforms is close to 1, and 0N and 1N isoforms are slightly overexpressed, compared to 2N isoforms, 37%, 54% and 9%, respectively (Goedert & Jakes, 1990; Hong *et al.*, 1998). In the PNS, the alternative splicing and transcription of the large exon 4a results in the expression of higher molecular weight proteins, named 'big tau' (120 kDa) (Couchie *et al.*, 1992; Georgieff *et al.*, 1991; Goedert *et al.*, 1992c). The expression of tau differs between species. In fetal rodent brain, the shortest 3R isoform (0N3R) is expressed, like in human fetal brain. However, in adult rodent brain, only three tau isoforms, all containing four microtubule-binding repeats (0N4R, 1N4R and 2N4R) are detected (Goedert *et al.*, 1994; Götz *et al.*, 1995).

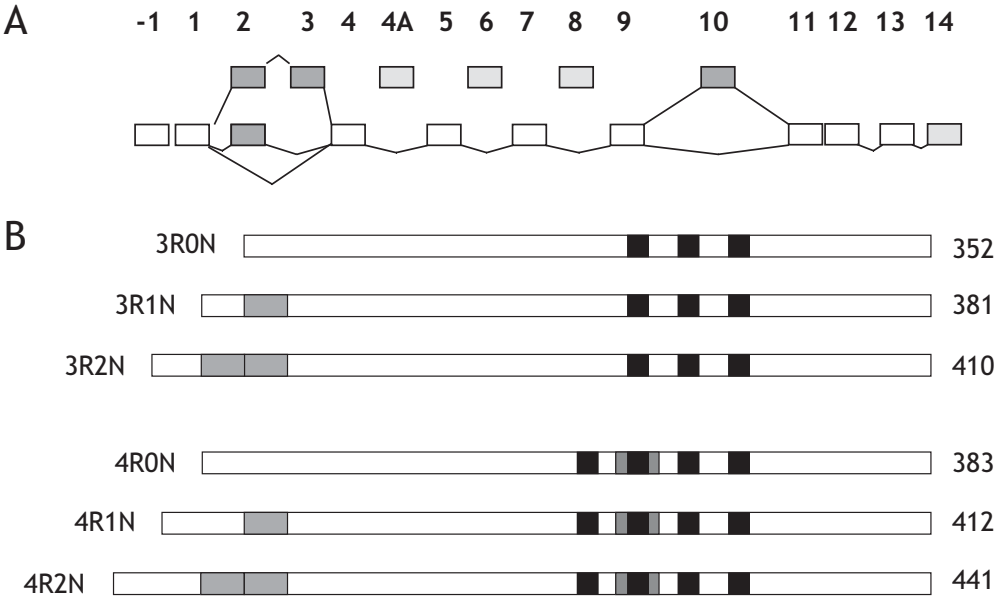


Figure 2: Schematic representation of the *tau* gene and the six different isoforms
(A) The human *tau* gene contains 16 exons, of which exons 2, 3 and 10 (dark grey boxes) are alternatively spliced. Exons 4A, 6 and 8 (light grey boxes) are not transcribed in the human CNS. (B) The six different CNS tau isoforms generated by alternative mRNA splicing of exons 2, 3 and 10 (dark grey boxes), varying in size from 352 to 441 amino acids. The black boxes represent the microtubule binding domains.

The class of MAP proteins, including the tau protein has been shown to play an important role in promoting the assembly of tubulin into microtubules and then maintaining their structural integrity (Cleveland *et al.*, 1977; Weingarten *et al.*, 1975). The four repeat domains of tau are the basic microtubule-interacting unit (Brandt & Lee, 1993; Goode & Feinstein, 1994; Gustke *et al.*, 1994). These motifs are composed of highly conserved 18-amino acid binding

elements separated by less conserved interrepeat regions composed of 13-14 amino acids. 4R isoforms are more efficient in promoting microtubule assembly than 3R isoforms (Butner & Kirschner, 1991; Himmler *et al.*, 1989; Lee *et al.*, 1989). Interestingly, the interrepeat sequence between the first and second microtubule-binding repeat has more than twice the binding affinity of any individual repeat (Goode & Feinstein, 1994). This region is only present in 4R isoforms and is believed to cause the difference in microtubule binding affinity (Goedert & Jakes, 1990).

The middle part of the tau protein is rich in proline residues (proline-rich region) and also required for efficient microtubule assembly (Brandt & Lee, 1993; Trinczek *et al.*, 1995). The aminoterminal region of tau projects from the microtubule surface and may interact with other cytoskeletal elements and plasma membrane components (Brandt *et al.*, 1995; Hirokawa *et al.*, 1988). Tau also plays a role in neuritic outgrowth and stabilization. Furthermore, tau interacts with the actin cytoskeleton and thereby plays a role in regulating cell shape, motility and microtubule-plasma membrane interactions. Tau may interact with actin directly, but more likely it associates with the actin cytoskeleton (Kempf *et al.*, 1996).

1.6 Tau phosphorylation

There are 79 potential serine (Ser) and threonine (Thr) phosphorylation sites described in the longest tau isoform. Phosphorylation at approximately 30 of these sites has been reported in normal tau proteins (Billingsley & Kincaid, 1997; Buee *et al.*, 2000). Most of these sites are clustered in regions flanking the microtubule binding repeats, and it is known that an increase in tau phosphorylation negatively regulates microtubule binding (Biernat *et al.*, 1993; Bramblett *et al.*, 1993). Tau phosphorylation is developmentally regulated; in such a way that fetal tau is more highly phosphorylated in embryonic CNS compared with tau in the adult CNS (Bramblett *et al.*, 1993; Kanemaru *et al.*, 1992; Watanabe *et al.*, 1993). The degree of phosphorylation of all six tau isoforms decreases with age, probably because of the activation of phosphatases (Mawal-Dewan *et al.*, 1994). The different stages of tau phosphorylation result from the activity of specific kinases and phosphatases to these sites.

A large number of Ser/Thr kinases have been implicated or suggested to play a role in regulating tau phosphorylation, including mitogen-activated protein kinase (MAPK) (Drewes *et al.*, 1992; Goedert *et al.*, 1992a), glycogen synthase kinase 3β (GSK-3β) (Hanger *et al.*, 1992), cyclin dependent kinases 2 and 5 (cdk2 and cdk5) (Baumann *et al.*, 1993; Kobayashi *et al.*, 1993), Ca^{2+}/calmodulin-dependent protein kinase II (CaMPKII) (Baudier & Cole, 1987) and microtubule-affinity regulating kinase (MARK) (Drewes *et al.*, 1997). In addition, several members of the family of stress-activated protein (SAP) kinases can also phosphorylate tau at multiple sites (Goedert *et al.*, 1997; Reynolds *et al.*, 1997). Notably, many of the studies to implicate the specific kinases in the phosphorylation of tau provide only *in vitro* evidence, and their *in vivo* role remains unclear. Although further *in vivo* experiments are necessary, GSK-3β and cdk5 are obvious and promising candidates. GSK-3β is expressed abundantly in brain and accociates with microtubules (Mandelkow *et al.*, 1992). Several co-transfection studies of tau and GSK-3β showed induction of tau hyperphosphorylation and loss of microtubule

binding (Lee *et al.*, 2003; Singh *et al.*, 1995). In addition, direct inhibition of GSK-3β reduces tau phosphorylation and affects microtubule stability (Lee *et al.*, 2001; Leost *et al.*, 2000). Cdk5 is highly expressed in neuronal cells and colocalizes with the cytoskeleton. Cdk5 forms a phosphorylation-dependent complex with tau, which promotes the binding to microtubules. Moreover, prephosphorylation of tau by cdk5 stimulates the rate and extend of subsequent GSK-3β-mediated phosphorylation (Noble *et al.*, 2003; Sengupta *et al.*, 1997).

Protein phosphatases are required for counterbalancing the effects of the tau protein kinases and have many direct and indirect effects. Several phosphatases are implicated in regulating tau phosphorylation, including PP1, PP2A, PP2B (calcineurin) and PP2C (Billingsley & Kincaid, 1997; Buee *et al.*, 2000). PP1 and PP2A were found to bind to tau, either by examining purified calf MTs, or by immunofluorescence microscopy techniques. Biochemical analysis and profiling revealed the two different phosphatases, which were associated with the MTs. The interaction to tau is believed to mediate the association with microtubules (Liao *et al.*, 1998; Sontag *et al.*, 1995). PP2A has been demonstrated to bind directly to tau and to microtubules, examined *in vitro* at a submolecular level. This interaction seems to regulate the phosphatase activity of PP2A (Sontag *et al.*, 1999). Furthermore, inhibition of either PP1 or PP2A in cultured human neuronal cells results in increased tau phosphorylation, accompanied by decreased tau binding to microtubules, selective destruction of stable microtubules and rapid degeneration of axons (Merrick *et al.*, 1997). For both kinases, implicated in the phosphorylation of tau, and phosphatases, further studies are necessary to define the *in vivo* regulation of the phosphorylation state of tau and the role of individual kinases and phosphatases. Phosphorylation of tau at different sites could affect not only its interaction with microtubules, but also its capacity to aggregate or interact with other proteins. The more phosphorylated tau is, the less able it is to bind to microtubules and to promote microtubule assembly (Bramblett *et al.*, 1993; Yoshida & Ihara, 1993). Some sites, in particular Ser 262 and Ser 396 might play a dominant role in the reduced binding capacities (Biernat *et al.*, 1993; Bramblett *et al.*, 1993). The delicate balance of kinase and phosphatase activities results in a continuing flow of phosphorylation and dephosphorylation, different in each individual protein.

1.7 Tau pathology

Filamentous neuronal or glial tau inclusions associated with the degeneration of affected brain regions are the defining neuropathological features of not only FTDP-17, but also the other tauopathies.

Alzheimer's disease

In Alzheimer's disease neurofibrillary tangles (NFT) are found in conjunction with senile plaques (SP). Clinically, AD is characterized by presenting symptoms of memory loss, aphasia, apraxia and behavioural disturbances, followed later in the disease by defects in cognitive functions and dementia (Suh & Checler, 2002). Alois Alzheimer first described the neuropathological hallmarks of the disease in 1911, but it is only since the last 15 years that the chemical composition has been elucidated (Alzheimer, 1911; Grundke-Iqbal *et al.*, 1986a;

Grundke-Iqbal *et al.*, 1986b). The key protein responsible for the generation of senile plaques is the amyloid precursor protein (APP), a fragment of which ($A\beta^{42}$ peptide) is neurotoxic and tends to aggregate in extracellular pleated structures throughout the brain (Glenner & Wong, 1984; Masters *et al.*, 1985). Mutations in the APP gene are found in cases with familial AD, strongly emphasizing its importance to the etiology of the disease (Goate *et al.*, 1991; Suh & Checler, 2002). The neurofibrillary lesions are formed intracellular within nerve cells of the cerebral cortex, the hippocampal formation and some subcortical nuclei. Ultrastructurally, the dominant components of the neurofibrillary lesions in AD are paired helical filaments (PHF) and straight filaments (SF) (Kidd, 1963). PHFs are composed of two strands of tau filaments twisted around one another with a periodicity of 80 nm and a diameter varying from 8 to 20 nm. SFs have a diameter of 15 to 18 nm and lack the helical periodicity (Crowther & Wischik, 1985). Both PHFs and SFs are composed predominantly of abnormally hyperphosphorylated tau proteins, immunoreactive to antibodies against the N- and C-terminal regions of the tau protein (Wischik *et al.*, 1988). Extraction of PHFs purified from brain and analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) reveals a profile that is often disease-specific. Tau protein derived from AD brain consists of three major bands of approximately 60, 64 and 68 kDa, as well as a minor band of 72 kDa (Figure 3A) (Greenberg & Davies, 1990). Several groups tried to elucidate which tau isoforms compose each PHF-tau band. The longest (4R2N) and the shortest (3R0N) tau isoforms make up the 72 kDa and the 60 kDa bands, respectively. The 64 kDa band is composed of a mixture of 3R1N, 3R2N, 4R1N and 4R2N tau isoforms. The 68 kDa consists of 3R2N and 4R2N tau isoforms, all hyperphosphorylated in a different state (Mulot *et al.*, 1994; Sergeant *et al.*, 1997; Spillantini *et al.*, 1997). After dephosphorylation, six bands are resolved that correspond to the six isoforms of tau found in adult human brain (Greenberg *et al.*, 1992). The relative proportions of the tau isoforms found in AD PHFs are similar to those found in normal adult human brain (Trojanowski & Lee, 1994). Evidence in support of a causative role of tau protein in neurodegeneration is provided by recent studies of other tauopathies, such as PSP, CBD and PiD, where tau pathology is the most prominent neuropathological feature.

Progressive supranuclear palsy

Clinically, progressive supranuclear palsy (PSP) is characterized by supranuclear gaze palsy as well as prominent postural instability (Steele, 1964). Neuropathologically, PSP is characterized by neuronal loss and gliosis in basal ganglia, subthalamus and brainstem. Within these brain regions, multiple NFTs, tufted astrocytes, coiled bodies and neuropil threads (NT) are found (Litvan *et al.*, 1996). Ultrastructural analysis revealed predominantly 15 to 18 nm straight filaments and filaments with a long periodicity (Roy *et al.*, 1974). Biochemical analysis showed two bands of 64 and 68 kDa, while the 72 kDa band is variably detected, all representing 4R tau isoforms (Figure 3B) (Vermersch *et al.*, 1994). A relative increase of 4R tau mRNA is found in brainstem, consistent with the distribution of the neurofibrillary pathology (Chambers *et al.*, 1999).

Pick's disease

Pick's disease (PiD) is defined neuropathologically by the presence of tau immunoreactive Pick bodies and frontotemporal atrophy associated with marked neuronal loss (Constantinidis *et al.*, 1974; Dickson, 1998). Ultrastructurally, Pick bodies are composed of a mixture of

15nm straight filaments and twisted filaments with a long periodicity (Dickson, 1998; Munoz-Garcia & Ludwin, 1984). Biochemical analysis showed that the composition of the insoluble tau is different from that in AD and PSP. Two major bands of 60 and 64 kDa can be detected, and a minor band of 68 kDa (Figure 3C) (Delacourte *et al.*, 1996). These two major bands specifically lack the microtubule binding repeat encoded by exon 10, thus are predominantly composed of 3R tau (Mailliot *et al.*, 1998). Phosphorylation of Ser 262, as detected by anti-tau antibody 12E8, is reported to be heterogeneous in PiD (Lieberman *et al.*, 1998; Probst *et al.*, 1996).

Numerous protein kinases and phosphatases have been implicated in the dysregulation of tau phosphorylation in neurodegenerative brains, mainly in AD (Billingsley & Kincaid, 1997). It was suggested that cdk5 plays a role in this process. It was shown that p25, a truncated form of p35, accumulates in neurons in the brains of AD patients (Patrick *et al.*, 1999). The accumulation of p25 correlated with the increased cdk5, forming a complex. Finally, expression of the p25/cdk5 complex in cells increased tau phosphorylation and disrupted the cytosceletal network (Kusakawa *et al.*, 2000; Lee *et al.*, 2000). This complex may very well play a role in the conversion of normal tau into PHF tau.

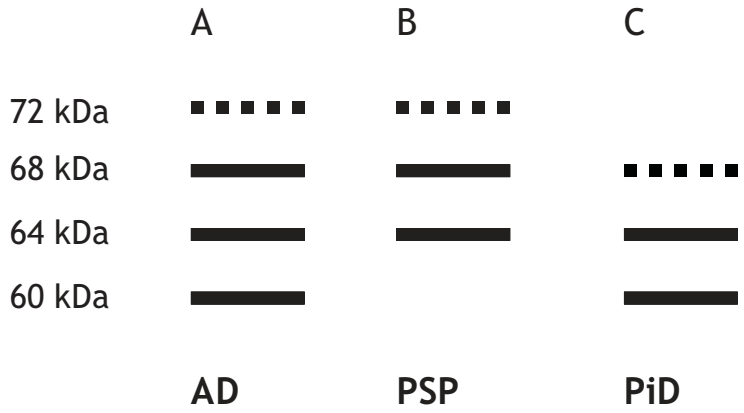


Figure 3: Schematic representation of Western blot banding patterns of insoluble tau from different tauopathies.
(A) Phosphorylated insoluble tau from brain of AD patients run as three major bands of 60, 64 and 68 kDa, and a minor, variable band of 72 kDa. (B) Phosphorylated insoluble tau from brain of PSP patients run as two major bands of 64 and 68 kDa. The 72 kDa band is variable detected. (C) In brain from PiD patients two major bands of 60 and 64 kDa are observed, and a minor, variable band of 68 kDa. Major tau bands are depicted by solid bars, and dashed bars are used to depict the minor and variable tau bands.

1.8 Tau as a risk factor

After the involvement of tau in the onset of FTDP-17, many studies were performed to investigate the involvement in other tauopathies. With a few exceptions no mutations were found in families with other neurodegenerative disorders. But recent studies showed that tau can be a risk or modifying factor in the development of several disorders. Genetic

changes in the *tau* gene may contribute to the risk of developing PSP because a polymorphic dinucleotide repeat in the intron between exon 9 and 10 of the *tau* gene has been associated with PSP (Conrad *et al.*, 1997). Subjects with the homozygous *tau* allele A0, characterized by 11 TG repeats, were found to be overrepresented in PSP patients (95.5%) compared with controls (57.4%) and AD patients (49.7%) in a Caucasian population. Subsequent studies have confirmed this correlation in other Caucasian populations, as well as its association with an earlier age at onset (Bennett *et al.*, 1998; Hoenicka *et al.*, 1999; Molinuevo *et al.*, 2000). More detailed studies using multiple tau polymorphisms spanning the whole *tau* gene including the promoter region showed association with an extended haplotype (H1/H1) in complete linkage disequilibrium with the *tau* gene. The more common haplotype H1 is significantly overrepresented in unrelated Caucasian PSP patients (Bonifati *et al.*, 1999; de Silva *et al.*, 2001; Pastor *et al.*, 2002). CBD shows a similar association with the A0 allele of the *tau* gene as well as with the H1 haplotype, like PSP, suggesting it to be a risk factor for developing CBD (Houlden *et al.*, 2001).

Extensive association studies on pathologically confirmed PiD cases showed no apparent association with *tau* haplotypes, suggesting that the *tau* gene is not a risk factor for PiD (Morris *et al.*, 2002; Russ *et al.*, 2001). The biological relevance of these associations is unknown; it is unclear whether the tau gene is simply a marker for a mutation in a nearby gene or whether the *tau* gene itself is responsible for the pathogenic changes. Chambers *et al.* analyzed tau mRNA isoform expression in selective affected and unaffected brain region in PSP patients, compared to control brain. They showed an increased production of 4R tau isoforms in the brainstem, a region heavily affected with NFTs in PSP, thereby mimicking the situation of splice mutations in FTDP-17 (Chambers *et al.*, 1999). Recently, also mutations in the *tau* gene were described in patients diagnosed with PSP, thereby widening the spectrum of neurodegenerative disorders caused by mutations in the *tau* gene (Morris *et al.*, 2003; Stanford *et al.*, 2000). Both of these studies are in favor of the hypothesis that the *tau* gene itself is involved in other tauopathies, rather than simple being a marker.

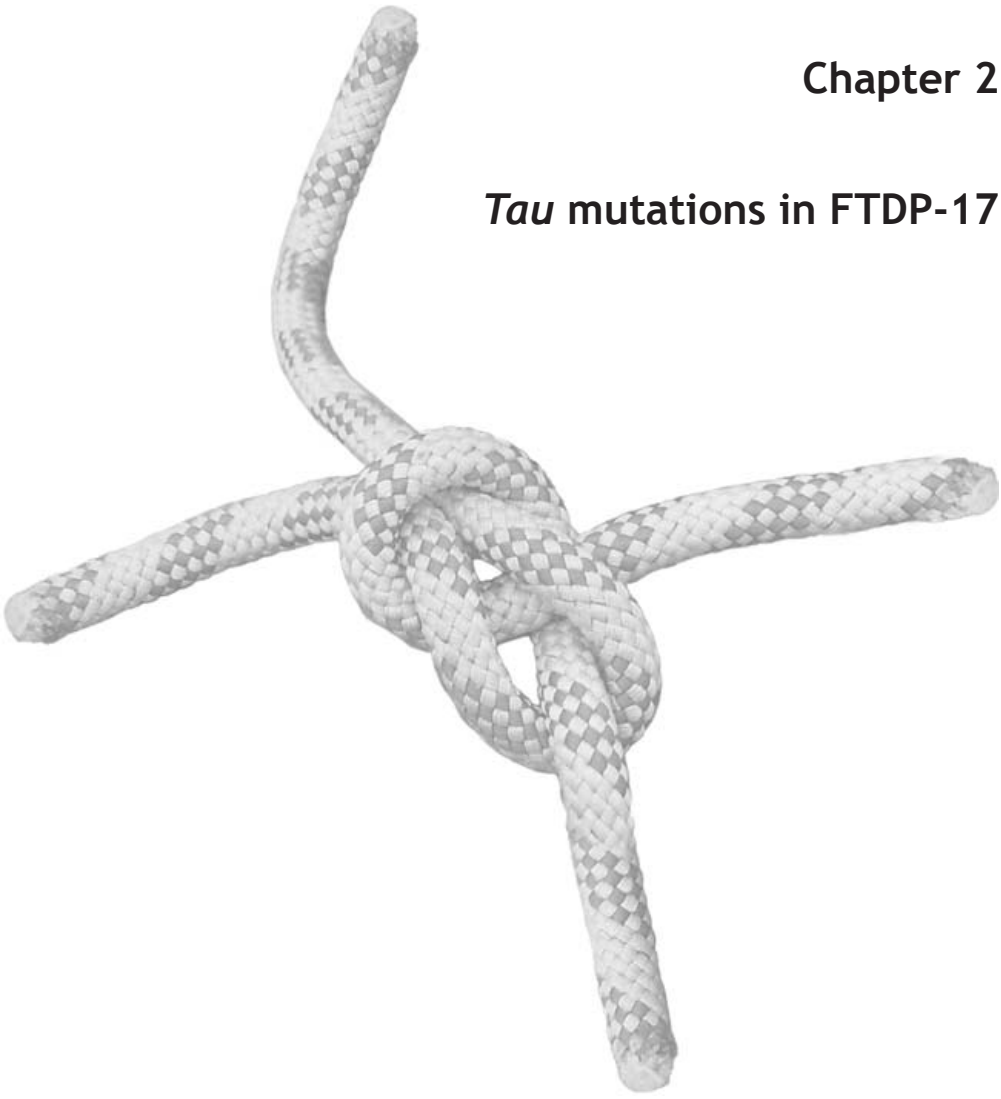
1.9 Scope of the thesis

The project described in this thesis started at the moment the first mutations in the *tau* gene were identified in families and patients with FTDP-17. At that moment, only little was known about the functional effects and frequencies of the mutations and of the molecular and cellular pathways that were involved. Therefore, we focused on two main research topics.

The first part of my thesis (**Chapters 2, 3 and 4**) focuses on *tau* mutations identified in patients with FTDP-17. Identification of novel *tau* mutations can lead to a better understanding of the disease, and widens the spectrum of clinical entities associated with *tau* mutations. After an introduction and overview of the literature (**Chapter 2**), two novel *tau* mutations are reported, with detailed description of clinical features, neuropathology, biochemical analysis and functional studies. The novel S320F *tau* mutation was described in a single patient with familial FTDP-17 and was the first mutation in exon 11 of the *tau* gene (**Chapter 3**). Tau neuropathology with inclusions resembling Pick's disease was observed.

The L315R mutation was described in two families with FTDP-17 in **Chapter 4**. Both families showed a large variation in disease expression, including non-penetrance in an 82-years-old mutation carrier.

The second part of my thesis (**Chapters 5, 6 and 7**) concentrates on model systems and functional studies. Model systems are essential to study the onset and the molecular mechanisms of this neurodegenerative disorder. The first chapter of this part (**Chapter 5**) gives an overview of cellular and animal models, tau interacting proteins and the degradation of the tau protein. In **Chapter 6** we describe the generation and identification of transgenic mice, carrying a human *tau* mutation under the control of a brain-specific promoter. In **Chapter 7** the degradation pathways of tau were studied, using a mouse model that is mimicking the human situation. Finally, the main findings of the studies are presented and discussed in **Chapter 8**.



Chapter 2

Tau mutations in FTDP-17

Tau mutations in FTDP-17

2.1 Identification and neuropathology

In 1994, a genetic-epidemiological study on FTD was started in the Netherlands. Three large families were collected and examined, and showed linkage to the same region of chromosome 17, 17q21-22 (Heutink *et al.*, 1997). As described in chapter 1.4, a consensus conference at Ann Harbor in 1996 was organized by geneticists and clinicians with FTD families, to establish whether these families would constitute a new distinct group with comparable clinical and neuropathological features (Foster *et al.*, 1997). The observation that all families were linked to the same region on chromosome 17q21-22, strongly suggested that a single locus or gene is responsible for this new disorder called frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (Foster *et al.*, 1997). Because the tau gene had been localized to chromosome 17q21-22 and hyperphosphorylated tau filaments were present in brains of affected individuals, it was an obvious candidate gene.

In 1998 our research group, followed by several other groups identified pathogenic mutations in the tau gene that co-segregated with FTDP-17 (Dumanchin *et al.*, 1998; Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998b). Until now, more than 30 distinct pathogenic mutations in the tau gene have been identified in a large number of families with FTDP-17 (see figure 1 and table 1). The first mutations described were three exonic mutations (G272V, P301L and R406W), in exons 9, 10 and 13 respectively, and three intronic mutations, all located in the intron following exon 10, disrupting the 5' splice site of exon 10 (Hutton *et al.*, 1998). All tau mutations identified until now are either missense, silent or deletion mutations in the coding region, or intronic mutations located close to the splice-donor site of the intron following exon 10 (Rosso & Van Swieten, 2002).

All missense mutations, except the two recently identified mutations in exon 1 (Hayashi *et al.*, 2002; Poorkaj *et al.*, 2002), are located in or near the microtubule binding domain of tau that consists of the repeats and interrepeat flanking regions. Mutations in exons 1, 9, 11, 12 and 13 affect all six tau isoforms, while mutations in exon 10 affect only tau isoforms with four microtubule-binding repeats. The recently identified mutations in exon 1, R5H and R5L, are found in a patient with late-onset (75 years of age) frontotemporal dementia and a patient with clinical and neuropathological PSP, respectively (Hayashi *et al.*, 2002; Poorkaj *et al.*, 2002). Surprisingly, the first mutation in exon 11 (S320F) was only described in 2002 (Rosso *et al.*, 2002), followed in 2003 by the second (L315R) (Van Herpen *et al.*, 2003). Detailed descriptions of these mutations can be found in chapters 3 and 4, respectively. The relative underrepresentation of mutations is this exon, containing the third microtubule binding repeat, cannot yet be explained. Intronic mutations were found in the intron after exon 10 (+3, +11, +12, +13, +14, +16), with the first nucleotide of the splice donor site taken as +1 (Lee *et al.*, 2001; Rizzu *et al.*, 1999). In addition a possible mutation was found in the intron after exon 9 (+33), but no functional data are available.

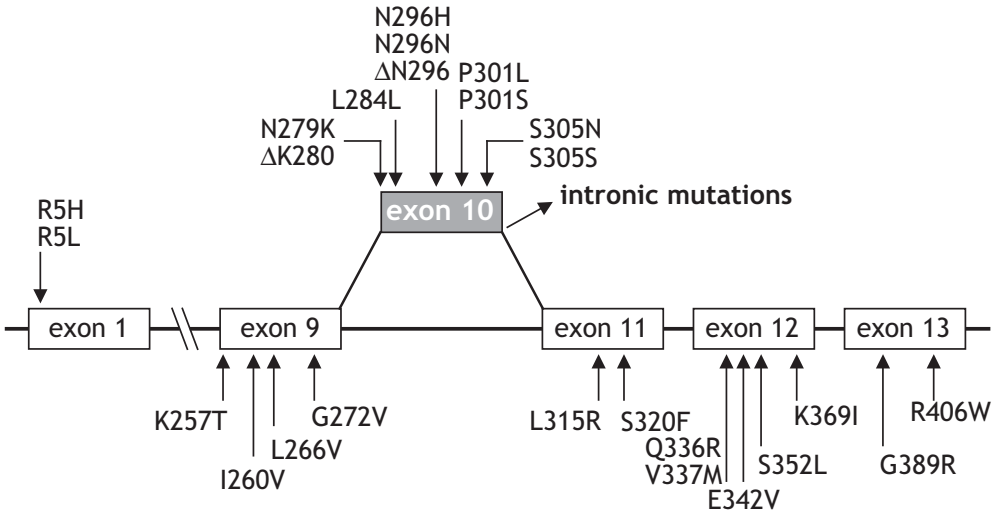


Figure 1: Schematic overview of mutations in the *tau* gene, identified in FTDP-17
All missense mutations identified up until now are located in exons 1 and 9 to 13. The alternatively spliced exon 10 is depicted in grey. Mutations are numbered according to codon number of the longest tau isoform (441 amino acids).

All cases of tau mutations in FTDP-17 patients examined showed the presence of filamentous aggregates containing hyperphosphorylated tau proteins (Goedert *et al.*, 1998b; Spillantini *et al.*, 1998b). The morphology of the filaments, their isoform composition and the distribution seems to differ by the location and effect of the specific *tau* mutation.

Coding region mutations located outside exon 10 affect all six isoforms (Murrell *et al.*, 1999; van Swieten *et al.*, 1999). Those mutations lead to a tau pathology that is largely neuronal, without a significant glial component. For some mutations in exon 13 (V337M, R406W) the morphologies and biochemical characteristics of tau filaments (both PHF and SF and containing all six tau isoforms) are indistinguishable from those of AD (Hong *et al.*, 1998; van Swieten *et al.*, 1999). By contrast, another mutation in exon 13, G389R, leads to a pathology more closely resembling Pick's disease, with large numbers of Pick-like inclusions, although the majority of the filaments look much like the SF found in AD (Murrell *et al.*, 1999). Other coding mutations (K257T, L266V, G272V, L315R, S320F, E342V and Q336R) give rise to a pathology characteristic of PiD, with large numbers of tau-immunoreactive Pick body-like and axonal inclusions (Kobayashi *et al.*, 2003; Lippa *et al.*, 2000; Rizzini *et al.*, 2000; Rosso *et al.*, 2002; Spillantini *et al.*, 1998a; Van Herpen *et al.*, 2003; Pickering-Brown *et al.*, 2004). These mutations show a large variation in the composition and structure of the tau filaments. The L266V, L315R, S320F and K369I mutations all lead to the formation of both straight and twisted filaments composed of 3R and 4R tau. Filaments produced by the K257T mutation are mainly twisted, but are composed predominantly of 3R tau (Rizzini *et al.*, 2000). The E342V mutation is different again, resulting in filaments resembling PHF in AD, but composed predominantly of 4R0N tau (Lippa *et al.*, 2000). A lack of fresh brain material from patients with the G272V mutation has prevented biochemical analysis until now.

Table 2: **Overview of *tau* mutations**

Mutation	Tau pathology	Biochemical analysis	Ultrastructural analysis	Additional studies	Reference
R5H	Glial-predominant inclusions	↑ 4R	Straight filaments	↓ microtubule binding	(Hayashi *et al.*, 2002)
R5L	Neuronal NFTs and astrocytic inclusions	↑ 4R	Straight filaments	↓ microtubule binding	(Poorkaj *et al.*, 2002)
K257T	Pick-like inclusions	3R > 4R	Narrow twisted	↓ microtubule binding	(Rizzini *et al.*, 2000)
I260V	Neuronal and glial tau pathology	4R isoforms	Straight filaments	↓ microtubule binding (only 4R)	(Grover *et al.*, 2003)
L266V	Pick-like and astrocytic inclusions	3R0N; 3R1N; 4R0N; 4R1N	NA	↓ microtubule binding	(Kobayashi *et al.*, 2003)
G272V	Pick-like inclusions	NA	NA	NA	(Hutton *et al.*, 1998)
+33 (5' splice ex 9)	NA	NA	NA	NA	(Rizzu *et al.*, 1999)
N279K	Neuronal and glial inclusions	↑ 4R	Straight and twisted filaments	↑ 4R (exon trapping)	(Delisle *et al.*, 1999; Wszolek *et al.*, 2000)
ΔK280	NA	NA	NA	↑ 3R (exon trapping)	(D'Souza *et al.*, 1999; Rizzu *et al.*, 1999)
L284L	Neuronal and glial inclusions + Aβ deposits	NA	NA	↑ 4R (exon trapping)	(D'Souza *et al.*, 1999)
N296H	Glial inclusions	↑ 4R	Variable modalities	↑ 4R (exon trapping) ↓ micro-tubule binding	(Grover *et al.*, 2002; Iseki *et al.*, 2001)
N296N	Corticobasal-like inclusions	NA	NA	↑ 4R (exon trapping)	(Grover *et al.*, 2002; Iseki *et al.*, 2001)
ΔN296	NA	NA	NA	↓ micro-tubule binding	(Grover *et al.*, 2002; Iseki *et al.*, 2001)
P301L	Neuronal and glial inclusions	↑ 4R	Twisted ribbons	↓ microtubule binding	(Dumanchin *et al.*, 1998; Hasegawa *et*

Mutation	Tau pathology	Biochemical analysis	Ultrastructural analysis	Additional studies	Reference
					al., 1998; Hutton *et al.*, 1998; Mirra *et al.*, 1999; Spillantini *et al.*, 1998a)
P301S	Neuronal and glial inclusions	NA	Straight filaments	↓ microtubule binding	(Bugiani *et al.*, 1999; Sperfeld *et al.*, 1999)
S305N	Neuronal and glial inclusions	NA	Straight filaments	↑ 4R (exon trapping)	(Hasegawa *et al.*, 1998; Iijima *et al.*, 1999)
S305S	Neuronal and glial inclusions	NA	Straight and twisted filaments	↑ 4R (exon trapping)	(Stanford *et al.*, 2000; Wszolek *et al.*, 2001)
+3 (5' splice ex 10)	Neuronal and glial inclusions	↑ 4R	Twisted filaments	↑ 4R (exon trapping)	(Spillantini *et al.*, 1998b; Tolnay *et al.*, 2000)
+11 (5' splice ex 10)	Neuronal and glial inclusions	NA	NA	↑ 4R (exon trapping)	(Kowalska *et al.*, 2002; Miyamoto *et al.*, 2001)
+12 (5' splice ex 10)	Neuronal and glial inclusions	↑ 4R	Twisted filaments	↑ 4R (exon trapping)	(Yasuda *et al.*, 2000)
+13 (5' splice ex 10)	NA	NA	NA	NA	(Hutton *et al.*, 1998)
+14 (5' splice ex 10)	Neuronal and glial inclusions	↑ 4R	Twisted filaments	↑ 4R (exon trapping)	(Hutton *et al.*, 1998)
+16 (5' splice ex 10)	Neuronal and glial inclusions	↑ 4R	Twisted filaments	↑ 4R (exon trapping)	(Goedert *et al.*, 1999b; Hutton *et al.*, 1998; Morris *et al.*, 2003)

Mutation	Tau pathology	Biochemical analysis	Ultrastructural analysis	Additional studies	Reference
+19 (5' splice ex 10)	NA	NA	NA	↑ 3R (exon trapping)	(Stanford *et al.*, 2003)
+29 (5' splice ex 10)	No tau inclusions	No insoluble tau	NA	↑ 3R (exon trapping)	(Stanford *et al.*, 2003)
L315R	Pick-like and astrocytic inclusions	↓ 3R0N	Straight and twisted filaments	↓ microtubule binding	(Van Herpen *et al.*, 2003)
S320F	Pick-like inclusions	↓ 3R0N + 4R2N	Straight and twisted filaments	↓ microtubule binding	(Rosso *et al.*, 2002)
Q336R	Pick-like inclusions	NA	Straight and twisted filaments	↑ microtubule binding; ↑ filament formation	(Pickering-Brown *et al.*, 2004)
V337M	Neuronal NFTs	AD like	Straight and twisted filaments	↓ microtubule binding	(Hasegawa *et al.*, 1998; Hong *et al.*, 1998; Poorkaj *et al.*, 1998)
E342V	Pick-like inclusions with NFTs	↑ 4R0N	Paired helical filaments	↑ mRNA with 4R	(Lippa *et al.*, 2000)
S352L	Neuronal inclusions	NA	NA	↓ microtubule binding	(Nicholl *et al.*, 2003)
K369I	Pick-like inclusions	AD like	Twisted ribbons	↓ microtubule binding	(Neumann *et al.*, 2001)
G389R	Pick-like inclusions	3R0N; 3R1N; 4R0N; 4R1N	Straight and twisted filaments	↓ microtubule binding; ↑ calpain digestion	(Murrell *et al.*, 1999; Pickering-Brown *et al.*, 2000)
R406W	Neuronal NFTs	AD like	Paired helical filaments	↓ microtubule binding	(Hutton *et al.*, 1998; Saito *et al.*, 2002)

NA = non available; NFTs = neurofibrillary tangles; Aβ = β amyloid; AD = Alzheimer's disease

The P301L and P301S mutations within exon 10 lead to a neuronal and glial tau pathology. Analysis of insoluble tau from brain of several P301L patients has revealed the presence of twisted filaments composed predominantly of 4R tau isoforms, resembling those found in PSP (Spillantini *et al.*, 1998a). Intronic mutations that influence the splicing of exon 10 also show widespread neuronal and glial tau pathology. Ultrastructurally, twisted filaments are observed, composed mainly of only of 4R tau isoforms, resembling those found in CBD (Goedert *et al.*, 1999b; Miyamoto *et al.*, 2001; Pickering-Brown *et al.*, 2002; Spillantini *et al.*, 1997; Yasuda *et al.*, 2000).

There is a great variability in the morphology of the filaments resulting from different mutations. Initially, it was believed that filament morphology reflected the tau isoform composition. Although this could be generally true, recent described *tau* mutations have shown that there are exceptions and that other factors might be involved (Ingram & Spillantini, 2002).

2.2 Functional effects

The location of most coding mutations to the carboxyterminal part of tau, where the microtubule binding repeats are located, suggests an effect of the coding mutations on binding of tau to tubulin and the promotion of microtubule assembly. Several studies using *in vitro* microtubule assembly and binding assays were performed to assess the functional consequences of the mutations. It was shown that certain *tau* mutations reduce the binding of tau to microtubules and decrease its ability to promote microtubule assembly in *in vitro* assays (Bugiani *et al.*, 1999; Hasegawa *et al.*, 1998; Hayashi *et al.*, 2002; Kobayashi *et al.*, 2003; Murrell *et al.*, 1999; Neumann *et al.*, 2001; Poorkaj *et al.*, 2002; Rizzini *et al.*, 2000; Rizzu *et al.*, 1999; Rosso *et al.*, 2002; Sperfeld *et al.*, 1999). Various degrees of reduced microtubule binding and defects in microtubule assembly were observed for different *tau* mutations in different assays. Even if some of the missense mutations cause only a modest reduction in microtubule binding affinity, the increased cytoplasmic concentrations of unbound mutant tau proteins may facilitate aggregation of these abnormal proteins into filamentous inclusions.

Furthermore, an overlapping subset of missense mutations promotes heparin- or arachidonic acid-induced tau filaments formation *in vitro* relative to wildtype (WT) tau (Arrasate *et al.*, 1999; Goedert *et al.*, 1999a; Nacharaju *et al.*, 1999). Many mutations have an effect on both the microtubule binding and aggregate formation, as can be seen in table 2. An *in vitro* microtubule binding assay showed that both exon 1 mutations slightly reduce the binding of tau to microtubules (15-20%) (Hayashi *et al.*, 2002; Poorkaj *et al.*, 2002). It is surprising that a substitution at the aminoterminus of the tau protein would affect the interaction with microtubules, because deletion analysis showed that only the microtubule binding region is required for binding. These mutations may act by a gain of function, resulting in an interaction between the amino terminal segment and the microtubule binding domain of tau, causing reduced microtubule binding and a conformational change. However, recently it was described by Gamblin *et al* that the aminoterminal domain of tau is a contributor to the

polymerization process *in vitro* (Gamblin *et al.*, 2003). Another option can still be that these amino acid changes are not pathogenic by themselves, meaning that the functional assays performed *in vitro* do not represent the *in vivo* situation.

Mutations in the intron following exon 10 influence its alternative splicing leading to a change in the ratio of tau 3R and 4R isoforms, but do not change the protein itself. The intronic mutations, clustered around the 5' splice site of exon 10, as well as several mutations within exon 10 (N279K, L284L, N296H, N296N, ΔN296, S305N and S305S) increase the ratio of 4R to 3R tau (Delisle *et al.*, 1999; D'Souza *et al.*, 1999; Grover *et al.*, 2002; Grover *et al.*, 1999; Hasegawa *et al.*, 1999; Hong *et al.*, 1998; Hutton *et al.*, 1998; Spillantini *et al.*, 1998b). The ΔK280 mutation has an opposite effect, in increasing the ratio of 3R to 4R tau. The proposed mechanism for these mutations is destabilizing a short stem-loop structure that spans the 5' splice site (see figure 2). This stem-loop is proposed to compete with the U1 snRNP for binding to the splice site of exon 10 (Hutton *et al.*, 1998). As a result of these mutations, there is a relative increase of exon 10-containing tau mRNA's as demonstrated in exon trapping experiments. Biochemical analysis of insoluble tau extracted from brain tissue of patients with these mutations reveals increased production of 4R tau transcripts. 4R tau protein levels are increased in both affected and unaffected regions of FTDP-17 brains (Hong *et al.*, 1998; Spillantini *et al.*, 1998b).

The regulation of the splicing of exon 10 in the *tau* gene appears to be complex and may involve multiple *cis*-acting regulatory elements that either enhance or inhibit the utilization of the exon 10 5' splice site (D'Souza *et al.*, 1999; D'Souza & Schellenberg, 2000; Gao *et al.*, 2000; Grover *et al.*, 2002; Grover *et al.*, 1999). Recently, it was shown that sequences in both introns after exons 9 and 10 are involved in exon 10 splicing, by a combination of enhancing and silencing sequences, from at least six different modulating elements. Furthermore, determination of a three-dimensional structure of a 25-nucleotide-long RNA from the E10 5'-intron junction by nuclear magnetic resonance (NMR) spectroscopy has shown that this sequence forms a stable, folded stem-loop structure. It was shown that it consists of upper and lower stems that are separated by a bulging A, with an apical stem of six nucleotides (Varani *et al.*, 1999; Varani *et al.*, 2000).

Known intronic mutations and the mutations in codon 305 (S305N and S305S) are located in the upper part of the stem and reduce the thermodynamic stability of the stem loop (Grover *et al.*, 1999; Varani *et al.*, 1999). The S305N mutation and the +3 intronic mutation may also enhance exon 10 splicing by increasing the strength of the splice site (Iijima *et al.*, 1999).

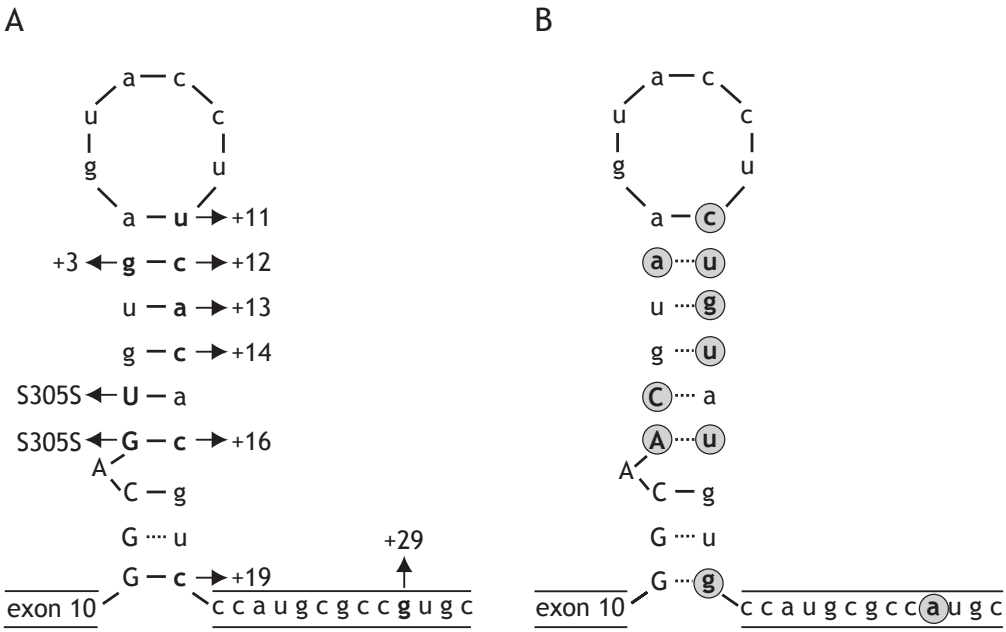


Figure 2: Schematic representation of the predicted stem-loop structure at the junction of exon 10 and the following intron and identified mutations in FTDP-17
Exonic RNA sequence is indicated with upper-case letters, and intronic sequence with lower-case letters. (A) Mutations reducing the stability of this structure are indicated with arrows. Exonic mutations are numbered according to codon number of the longest tau isoform, and intronic mutations are labeled according to nucleotide number, with the first nucleotide of the intron taken as +1. (B) In grey circles are depicted the mutated nucleotides of the stem-loop structure. The dashed bars between the base pairs indicate the destabilized structure of the predicted stem-loop. A destabilized stem-loop structure results in increased exon 10 containing transcripts.

Other pathogenic FTDP-17 mutations may alter exon 10 splicing by affecting one of the regulatory elements. The N279K (AAT to AAG) mutation, for example, creates a purine-rich exonic splice enhancer (ESE) sequence (D'Souza & Schellenberg, 2000). Moreover, the thymidine nucleotide present in the wildtype sequence may function as an inhibitor of splicing (Tanaka *et al.*, 1994). The ΔK280 mutation is of particular interest, because it has a strong effect in microtubule interaction *in vitro*. The reduction in promoting microtubule binding assembly is even stronger than the P301L mutation. However, this effect might be compensated by the observation that this deletion of three purine residues (AAG) abolishes exon 10 splicing almost completely (D'Souza *et al.*, 1999). The L284L mutation disrupts a sequence that might act as an exonic splicing silencer (ESS), thereby enhancing exon 10 splicing. However, because mutation of this consensus sequence does not increase exon 10 splicing, a second possibility is that the mutation lengthens the AC-rich element within the ESE. Thus, this mutation may affect either an inhibiting or an enhancing regulatory splicing element (D'Souza *et al.*, 1999; D'Souza & Schellenberg, 2000; Spillantini *et al.*, 2000). The effect of the codon 296 mutations (N296H, N296N and Δ296) on the increased splicing of exon 10 is probably due to disruption of an ESS, as showed by D'Souza (D'Souza & Schellenberg,

2000). However, Grover *et al* showed that the two point mutations indeed increases the ratio of exon 10 splicing, but the deletion mutant showed no significant change, suggesting that new splice enhancer sequences are created, rather than destroying the putative ESS (Grover *et al.*, 2002). The +33 mutation in the intron following exon 9 (G to A transition) is thought to disrupt one of the several GGG repeats that may play a role in the regulation of the alternative splicing of exon 10, but this was never confirmed by *in vivo* experiments (Cogan *et al.*, 1997).

The +33 mutation in the intron following exon 9 and the ΔK280 mutation in exon 10 were observed only in two individual patients from small families with no other affected family members available for study. Thus, the evidence that these are pathogenic mutations can be obtained only from *in vitro* assays. The study of brain material from autopsied patients will be of great value for understanding the disease process in these cases (Rizzu *et al.*, 1999).

The +19 and +29 mutations show a novel splicing effect by downregulating the splicing of exon 10, switching to more 3R tau isoforms (Stanford *et al.*, 2003). Together with the ΔK280 mutation these are the only known mutations negatively regulating exon 10 splicing (D'Souza *et al.*, 1999; Stanford *et al.*, 2003). The +19 mutation is associated with an intron silencer modulator (ISM) sequence element. Previously, the +29 mutation has been reported as a rare polymorphism, but recently Stanford *et al.* suggest it to be a pathogenic mutation, because it cosegregates with the disease phenotype in multiple generations. No insoluble tau depostions were found in brain material from a patient with this mutation but interestingly an increase in tau degradation products was observed in this patient (Stanford *et al.*, 2003). This observation would suggest another disease mechanism in this patient, without any tau filament formation but only impaired tau degradation, although the amount of data is insufficient to be conclusive.

All mutations, exonic as well as intronic, that affect the splicing of exon 10 have in common that the ratio between 3R and 4R tau is changed. The proteins themselves are unchanged and functional; showing that only disturbance of the precious balance of the different isoforms can result in neurodegeneration. A possible explanation can be that because 3R and 4R tau bind to distinct sites on the microtubules (Goode & Feinstein, 1994), a specific ratio of tau isoforms is needed for normal microtubule stability and function. In addition, overproduction of 4R tau may lead to an excess of free tau in the cytoplasm, leading to its hyperphosphorylation and assembly into filaments. For the mutations with the opposite effect on the splicing of exon 10 (+19 in the intron after exon 10) an increased amount of 3R tau isoforms are present in the cytoplasm, no tau aggregates are formed but an increased proteolysis and activation is observed, suggesting another disease mechanisms based on apoptotic cell death (Stanford *et al.*, 2003). Experiments on several mutations in and around exon 10 show that a single *tau* mutation can cause pathogenic changes at both mRNA and protein levels, emphasizing that the relative effect of a mutation *in vitro* does not necessarily correlates with the *in vivo* situation (D'Souza *et al.*, 1999; D'Souza & Schellenberg, 2002; Grover *et al.*, 2002).

2.3 Frequency of *tau* mutations

The contribution of *tau* mutations in the general FTD population was estimated in a large group of patients in a genetic epidemiological study in the Netherlands in 1999. A mutation in the *tau* gene was found in 17.8% of patients with FTD (Rizzu *et al.*, 1999). In later studies the frequency of *tau* mutations varied considerably in different FTD populations, ranging from 5.6 to 18% (Binetti *et al.*, 2003; Houlden *et al.*, 1999; Poorkaj *et al.*, 2001; Sobrido *et al.*, 2003). The chance of identifying *tau* mutations will greatly depend on the population that is studied (Fabre *et al.*, 2001; Houlden *et al.*, 1999). A recent study of Rosso *et al.* in the Netherlands, partly overlapping with a previous study of Rizzu *et al.*, showed a frequency of *tau* mutations in 14% (34 patients) of the total FTD population included in the study (245 patients), and 32% of patients with a positive family history (Rosso *et al.*, 2003). From this Dutch FTD population, more than 80 patients without a positive family history are screened for mutations in the *tau* gene, but up until now no mutations are detected (P. Heutink, personal communication). So, unless there is a strong family history of dementia or tau-related neuropathological findings, screening patients with dementia for *tau* mutations will have a very low yield of positive results. However, discovering a patient with a known or even novel *tau* mutation has important consequences for research purposes and for the family if they request genetic counseling.

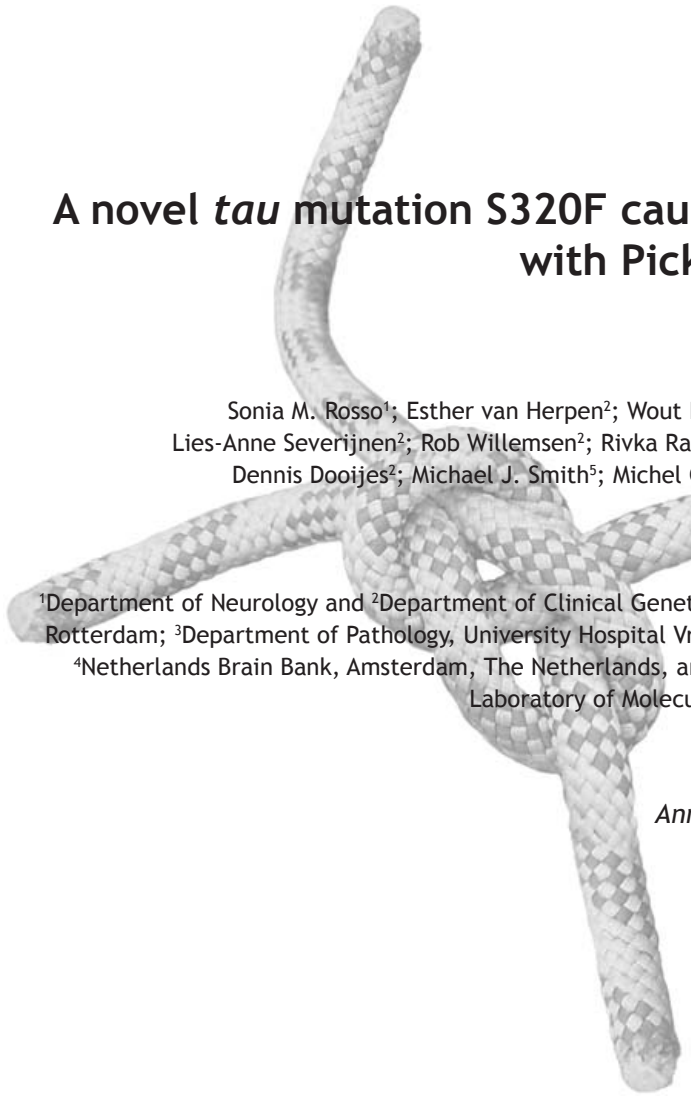
Up until today, segregation of nearly all mutations in *tau* is consistent with an autosomal dominant pattern of inheritance with almost complete penetrance. Recently, two pedigrees have been reported with a recessive form, with patients homozygous for a specific *tau* mutation. The first one is a PSP patient homozygote for the ΔN296 mutation, whereas heterozygote carriers being less severely affected (late onset parkinsonism) (Pastor *et al.*, 2001). The second one is the novel S352L mutation associated with apparent recessive inheritance and extensive tau neuropathology. Heterozygous carriers are not affected. The presenting symptoms were respiratory hypoventilation, probably due to the involvement of medullary neurons (Nicholl *et al.*, 2003). On the other hand, recently we have shown incomplete penetrance in a family with the L315R mutation, where an 82 years old mother of two affected sons was mentally normal (see Chapter 4) (Van Herpen *et al.*, 2003). Previously, the issue of incomplete penetrance was raised in connection with several other *tau* mutations, like K257T, G389R and the +16 intronic mutation, but it could never be demonstrated in an individual that was available for examination (Janssen *et al.*, 2002; Murrell *et al.*, 1999; Pickering-Brown *et al.*, 2002; Rizzini *et al.*, 2000). This observation will have a big impact on the predictive value based on presymptomatic DNA testing.

The frequency of *tau* mutations combined with the prevalence data suggests that sporadic FTD cases still remain idiopathic, and that only a few of the cases are associated with tau pathology or with any of the known *tau* mutations. *Tau* mutations are a rare finding in familial FTD cases in which neuropathological examination fails to reveal tau filamentous abnormalities. Moreover, when filamentous tau inclusions are present in brains of familial FTD cases, *tau* mutations are detected in a minority of the cases. Not all the FTDP-17 families previously linked to 17q21-22 presented mutations in the *tau* gene. Negative results were obtained in at least three families, including the Dutch family III (Fabre *et al.*, 2001;

Kertesz *et al.*, 2000; Rosso *et al.*, 2001; Savioz *et al.*, 2000). It cannot be ruled out that some mutations in the *tau* gene have remained undetected, for example intronic sequences outside the primers used to amplify the product or unknown elements in the promoter region. However, these families lack any obvious tau pathology, suggesting a hypothetical *tau* mutation to cause the disease through a different pathogenic mechanism (Kertesz *et al.*, 2000; Rosso *et al.*, 2001; Savioz *et al.*, 2000). Extension of the family by our group revealed new recombinants, which exclude the *tau* gene. Subsequently, the existence of many cases of familial FTD that have neither *tau* mutations nor tau pathology is strong evidence that additional genes for familial FTD are likely. Besides chromosome 17, genetic linkage to chromosome 3 and 9 has been found in several FTD families (Ashworth *et al.*, 1999; Hosler *et al.*, 2000; Kovach *et al.*, 2001).

Moreover, Zhukereva *et al* reported that patients from a family with a subtype of FTD, hereditary dysphasic disinhibition dementia (HDDD2), linked to chromosome 17q21-22, as well as a number of sporadic FTD patients with Dementia Lacking Distinctive Histology (DLDH), were characterized by a selective reduction of soluble brain tau. Also no insoluble tau or fibrillary tau inclusions were detected, but the loss of protein is not reflected at the mRNA level, suggesting that the abundance of tau protein may be controlled posttranscriptionally (Zhukareva *et al.*, 2001). The suggested explanations by the authors, such as decreased mRNA stability or misregulation of transcription, are difficult to explain with the current biological knowledge. The observation that, until now no other research group reported a family or case like this raises questions concerning the technical procedures that were used. A recent follow-up study revealed phenotypic heterogeneity within the HDDD2 family, by showing one patient with substantial amounts of pathological and insoluble tau (Zhukareva *et al.*, 2003).

Although the biochemical and structural characteristics of the tau aggregates in FTDP-17 appear to be somewhat predictable, based on our understanding of the function of tau and the *tau* gene splicing, the differences in clinical phenotypes and topographical distributions remain unclear. The clinical and neuropathological phenotype of individual patients range from FTD, including subtypes like PiD, CBD and PSP, to multisystem neurodegeneration. However, some *tau* gene mutations cause a similar phenotype in different families or in individual members of one family, for instance the N279K mutation (Delisle *et al.*, 1999; Yasuda *et al.*, 1999). Several clinical and neuropathological descriptions of families with the P301L mutation demonstrate a great clinical variation, ranging from PSP to CBD to PiD (Bird *et al.*, 1999; Mirra *et al.*, 1999; Nasreddine *et al.*, 1999; Spillantini *et al.*, 1998a). Furthermore, in a family with the P301S mutation, one individual presented with FTD whereas his son presented clinically with CBD (Bugiani *et al.*, 1999). The overlap between the various tau-related disorders and the pathologic distinctions may be due to other genetic and/or epigenetic factors that modify the effects of the primary mutation. This is currently an active field of investigation, the collection of large families with phenotypic variation, identification of founder effects of a common mutation (N279K and P301L) (Kobayashi *et al.*, 2002; Tsuboi *et al.*, 2002a; Tsuboi *et al.*, 2002b) and the generation of animal models or other model systems may facilitate this research.



Chapter 3

A novel *tau* mutation S320F causes a tauopathy with Pick-like inclusions

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Abstract

Mutations in the *tau* gene cause familial frontotemporal dementia and parkinsonism linked to chromosome17. In this article, we describe a novel missense mutation, S320F, in the *tau* gene in a family with presenile dementia. To our knowledge, it is the first mutation to be described in exon 11 of *tau*. The proband died at age 53 years, after a disease duration of 15 years, and autopsy revealed a neuropathological picture similar to Pick's disease. Recombinant tau protein with the S320F mutation showed a greatly reduced ability to promote microtubule assembly.

Introduction

The identification of different types of mutations in the *tau* gene in familial frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), and its association with a spectrum of filamentous tau pathology, has established the important role of the *tau* gene in causing neurodegeneration (Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998b). The primary effect of intronic and some coding region mutations in exon 10 is at the mRNA level, resulting in a change in ratio of 3- to 4-repeat tau isoforms. By contrast, most missense mutations reduce the ability of mutant tau to interact with microtubules and other molecules, and some also stimulate the *in vitro* assembly of tau into filaments.

In this article, we report a novel missense mutation (S320F) in *tau* in a family with presenile dementia. It constitutes the first known mutation in exon 11 of *tau*. Experimentally, the S320F mutation resulted in a markedly reduced ability of tau to promote microtubule assembly.

Patient and Methods

Clinical history of the proband

The proband, a traveling salesman, presented at age 38 with complaints of mild memory problems and spatial disorientation. Neuropsychological examination, computed tomography, and electroencephalography were normal at this time. Nine years later, at age 47 years, memory problems and naming difficulties had evidently worsened. Furthermore, he had become introverted, mentally inflexible, and disinterested. Psychometric evaluation revealed fluent aphasia, word finding difficulties, impairment of comprehension, and abstract thinking. Extrapyramidal signs and motor neuron disease were absent. Magnetic resonance imaging of the brain showed moderate bilateral temporal atrophy. The patient died at age 53 years; the proband's mother had also died of a similar dementing illness at age 53 years. Neither of the mother's parents (ages at death, 57 and 90 years) nor any of her seven siblings were reported to have developed dementia.

Immunohistochemistry

Immunohistochemistry with phosphorylation-dependent (AT8, AT180, AT270, PHF1, MC1 and 12E8 (1: 500, donated by P. Seubert, Elan Pharmaceuticals, San Francisco), and phosphorylation-independent

tau antibodies (BR01, Tau 2) was performed, as well as with antibodies directed against ubiquitin, β-amyloid, α-synuclein, and β-crystallin, as described previously (Rosso *et al.*, 2001).

DNA extraction and mutational analysis

Genomic DNA of the proband was extracted, and exons 9 to 13 of *tau* were amplified and sequenced as described (Rizzu *et al.*, 1999). Exon 11 of *tau* was also sequenced from the genomic DNA of a healthy maternal uncle of the proband (age 84 years), as well as 50 control individuals.

Tau extraction, immunoblotting, and electron microscopy

Sarkosyl-soluble and -insoluble tau was extracted, dephosphorylated, and analyzed as described previously (Goedert *et al.*, 1992b), and incubated with BR01 tau antibody (1: 2,000). The ratio of soluble 3- to 4-repeat tau was assessed using Image Master 1D elite software (Amersham Pharmacia Biotech, United Kingdom). Dispersed filaments from the sarkosyl-insoluble fraction were processed for electron microscopy and immunolabeled with tau-antibodies BR01 and AT8, as described previously (Goedert *et al.*, 1992b).

Microtubule assembly

Site-directed mutagenesis was used to change S320 to phenylalanine in the 3-repeat 381 and 4-repeat 412 amino acid isoforms of human tau (numbering of 441 amino acid isoform of human tau), expressed from cDNA clones htau37 and htau46, respectively. Wild-type and mutant tau proteins were expressed in *Escherichia coli* BL21(DE3), purified, and incubated with bovine brain tubulin as described previously (Hasegawa *et al.*, 1998). Assembly into microtubules was monitored over time by change in turbidity at 350nm.

Results

Sequencing of the proband's genomic DNA showed a C to T transition in exon 11 at the second base position of codon 320 (TCC to TTC), which results in the substitution of serine by phenylalanine (S320F). This change was not observed in the healthy maternal uncle of the proband or in 100 control chromosomes.

At autopsy, the proband's brain (weight 1,200g) showed focal bilateral atrophy of the anterior temporal lobes, with only very mild frontal atrophy. Severe neuronal loss and gliosis were present in the temporal cortex, cingulate gyrus, entorhinal cortex, and hippocampus. The substantia nigra was not affected. A few Pick cells were seen in the temporal cortex. Bodian silver staining did not show any Pick bodies or neurofibrillary tangles.

Immunohistochemical staining showed extensive tau pathology in the form of Pick-like bodies and more diffuse cytoplasmic staining in neurons of the frontal, temporal, and parietal cortices; the dentate gyrus; the amygdala; and the ventral striatum (Figure 1A-E). The Pick-like bodies were immunoreactive with all anti-tau antibodies tested, with the exception of antibody 12E8. A few glial cells, probably oligodendrocytes, in affected regions also contained tau-positive inclusions. Staining with β-amyloid and α-synuclein was negative.

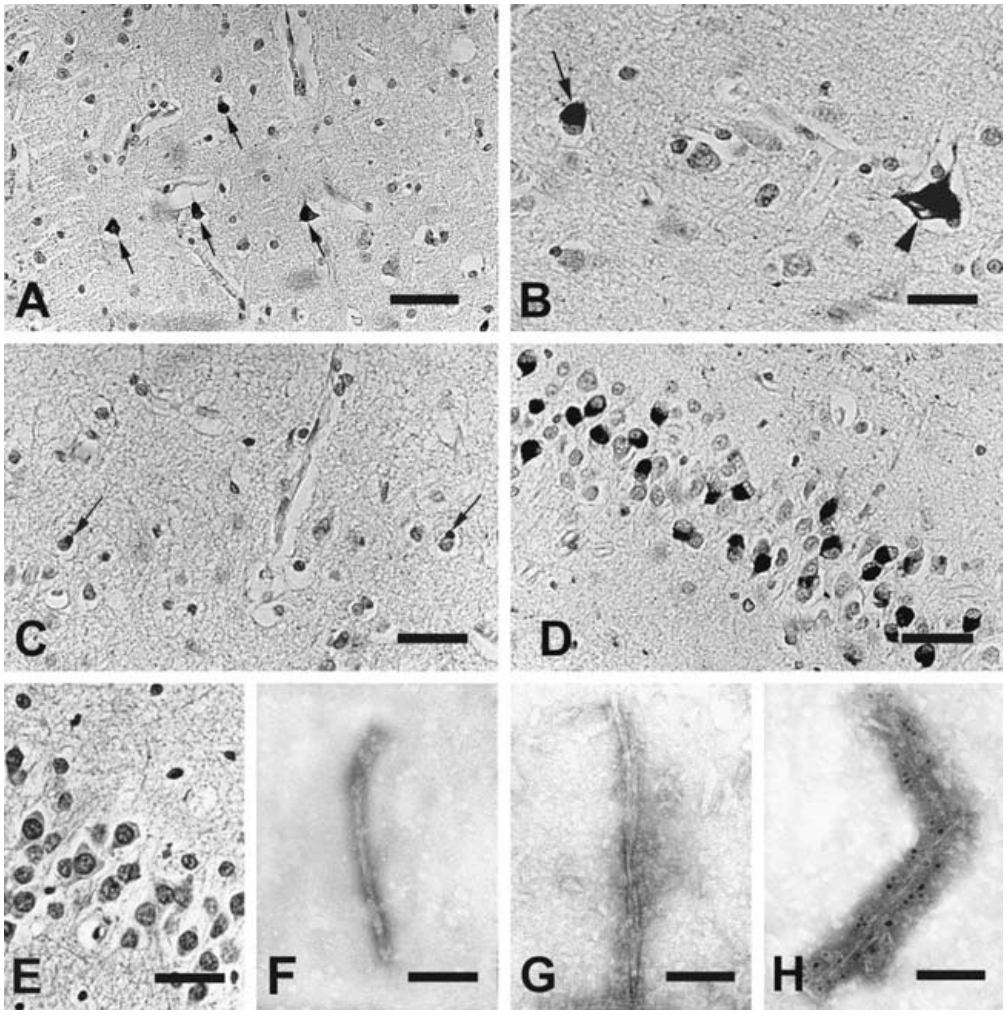

Figure 1. Neuropathological findings in the proband's brain.
(A,B) Immunostaining of the frontal cortex with the phosphorylation-dependent anti-tau antibody AT8 shows multiple tau-positive inclusions (A), some of which resemble Pick bodies (arrow in B), while others show a more diffuse staining of the cytoplasm (arrowhead in B). (C) A small number of AT8-positive glial cells are seen in the frontal cortex. (D,E), The granule cells of the dentate gyrus of the hippocampus contain numerous inclusions resembling Pick bodies that are immunoreactive with AT8 (D) but not with the phosphorylation-dependent anti-tau antibody 12E8 (E). (F-H) Electron micrographs of tau filaments isolated from the proband's brain, showing unlabeled straight (F) and twisted (G) filaments as well as a twisted filament labeled by AT8 (H). Scale bars: 150 µm (A), 100 µm (B-E) and 80 nm (F-H).

By immunoblotting, sarkosyl-insoluble tau ran as two major bands of 60 and 64 kDa (Figure 2A). Following dephosphorylation, these bands resolved into four bands that aligned with human tau isoforms 4R0N, 3R1N, 4R1N, and 3R2N, except in the temporal cortex, where the 3R2N band was not observed (Figure 2B). Following dephosphorylation, soluble tau gave a pattern similar to that seen in Alzheimer's disease (AD; Figure 2C), with a ratio of 3- to 4-repeat tau isoforms of 0.92, compared with 1.01 in the control brain.

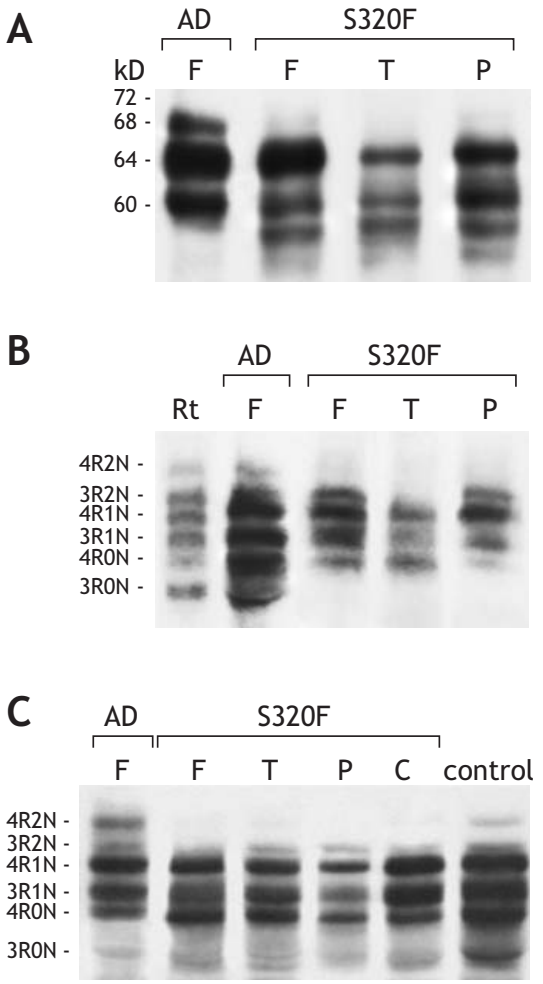

Figure 2. Sarkosyl-insoluble tau and soluble tau from the proband's brain.
(A,B) Immunoblot of sarkosyl-insoluble tau before (A) and after (B) alkaline phosphatase treatment. (C), Immunoblot of soluble tau after alkaline phosphatase treatment. Immunoblotting was done using the phosphorylation-independent anti-tau antibody BR01. F = frontal cortex; T = temporal cortex; P = parietal cortex; C = cerebellum; AD = Alzheimer's disease; Rt = recombinant tau.

Electron microscopy of preparations of sarkosyl-insoluble filaments showed filaments with two distinct morphologies (Figure 1F, G). The major species (approximately 80% of filaments) was a straight filament, very similar to the filaments seen in AD brain. The minor species (approximately 20%) was an irregularly twisted filament with a crossover spacing of 110 to160nm and a diameter of 6 to 8nm in its narrow part. Both types of filament were decorated by BR01 and AT8 antibodies (Figure 1H).

Recombinant 3-repeat htau37 and 4-repeat htau46 with the S320F mutation showed a markedly reduced ability to promote microtubule assembly when compared with the corresponding wild-type proteins (Figure 3A,B). Thus, the S320F mutation led to a 90 to 95% reduction in the rates of microtubule assembly when expressed as the optical density at 2 minutes (Figure 3C).

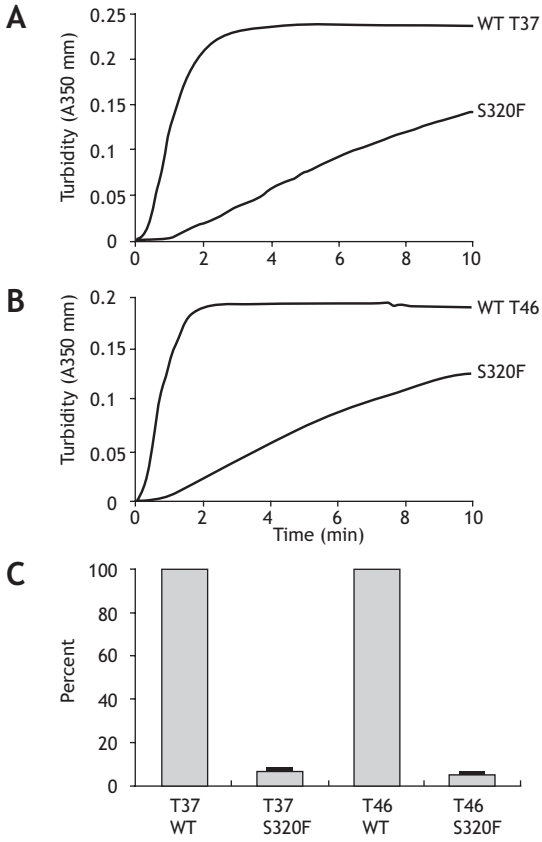

Figure 3. Effects of the S320F mutation on the ability of three-repeat htau37 (381 amino acid isoform of human tau) and four-repeat htau46 (412 amino acid isoform of human tau) to promote microtubule assembly.

(A) Polymerization of tubulin induced by wild-type htau37 and htau37S320F. (B) Polymerization of tubulin induced by wild-type htau46 and htau46S320F. Microtubule assembly was monitored over time by turbidimetry. (C) Optical densities for wild-type and mutant htau37 and htau46 at 2 minutes (expressed as percent of wild-type htau37 and htau46 taken as 100%). Each result is expressed as the mean ± the standard error of the mean (n = 5).

Discussion

This study describes a novel mutation in exon 11 of the *tau* gene in a patient with presenile dementia. S320F is the first mutation to be described in exon 11 of *tau*. The initial clinical diagnosis was AD, but neuropathological findings closely resembled Pick's disease (PiD). The inclusions in S320F brain were similar to those described in sporadic PiD (Dickson, 1998) and in some other cases with *tau* mutations (Lippa *et al.*, 2000; Murrell *et al.*, 1999; Neumann *et al.*, 2001; Pickering-Brown *et al.*, 2000; Rizzini *et al.*, 2000; Spillantini *et al.*, 1998a), except that they were undetectable with Bodian silver staining. They were immunoreactive with all anti-tau antibodies used, with the exception of antibody 12E8, which recognizes tau phosphorylated at S262, S356, or both. This 12E8-negative staining of Pick bodies has also been described in sporadic PiD (Probst *et al.*, 1996) as well as K257T, G272V, and K369I mutations (Neumann *et al.*, 2001; Rizzini *et al.*, 2000; Spillantini *et al.*, 1998a), indicating that these epitopes are not substantially hyperphosphorylated in most cases with Pick-like pathology. However, the 12E8-positive staining in G389R mutation suggests that nonphosphorylation of these sites is not required for the formation of Pick bodies (Murrell *et al.*, 1999).

Sarkosyl-insoluble tau extracted from S320F brain resolved into two major bands of 60 and 64 kDa, like the pattern seen in sporadic PiD (Dickson, 1998) and in K257T and G389R mutations (Murrell *et al.*, 1999; Pickering-Brown *et al.*, 2000; Rizzini *et al.*, 2000). However, following dephosphorylation, the normally abundant band corresponding to tau isoform 3R0N was missing. Four major bands aligning with isoforms 4R0N, 3R1N, 4R1N and 3R2N were observed instead. As the 60 kDa band corresponds to the 3R0N tau isoform in AD brain (Goedert *et al.*, 1992b), the presence of a 60 kDa band in the absence of 3R0N tau in the case described herein implies that the isoform composition of sarkosyl-insoluble tau differed from that of AD. Previously, unexpected tau isoform patterns have been observed in the E342V mutation and in one of two families with a G389R mutation (Lippa *et al.*, 2000; Pickering-Brown *et al.*, 2000). As in the present case, it remains to be seen whether these patterns are a direct and general result of the *tau* mutations, or whether they are limited to the individual cases within each family studied so far. Soluble tau from S320F brain consisted of all six isoforms, similar to that seen in AD and other missense mutations in *tau*. The two distinct filament morphologies, straight and twisted, have also been described in some cases of sporadic PiD and in some other cases with *tau* mutations and a Pick-like phenotype (Dickson, 1998; Murrell *et al.*, 1999).

The S320F mutation is located within the highly conserved third microtubule-binding domain of tau. A serine residue is found at this position in all known tau sequences, as well as in related proteins MAP2 and MAP4. Accordingly, recombinant tau with the S320F mutation showed a greatly reduced ability to promote microtubule assembly, suggesting that this may be its primary effect. It is conceivable that this mutation has additional effects. It is located within the core region of the paired helical filament of AD, two residues amino-terminal of C322, which is known to be required for the dimerization of tau (Friedhoff *et al.*, 2000; Jakes *et al.*, 1991). The S320F mutation removes a potential phosphorylation site in tau. *In vitro* studies have shown that microtubule-affinity regulating kinase, protein kinase N, and cyclic adenosine monophosphate-dependent protein kinase (in presence of heparin)

can phosphorylate S320 (Schneider *et al.*, 1999; Taniguchi *et al.*, 2001). It has even been suggested that phosphorylation of this site may inhibit the assembly of tau protein into filaments (Schneider *et al.*, 1999). However, at present, there is no evidence to suggest that S320 is phosphorylated in either normal or pathological tau *in vivo*.

In conclusion, the present study describes a novel *tau* mutation that causes a syndrome similar to Pick's disease. It further underlines the relevance of tau protein dysfunction in the etiology and pathogenesis of frontotemporal dementia in general, and Pick's disease in particular.

Acknowledgements

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Chapter 4

Variable phenotypic expression and extensive tau pathology in two families with the novel *tau* mutation L315R

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Abstract

Mutations in the *tau* gene cause familial frontotemporal dementia and parkinsonism linked to chromosome 17. Here, we describe two Dutch families with familial frontotemporal dementia associated with the novel missense mutation L315R in exon 11 of *tau*. The age at onset of disease showed a large variation within each family, ranging from 25 to 64 years. Incomplete penetrance was established in an 82-year old mutation carrier with no signs of dementia and appeared probable in two additional subjects. The brains of two affected subjects were studied and showed extensive tau pathology in neurons (Pick-like inclusions) and astroglial cells, particularly in the frontotemporal cortex and the hippocampal formation. Sarkosyl-insoluble tau extracted from the cerebral cortex showed the presence of straight and twisted tau filaments and a pattern of pathological tau bands similar to that of Pick's disease. Upon dephosphorylation, only five of the six brain tau isoforms were observed, with the shortest isoform being undetectable. All six tau isoforms were present in soluble brain tau. Recombinant tau proteins with the L315R mutation showed a reduced ability to promote microtubule assembly.

Introduction

Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) is a neurodegenerative disease, in which mutations in the *tau* gene (Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998b) lead to the assembly of tau protein into abnormal filaments (Heutink, 2000; Lee *et al.*, 2001; Rosso & van Swieten, 2002). Tau filaments are also characteristic of other neurodegenerative diseases, such as Alzheimer's disease (AD) and several other disorders. The study of FTDP-17 continues to throw light on some of these disorders, as exemplified by a clinical picture of progressive supranuclear palsy in cases with the R5L and ΔN296 tau mutations and the recently identified L266V mutation (Kobayashi *et al.*, 2003; Pastor *et al.*, 2001; Poorkaj *et al.*, 2002).

Six tau isoforms are produced in the adult human brain by alternative mRNA splicing of exons 2, 3 and 10 (Goedert *et al.*, 1989a). Three isoforms with 3 repeats (3R), and three isoforms with 4 repeats (4R), differing from each other by the presence of exon 10, are found in equal amounts in normal brain (Goedert & Jakes, 1990). These repeats constitute the microtubule binding domains of tau (Kar *et al.*, 2003; Lee *et al.*, 1989). Missense or deletion mutations are located in the repeat region or close to it and reduce the ability of mutant tau to promote microtubule assembly (Hasegawa *et al.*, 1998; Hong *et al.*, 1998). Some of these mutations also result in the enhanced assembly of tau into filaments *in vitro* and its reduced ability to bind to protein phosphatase 2A (Goedert *et al.*, 1999a; Goedert *et al.*, 2000; Nacharaju *et al.*, 1999). Intronic mutations located close to the splice-donor site of the intron after exon 10 and some mutations in exon 10 have their primary effect at the RNA level and change the ratio of 3R to 4R tau, leading to the relative overproduction of 4R tau isoforms.

In FTDP-17, filamentous tau deposits are present in both neurons and glial cells (Heutink, 2000; Lee *et al.*, 2001; Rosso & van Swieten, 2002). Neuronal and glial inclusions, as found

in cases with mutations in exon 10 or the intron after exon 10, correlate with the expression of mutant 4R tau or the relative overproduction of 4R tau. In contrast, the presence of tau inclusions in neurons correlates with the expression of mutant 3R and 4R tau. However, recently described mutations (R5H, R5L and L266V) do not appear to fit into this scheme, because they lead to a neuronal and glial tau pathology, despite all six tau isoforms being mutated (Hayashi *et al.*, 2002; Kobayashi *et al.*, 2003; Poorkaj *et al.*, 2002).

The novel mutation (L315R) in exon 11 of *tau* in two families with frontotemporal dementia that we describe here was associated with a severe neuronal and glial tau pathology. Clinically, it showed incomplete penetrance. Experimentally, the L315R mutation reduced the ability of recombinant tau proteins to promote microtubule assembly, whereas it had no significant effect on heparin-induced assembly of tau into filaments.

Subjects and methods

Case reports

Two families (A and B), originating from the same genetically isolated part of the Netherlands, were studied. Four affected subjects in five generations of family A were identified (Table 1; Figure 1A). Behavioural changes, memory problems, or word-finding difficulties started in three subjects at age 53, 55 and 56 years, respectively, whereas the onset could not be determined in the fourth patient (A IV:1), who died from a similar dementing disease at age 61 years. Perseverations, impaired executive function, and decreased attention and abstraction were found in the two neuropsychologically tested patients (A V:1, A V:3). The mother (A IV:4) of two siblings (A V:2 and A V:3) was cognitively normal at age 82 years. Neuroimaging showed asymmetric temporal atrophy (A V:2, A V:3), frontotemporal atrophy (A V:1) or no abnormalities (A IV:4). An autopsy was performed on patient A V:1, who died at age 61 years after a disease duration of 8 years.

Four affected subjects were identified in four generations of family B (Table 1; Figure 1B). Frontal lobe symptoms developed in two patients (B III:3, B III:4) at age 64 and 56 years, but at a much younger age (25 and 39 years) in two other patients (B III:1, B IV:1). Depression with suicidal tendencies and apathy were the predominant features in patient B III:3. Patient B III:1, who was unable to complete primary school, but reached psychomotor milestones at normal age, developed word finding problems, dysarthria, and apathy at age 25 years and died at the age of 33 years, and an autopsy was performed. One parent (B II:2) of patient B III:1 who according to the pedigree should have been the mutation carrier, died at age 70 years without signs of cognitive impairment. Patient B IV:1 presented with depression and impaired language comprehension at age 39 years. Neuroimaging showed temporal (B III:4, B IV:1), frontotemporal (B III:3) or generalized (B III:1) atrophy (Table 1).

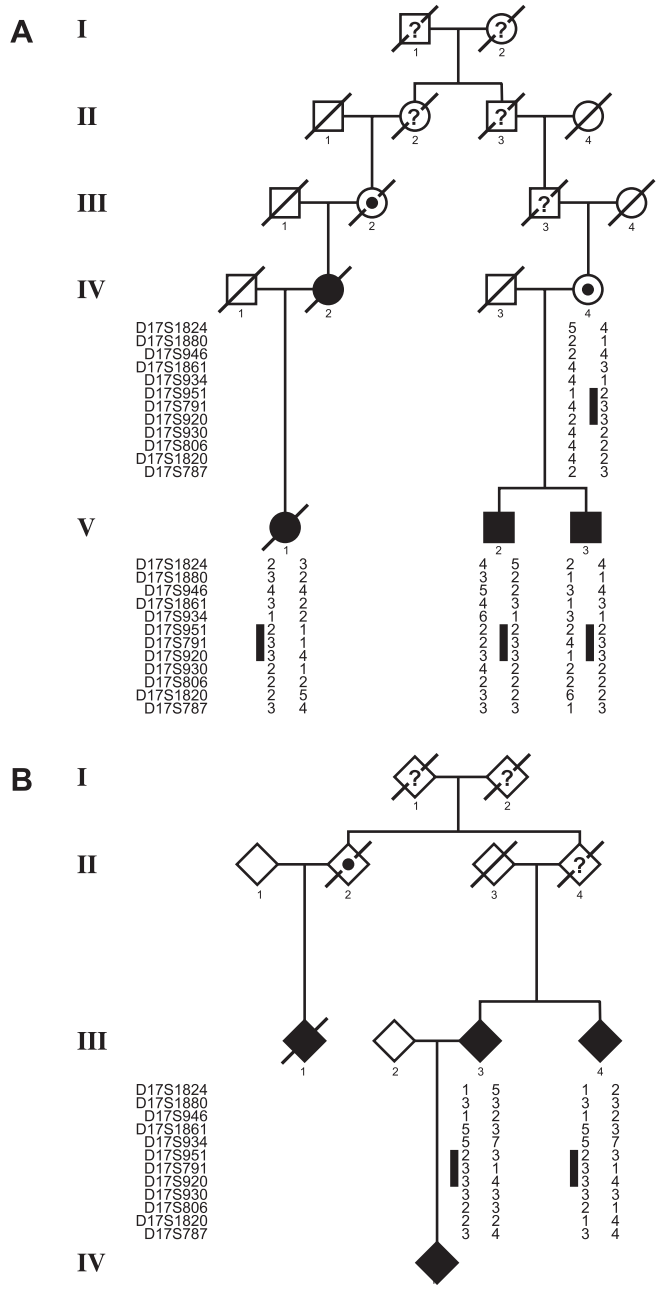

Figure 1. Pedigrees of the two families with haplotypes for the familial frontotemporal dementia and parkinsonism linked to chromosome 17 region.
Black bars indicate the shared alleles. Markers were obtained and ordered according to the Marshfield integrated linkage map and the April 2001 physical assembly of the Human Genome (http://genome.ucsc.edu).

Table 1. Overview of clinical features and neurological examination in affected individuals of families A and B

Patient	Onset	Death	Current age	Presenting symptoms	Neurological findings[a]	Atrophy on neuroimaging
A IV:2	59	61[b]	-	NA	NA	NA
A V:1	55	63[c]	-	word finding problems, restless, interest ↓	executive functions, attention ↓	frontotemporal R > L
A V:2	53	-	57	memory ↓, hyperorality, disinhibition	NA	temporal R
A V:3	56	-	56	behavioral changes and memory problems	perservations, loss of insight executive functions ↓	temporal R
B III:1	25	33[c]	-	word-finding problems, dysarthria	spontaneous speech ↓ motor stereotypes	generalized
B III:3	64	-	66	depression, apathy	perseverations	frontotemporal
B III:4	56	-	59	word finding problems, loss of insight, compulsive behavior	memory, executive functions ↓ perseverations, loss of insight	temporal L
B IV:1	39	-	41	depression, semantic difficulties	memory ↓ frontal signs	temporal

a Including neuropsychological examination

b Cause of death is not end-stage dementia

c Autopsy of the brain was performed, and material was available for further experiments.

NA Not available

DNA extraction and mutation analysis

Genomic DNA of patients and relatives was extracted, and all exons of the *tau* gene were amplified and sequenced as described (Rizzu *et al.*, 1999). Restriction enzyme digestion was used to investigate the presence of the base change in exon 11 in 200 control chromosomes, according to the manufacturer's (Invitrogen, San Diego, CA) instructions. *Hpa II* digests specifically the mutant allele of the exon 11 polymerase chain reaction product, whereas *Dde I* digests only the normal allele.

Tau haplotype analysis and apolipoprotein E genotyping

Twelve polymorphic short tandem repeat markers linked to the FTDP-17 locus were typed (Breedveld *et al.*, 2002): D17S1824, D17S1880, D17S946, D17S1861, D17S934, D17S951, D17S791, D17S920, D17S930, D17S806, D17S1820, and D17S787. The markers were obtained and ordered according to the Marshfield integrated linkage map and the April 2001 physical assembly of the Human Genome (http://genome.ucsc.edu). Fluorescently labeled markers were used as specified by the manufacturer and analyzed using an ABI3100 automated sequencer with Genemapper 2.0 software (Applied Biosystems, Foster City, CA). Amplification of the apolipoprotein E (ApoE) locus was performed using primers TTCAAGGAGCTGCAGGCGGCGCA and ACAGAATTCGCCCCGGCCTGGTACACTGCCA. The allelic setup was determined by genotyping codon 112 (SBE primer TTTTTTTTTTTTGGGCGCGGACAT GGAGGACGTG) and 158 (TTTTTTTTTTTTTTTTTTCGATGCCGATGACCTGCAGAAG) using the ABI Prism SNaPshot Multiplex Kit (Applied Biosystems) as specified by the manufacturer.

Histology and immunohistochemistry

Available brain material of two patients (A V:1 and B III:1) was processed for histology and immunohistochemistry. Adjacent sections (4 µm) were stained with hematoxylin and eosin and Bodian silver stain, and used for immunohistochemistry, as described previously (Rosso *et al.*, 2001). Phosphorylation-dependent anti-tau antibodies MC-1 (a gift from Dr. P. Davies, Albert Einstein College of Medicine, Bronx, NY(Jicha *et al.*, 1999; Jicha *et al.*, 1997)), AT8, AT180, AT270 (all from Innogenetics) (Goedert *et al.*, 1994; Goedert *et al.*, 1995), and 12E8 (a gift from Dr. P. Seubert, Elan Pharmaceuticals, San Fransisco, CA) (Seubert *et al.*, 1995) were used. Antibodies against glial fibrillary acidic protein, ubiquitin, β-amyloid (all from Dako, Glostrup, Denmark), and α-synuclein (Chemicon International, Temecula, CA) were also tested (Rosso *et al.*, 2001).

Tau protein extraction and immunoblotting

Sarkosyl-soluble and insoluble tau proteins were extracted from the cerebral cortex and cerebellum of patient A V:1, as described (Goedert *et al.*, 1992b). Dephosphorylation and immunoblotting using the phosphorylation-independent anti-tau antibody H-7 (Mercken *et al.*, 1992) were performed as described (Rizzu *et al.*, 2000; Rosso *et al.*, 2001). The ratio of soluble 3R/4R tau was assessed using Image Master1D elite software (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Microtubule assembly

Site-directed mutagenesis was used to change L315 to arginine in the 3R 381-amino acid isoform and in the 4R 412-amino acid isoform of human tau, expressed from cDNA clones

htau37 and htau46, respectively. Wild-type and mutant tau proteins were expressed in *Escherichia coli* BL21 (DE3), as described (Goedert & Jakes, 1990). 3R tau with the K257T mutation and 4R tau with the ΔK280 mutation served as controls. Tau proteins were purified, and their concentration was determined by densitometry (Hasegawa *et al.*, 1998). Purified recombinant and mutant 3R and 4R tau were incubated with bovine brain tubulin (Cytoskeleton, Denver, CO), as described previously (Yoshida *et al.*, 2002). Assembly of tubulin into microtubules was monitored over time by a change in turbidity at 350 nm. In all experiments, wild-type and mutant tau proteins were expressed and purified in parallel and five separate experiments were performed.

Tau filament assembly

Purified wild-type and L315R mutant forms of 3R and 4R tau were incubated in the presence of heparin (British Drug House, Poole, England), as described (Goedert *et al.*, 1996b). 3R tau with the K257T mutation and 4R tau with the P301S mutation served as controls. Assembly was monitored semiquantitatively by electron microscopy and quantitatively using thioflavin T fluorescence (Yoshida *et al.*, 2002). Each filament assembly experiment made use of newly prepared batches of wild-type and mutant tau proteins that had been purified in parallel and five separate experiments were performed.

Electron microscopy

Aliquots of sarkosyl-insoluble, dispersed filament preparations and of synthetic tau filament assemblies were processed for electron microscopy as described (Goedert *et al.*, 1992b). Anti-tau antibody AT8 was used at a dilution of 1 to 100.

Results

Both brains (A V:1 and B III:1; brain weights 818 and 863 gm, respectively) showed a severe atrophy of the frontal and temporal lobes of the cerebral cortex. Routine staining of brain A V:1 revealed massive neuronal loss and reactive gliosis in temporal cortex, hippocampus, substantia nigra, caudate/putamen and amygdala, with only moderate neuronal loss in the frontal cortex. The dentate gyrus of the hippocampus had almost completely degenerated, with only a few neurons with silver-positive Pick bodies remaining. Severe neuronal loss and reactive gliosis in frontal cortex and underlying white matter were seen in brain B III:1, with a less severe involvement of temporal cortex, hippocampus, substantia nigra, and caudate/putamen.

Staining of brain A V:1 with phosphorylation-dependent anti-tau antibodies revealed numerous Pick bodies of variable size and staining intensity in frontal cortex, with some tau deposits in astrocytic processes. In contrast, the more severely affected temporal cortex contained extensive tau deposits in astrocytes, with a radiating, elongated dendritic appearance. Only a few Pick bodies in the remaining neurons were found in conjunction with this severe astrocytic pathology. Numerous Pick bodies, immunoreactive with all anti-tau antibodies used, except 12E8, were present in the subiculum. In brain B III:1, extensive astrocytic tau deposits and some Pick bodies were seen in the frontal cortex, whereas a less severe glial tau

pathology and numerous Pick bodies were present in the temporal cortex (Figure 2A, C). Many Pick bodies were found in subiculum (Figure 2E), dentate gyrus granule cells (Figure 2F), caudate/putamen, locus coeruleus and olivary nuclei, whereas glial tau staining was most intense in the substantia nigra. Compared with AT8, antibodies AT180 and AT270 gave weaker staining, particularly of the glial tau pathological state (Figure 2B, G). Antibodies specific for ubiquitin, β-amyloid or α-synuclein gave no positive staining (for detailed overview see Table 2).

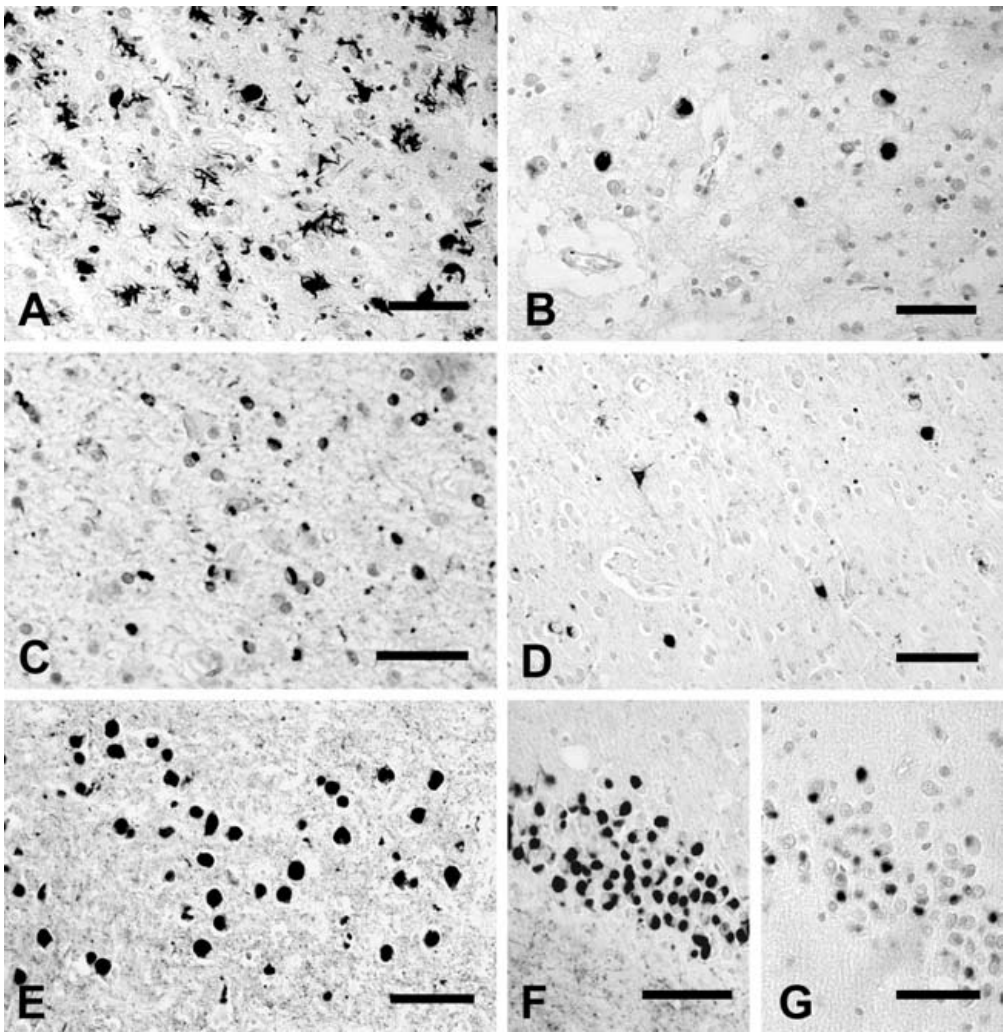

Figure 2. Microscopic neuropathology in cerebral cortex and hippocampal formation from patient B III:1. (A) Immunostaining of the frontal cortex with the phosphorylation-dependent anti-tau antibody AT8 shows some Pick-like inclusions and multiple tau-positive astrocytes. (B) Immunostaining of the frontal cortex with the phosphorylation-dependent anti-tau antibody AT180 shows only neuronal staining. (C) Immunostaining of the white matter of frontal cortex shows many AT8-positive astroglial cells. (D) Immunostaining of the temporal cortex with AT8 shows staining of Pick-like inclusions. (E) Immunostaining of the subiculum with AT8 shows numerous Pick-like inclusions. (F,G) Immunostaining of the dentate gyrus of the hippocampus shows Pick-like inclusions stained with AT8 (F) and AT180 (G). Scale bars in A through G = 100μm.

Table 2. Overview of neuropathological findings of two autopsied patients

Brain regions	Neuronal loss		Gliosis		Neuronal tau deposits (Pick bodies)		Astrocytic tau deposits	
	A V:1	B III:1	A V:1	B III:1	A V:1	B III:1	A V:1	B III:1
Frontal cortex	++	+++	+++	+++	+++	+	+	+++
Temporal cortex	+++	+	+++	+++	+	+++	+++	++
Parietal cortex	++	-	++	-	++	+	+	-
Hippocampus	+++	-	-	-	+++	+++	+	+
Substantia nigra	+++	+	+	+	+	+	-	-
Caudate nucleus	-	-	-	-	+++	+++	-	-
Putamen	-	-	-	-	-	++	-	-
Thalamus	-	-	+	-	-	+	-	-
Locus coeruleus	-	-	-	-	++	+++	-	-
Medulla	-	-	-	-	-	-	-	-

Sequence analysis of genomic DNA from all affected family members identified a T to G transition at the second base position in codon 315 in exon 11 of *tau* (CTG to CGG), resulting in a leucine to arginine substitution. The same nucleotide change was also present in the nondemented 82 year-old mother (A IV:4) of two affected siblings (A V:2 and A V:3) but not in 200 control chromosomes. Haplotype analysis (Figure 1) showed a shared allele (0.54 cM / 3.2 Mb) between markers D17S951 and D17S920 in the region of the *tau* gene in families A and B, strongly suggesting a common ancestor to both families. All analyzed mutation carriers had the apoE3/E3 genotype (data not shown).

Immunoblotting of sarkosyl-insoluble tau extracted from the cerebral cortex of patient A V:1 showed two major bands of 60 and 64 kDa and a minor band of 68 kDa (Figure 3A). After dephosphorylation, these three bands resolved into five bands that aligned with the recombinant human tau isoforms 4R2N, 3R2N, 4R1N, 3R1N and 4R0N (Figure 3B). Immunoblotting of dephosphorylated sarkosyl-soluble tau resolved into all six tau isoforms in a pattern similar to that found in AD and control brains, with a 4R to 3R isoform ratio of 1 to 1.2 (Figure 3C).

Electron microscopy of sarkosyl-insoluble material extracted from the frontal cortex of patient A V:1 showed a small number of filaments, that were marked with the anti-tau antibody AT8 (Figure 3D). The majority of filaments (approximately 80%) were irregularly twisted, with a diameter of 13 to 15nm and a periodicity of over 130nm. The remainder consisted of straight filaments.

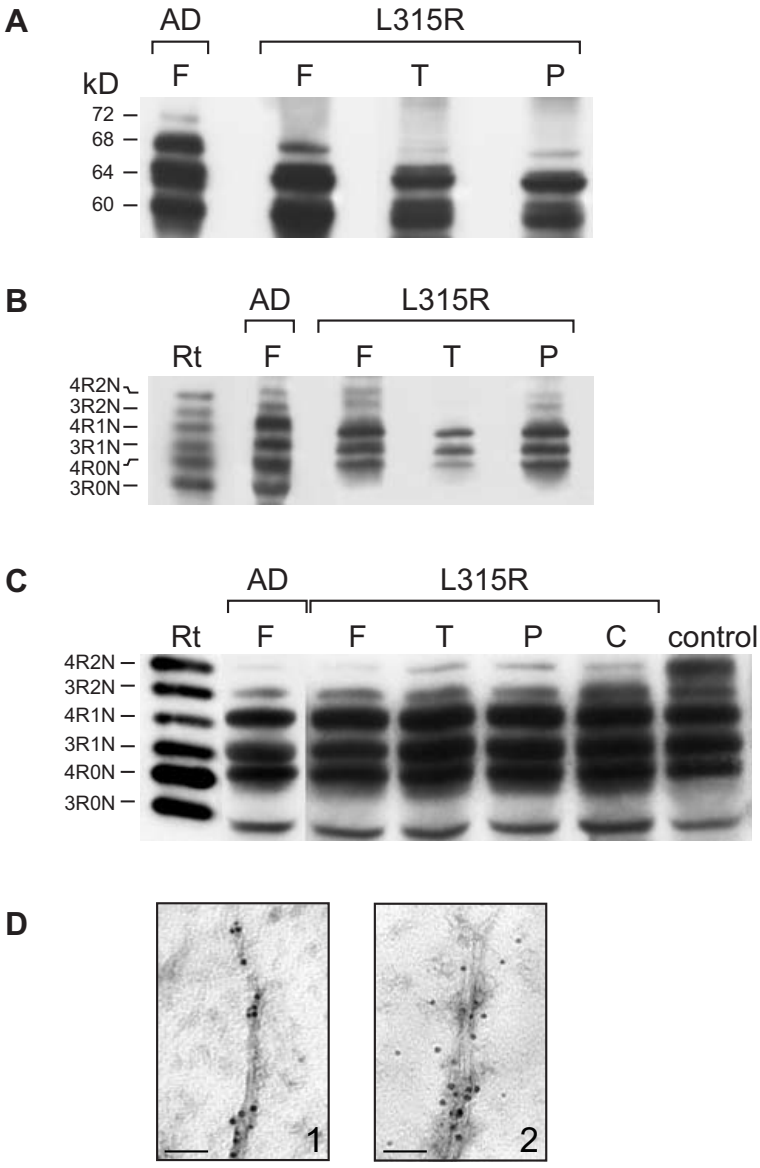

Figure 3. Sarkosyl-insoluble tau and soluble tau in cerebral cortex from patient A V:1.
(A,B) Immunoblot of sarkosyl-insoluble tau before (A) and after (B) alkaline phosphatase treatment. (C) Immunoblot of sarkosyl-soluble tau after alkaline phosphatase treatment. Immunoblotting was done using the phosphorylation-independent anti-tau antibody H-7. (D) Electron photomicrographs of tau filaments from the sarkosyl-insoluble fraction of the frontal cortex shows straight (D1) and twisted (D2) filaments marked by the phosphorylation-dependent anti-tau antibody AT8. AD = Alzheimer's disease; F = frontal cortex; T = temporal cortex; P = parietal cortex; C = cerebellum; Rt = recombinant tau isoforms. Scale bars in D = 50nm.

Recombinant 3R and 4R proteins with the L315R mutation showed a reduced ability to promote microtubule assembly (Figure 4A, B). The mutation led to a 20 to 30% reduction in the rates of microtubule assembly, when expressed as the optical density at 2 minutes (Figure 4C). No significant effect of the L315R mutation on filament assembly of recombinant 3R and 4R tau proteins was observed. Assembly was assessed quantitatively by thioflavin T fluorescence (Figure 5) and semi-quantitatively by electron microscopy (data not shown).

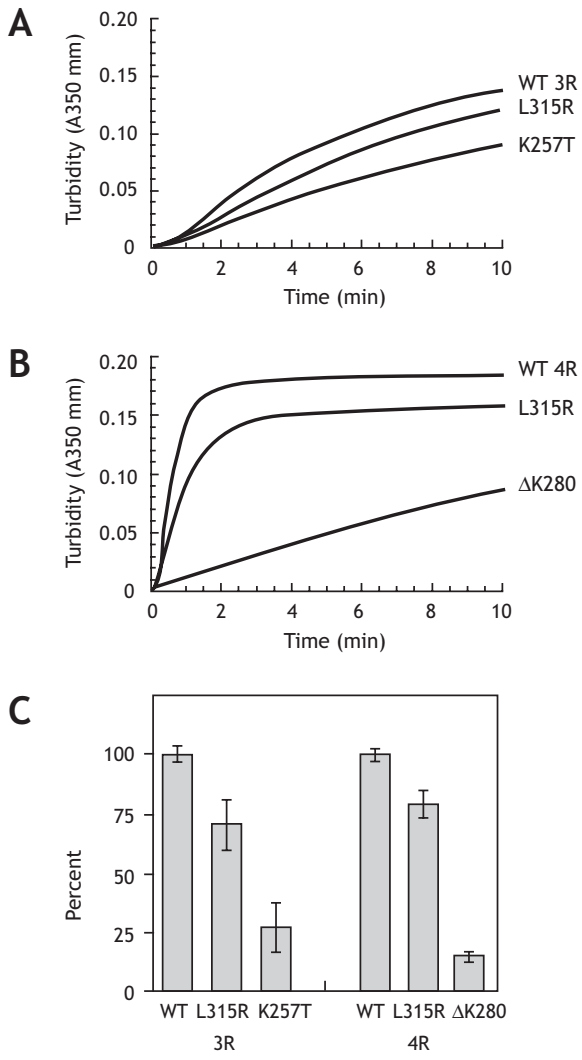

Figure 4. Effects of the L315R mutation on the ability of 3R htau37 (381 amino acid isoform of human tau) and 4R htau46 (412 amino asic isoform of human tau) to promote microtubule assembly (A) Polymerization of tubulin induced by wild-type (WT) 3R tau and 3R tau with the L315R or K257T mutations. (B) Polymerization of tubulin induced by wild-type 4R tau and 4R tau with the L315R or ΔK280 mutation. (C) Optical densities for wild-type and mutant 3R and 4R tau proteins at 2 minutes (expressed as percentage of wild-type 3R and 4R tau, taken as 100%). Each result is expressed as the mean ± S.E.M. (n = 5).

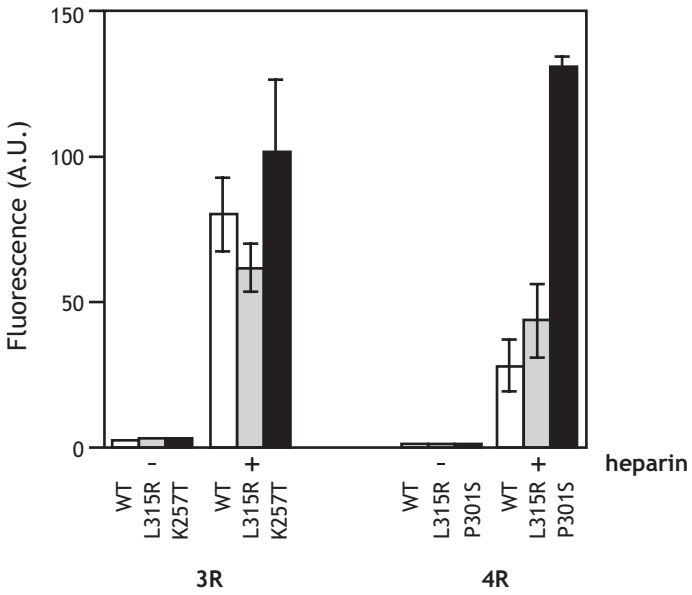

Figure 5. Effects of the L315R mutation on the ability of three-repeat (3R) htau37 (381-amino acid isoform of human tau) and four-repeat (4R) htau46 (412-amino acid isoform of human tau) to assemble into filaments in the presence of heparin.
Thioflavin T fluorescence of tau proteins in the absence (-) or the presence (+) of heparin. 3R tau with the K257T mutation and 4R tau with the P310S mutation served as controls. The fluorescence is given in arbitrary units (AU). The results are expressed as the mean ± S.E.M. (n = 5). WT = wild type.

Discussion

The novel *tau* mutation L315R was found to lead to a variable clinical expression. Proof of nonpenetrance was obtained in one subject, while the family history suggested nonpenetrance in two other subjects. Extensive tau pathology was present in neurons and astrocytes in the brains of two clinically affected subjects.

The two families with the L315R mutation could not be linked genealogically, because no archive material was available. This notwithstanding, both families probably had a common ancestor, because they originated from the same geographically and genetically isolated part of the Netherlands and because haplotype analysis showed a shared allele.

The clinical phenotype of patients with the L315R mutation highlights the issue of incomplete penetrance. The onset of clinical symptoms in a 25-year old patient (B III:1) was in sharp contrast with the normal mental state of a 82-year old mutation carrier (A IV:4). There is insufficient evidence for a link between early onset dementia and reduced cognitive abilities, described in patient B III:1, as suggested by Miyamoto and colleagues in a patient with mental retardation (Miyamoto *et al.*, 2001). Previously, the issue of incomplete penetrance was raised

in connection with other *tau* mutations (Bugiani *et al.*, 1999; Murrell *et al.*, 1999; Pickering-Brown *et al.*, 2002; Rizzini *et al.*, 2000; Sperfeld *et al.*, 1999). However, the present data provide much stronger evidence in favour of incomplete penetrance, because the mother (A IV:4) of two affected subjects was neurologically normal and had a normal computed tomography scan without lobar atrophy at age 82 years, despite carrying the mutation. Moreover, two additional cases of possible incomplete penetrance were present in families A and B, wherein subjects A III:2 and B II:2 died at 92 and 70 years of age, without clinical signs of a dementing illness. ApoE genotype, smoking, alcohol consumption, level of education, or exposure to toxic entities did not correlate with the clinical variability in these families.

Numerous Pick-like inclusions were present in cerebral cortex and hippocampus from both autopsied patients (A V:1 and B III:1). The inclusions were silver-positive and were stained with all anti-tau antibodies used, except 12E8. Similar findings have been reported previously for several other mutations in *tau* (Kobayashi *et al.*, 2003; Lippa *et al.*, 2000; Murrell *et al.*, 1999; Neumann *et al.*, 2001; Pickering-Brown *et al.*, 2000; Rizzini *et al.*, 2000; Rosso *et al.*, 2002; Spillantini *et al.*, 1998a). However, the L315R mutation also resulted in a severe astrocytic pathological condition, resembling the tufted astrocytes seen in progressive supranuclear palsy, in conjunction with the almost complete disappearance of neurons in some parts of the cerebral cortex. This severe astrocytic pathological finding was unexpected, because abundant tau inclusions in glial cells are normally associated with mutations that only affect 4R tau or that increase the relative amount of 4R tau (Heutink, 2000; Lee *et al.*, 2001; Rosso & van Swieten, 2002). The L315R mutation affects all six tau isoforms and does not change the ratio of 3R/4R tau. Similar neuronal and astrocytic pathological findings have been described recently for the L266V mutation in exon 9 of *tau* (Kobayashi *et al.*, 2003). There appeared to be an inverse relationship between the relative numbers of neuronal and glial tau inclusions in both brains examined. This finding suggests that astrocytic inclusions may develop later than neuronal deposits or that they may be longer lived. Phagocytosis of neuronal deposits by astrocytes (Bechmann & Nitsch, 2000) is also compatible with the observed staining pattern, which would imply the presence of 3R tau inclusions in astrocytes. Clarification will require the use of antibodies specific for 3R and 4R tau.

Sarkosyl-insoluble tau extracted from a L315R brain resolved into major bands of 60 and 64 kDa and a minor band of 68 kDa, similar to the pattern seen in sporadic Pick's disease (Delacourte *et al.*, 1996) and in patients with the K257T, L266V, S320F and G389R mutations in *tau* (Delacourte *et al.*, 1996; Kobayashi *et al.*, 2003; Murrell *et al.*, 1999; Pickering-Brown *et al.*, 2000; Rizzini *et al.*, 2000; Rosso *et al.*, 2002). After dephosphorylation, five bands aligning with isoforms 4R2N, 3R2N, 4R1N, 3R1N and 4R0N were observed. However, despite the presence of the 60kDa band, isoform 3R0N was missing from the sarkosyl insoluble tau of the L315R brain. Because the 60 kDa band from AD brain corresponds to isoform 3R0N (Goedert *et al.*, 1992b; Mulot *et al.*, 1994), this finding suggests that the isoform composition of sarkosyl-insoluble tau differed from that of AD. We cannot exclude that the amount of 3R0N was below the detection limit or that it became incorporated into another structure, which we did not analyze. Previously, a similar tau isoform pattern was described in the cerebral cortex from a patient with the S320F mutation in exon 11 of *tau* (Rosso *et al.*, 2002). This raises the intriguing possibility that the absence of 3R0N tau from the sarkosyl-insoluble fraction may

be a general characteristic of mutations in exon 11. The underlying mechanisms remain to be discovered. It may be significant that L315R and S320F are the only known mutations in *tau* that compose the epitope of conformation-sensitive antibodies, such as MC-1 (Jicha *et al.*, 1999; Jicha *et al.*, 1997; Rosso *et al.*, 2002). Soluble tau from L315R brain consisted of all six tau isoforms, similar to what is seen in AD and other cases of FTDP-17 (Lee *et al.*, 2001). Tau filaments similar to those present in sarkosyl-insoluble tau from the L315R brain have been described in some cases of sporadic Pick's disease and in cases of FTDP-17 with a Pick-like phenotype (Delacourte *et al.*, 1996; Murrell *et al.*, 1999; Rosso *et al.*, 2002).

A leucine residue at position 315 is found in all known vertebrate proteins with tau repeats, suggesting functional relevance (Yoshida & Goedert, 2002). Accordingly, recombinant tau with the L315R mutation showed a reduced ability to promote microtubule assembly. However, when compared with other mutations, the effect of the L315R mutation was relatively small. This might explain the reduced penetrance of this mutation, but is in apparent contradiction with the early onset of disease and the severe pathology observed in some patients.

Mutation L315R is located within the heptapeptide sequence DLSKVTS (residues 314-320) that has been reported to be essential for the polymerization of tau induced by fatty acids (Abraha *et al.*, 2000). It is also located close to the hexapeptide sequence VQIVYK (residues 301-311) that has been proposed to initiate heparin-induced tau filament assembly by forming a local β-structure (von Bergen *et al.*, 2000). Despite this observation, we failed to detect a significant effect of the L315R mutation on tau filament assembly.

In conclusion, the novel mutation in exon 11 of *tau* widens the clinical and pathological spectrum of FTDP-17. The observation of incomplete penetrance influences the clinical prediction of the disease based on presymptomatic DNA testing. These findings, in conjunction with the small functional effect of the L315R mutation, emphasise the need to investigate in detail the role of epigenetic factors, modifying genes or environmental influences in the neurodegenerative process of the tauopathies.

Acknowledgements

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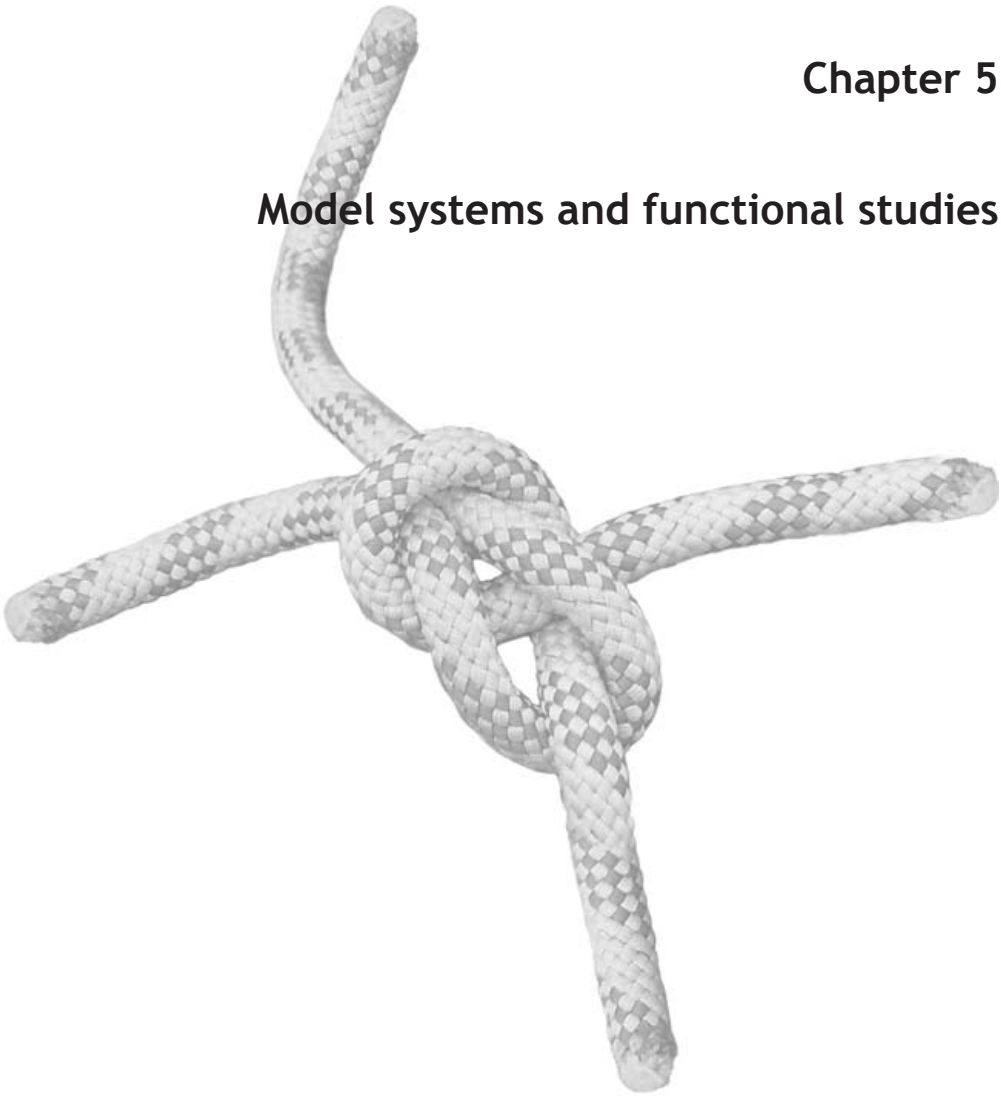
Chapter 5

Model systems and functional studies



Model systems and functional studies

To study the function of the tau protein and its role in neurodegenerative processes, different approaches have been used, often including animal or cellular models. Because the connection between tau and neurodegeneration was known before mutations in the *tau* gene were identified, the first experiments were carried out using wild type *tau* gene constructs. Several different mouse lines were generated (described in detail in chapter 5.2) to study aggregate formation, but no or only limited amounts of aggregates could be detected. After 1998, several studies were carried out with mutated *tau* gene constructs, using both mouse models and cellular over expressing systems (described in chapters 5.3 and 5.4). The individual experiments vary greatly in the choice of tau isoform, exact construct and specific mutation. Therefore, the results of individual studies are different and not always easy to compare. Aggregates, one of the key features of the neuropathology in human patients, were detected only in a few model systems. Most attention is focused on those model systems.

Chapter 5.6 describes tau interacting proteins, to gain more insight in the molecular and cellular pathways and how tau fits in the scheme. The last part of this chapter (5.7) focuses on the degradation of the tau protein. Disturbed degradation is becoming a common theme in the onset of several neurodegenerative disorders.

5.1 Tau knockout mice

The first mouse model system that was studied was a tau knockout (KO) mouse, generated in 1994 (Harada *et al.*, 1994). The inactivation of the *tau* gene by homologous recombination showed no obvious phenotype, indicating that tau was not essential for neuronal functioning, especially for axonal formation and microtubule functioning in the cell. The mice developed normally, survived well and did not develop major phenotypic changes. The nervous system of the tau-deficient mice was immunohistochemically normal and axonal elongation was not affected in cultured hippocampal neurons obtained from these animals. Only in some small-caliber axons microtubule density and stability were decreased (Harada *et al.*, 1994).

After the discovery of tau mutations in FTDP-17, the mice were re-examined, and behavioral and neurological tests were performed. The tau-deficient mice showed some muscle weakness, hyperactivity and impaired contextual fear conditioning (Ikegami *et al.*, 2000). Recently, another knockout mouse was developed which also appeared phenotypically normal and was able to reproduce. In contrast, primary cultures from these mice showed a significant delay in their axonal and dendritic extension formation (Dawson *et al.*, 2001). By mating the knockout mice with tau transgenic mice, a normal pattern of axonal growth and neuronal maturation was restored. The results of the two separate tau KO mouse models seemed to be conflicting. According to Dawson *et al* this could be explained by a difference in the wild type measurements. Due to suboptimal conditions of measuring neuronal growth in culture, the first study fails to observe a difference between wild type and KO cultures (Dawson *et al.*, 2001).

The data suggest that there is redundancy among the remaining microtubule associated proteins (MAPs), hiding a required function of tau during normal development in the whole animal. In fact, an increased expression of another MAP, MAP1A, has been found in the first described tau knockout mouse (Harada *et al.*, 1994). Supporting evidence for this comes from the generation and characterization of a double knockout mouse, deficient in both axonal MAP's, tau and MAP1B (Takei *et al.*, 2000). These mice died 4 weeks after birth and the phenotypic changes were more severe than those observed in the individual knockout mice (Takei *et al.*, 2000).

5.2 Transgenic mouse models for human wildtype tau

In order to overexpress a gene of interest in mice, a construct of the gene together with a suitable promoter can be integrated randomly in the mouse genome by m-injection technology. Transgenic animal models of tauopathies were developed to serve as informative systems for elucidating the role of tau in the onset and progression of a variety of neurodegenerative disorders. In early efforts this was addressed in a transgenic mouse model of β-amyloid deposition, caused by overexpression of mutated amyloid precursor protein (APP). The idea behind the production of indirect animal models with tau pathology was largely based on the hypothesis that extracellular Aβ pathology would induce intraneuronal tau pathology. Although various transgenic mice expressing mutated APP accumulate Aβ plaques, no somatodendritic staining of hyperphosphorylated tau was described in these mice (Games *et al.*, 1995; Hsiao *et al.*, 1996; Sturchler-Pierrat *et al.*, 1997).

Several animal models to study tau pathology were produced by overexpressing human tau proteins. In mouse brain only tau isoforms containing 4R are produced, which is in contrast with the human adult brain, having a ratio of 3R and 4R tau isoforms of approximately 1:1 (Goedert *et al.*, 1994). The first study expressing human tau protein in transgenic mice was published in 1995 by Götz *et al* and described the generation of transgenic mice expressing the longest human brain tau isoform (4R2N) under the control of the Thy1 promoter (Götz *et al.*, 1995). The transgenic mice showed strong somatodendritic tau expression and, in a subpopulation of cells, axonal staining with antibodies directed against hyperphosphorylated tau, suggestive of pretangle tau pathology. No filamentous tau inclusions were observed and the animals were phenotypically normal. The relative modest expression levels of human tau, approximately 10% above the endogenous mouse tau expression levels may explain the lack of filament formation (Götz *et al.*, 1995).

Subsequently, several other groups generated transgenic mice lines with the same human tau isoform (4R2N) and the same promoter (Thy1), but reaching much higher expression levels of human tau, between 1.5 and 6 times above the endogenous mouse tau expression levels (Probst *et al.*, 2000; Spittaels *et al.*, 1999). These mice developed numerous abnormal tau-immunoreactive nerve cell bodies and dendrites and large numbers of pathologically enlarged axons containing tau-immunoreactive spheroids. These changes were most prominent in the spinal cord, but were also seen in the brain. The animals showed motor disturbances in tasks involving balancing on a rod and hanging form an inverted grid. However, no filamentous tau

inclusions could be detected, and neuronal loss was not evident (Probst *et al.*, 2000; Spittaels *et al.*, 1999). To examine the role of tau hyperphosphorylation in disease pathogenesis, 4R2N animals were crossed with mice overexpressing glycogen synthase kinase 3β (GSK-3β), a putative tau kinase (Spittaels *et al.*, 2000). Double transgenic mice showed increased levels of tau phosphorylation and a marked reduction of axonopathy, motor deficits and amount of spheroids (Spittaels *et al.*, 2000). In this system, hyperphosphorylation of tau correlates inversely with pathology.

Although the adult mouse brain predominantly expresses 4R tau isoforms, two groups generated transgenic mice overexpressing the shortest human tau isoform (3R0N) to test the hypothesis that absence of 3R tau inhibits the development of fibrillary tangles. Brion *et al* described the first transgenic 3R0N model in 1999, using the 3-hydroxy-methyl-glutaryl CoA reductase promoter to drive the expression of the transgene (Brion *et al.*, 1999). Somatodendritic expression of tau was detected, but no tau filaments could be identified. Ishihara *et al* used the mouse *prion* promoter (PrP) to establish transgenic 3R0N tau mice; overexpressing tau 5-10 times compared to endogenous tau levels (Ishihara *et al.*, 1999). These mice showed an age-dependent increase in insoluble tau, motor neuron degeneration and tau positive inclusions in the brain and spinal cord.

In another approach to model the human tauopathies in mice Duff *et al* generated mice containing the entire human tau gene (Duff *et al.*, 2000). These mice produce all six human tau isoforms with an increased expression level of 3.5 times, in a realistic attempt to mimic the human situation. Interestingly, in the mouse brain, splicing of the human genomic tau transgene is altered in favor of the 3R tau isoforms, but these mice showed no abnormal tau pathology or behavioral changes. Also this, more sophisticated expression model of wildtype tau in mice, although showing pathological changes, does not resemble the disease characteristics of tauopathies such as AD or FTDP-17 (Duff *et al.*, 2000). However, a follow-up study of the highest expressing 3R0N line aged mice of over 18 months, modest numbers of filamentous tau tangles with similar properties as the ones found in AD could be detected (Ishihara *et al.*, 2001).

Another approach to mimic human disease was carried out by Higuchi *et al*. Transgenic mice overexpressing all three human 3R tau isoforms were generated using a minigene construct combined with the mouse α-tubulin promoter. The transgenes were expressed in neuronal and glial cells, but only glial tau pathology, resembling CBD astrocytic plaques was detected in aged animals (Higuchi *et al.*, 2002). All studies overexpressing wild type tau protein in mice, as described here, show no or limited amounts of filaments, suggesting that overexpressing wild type tau is not enough to produce filaments or aggregates. Although the tau isoform composition in mice and human differ, disruption of the delicate balance between the individual tau isoforms in mice does not have the same detrimental effect as in human FTDP-17 patients. Furthermore, the relevance of this mouse models to the disease mechanism in tauopathies in humans is questionable. An overview of all established transgenic mouse lines can be found in table 1.

5.3 Transgenic models for human mutated tau

The discovery of mutations in the *tau* gene in FTDP-17 has led to the generation of transgenic mouse lines expressing mutant human tau proteins in neurons and glial cells. In addition, they may be useful models for the development and testing of novel therapies, all depending on whether the characteristics of FTDP-17 patients are resembled in the mouse phenotypes. The human 4R tau isoform lacking the two aminoterminal inserts (4R0N) and carrying the P301L mutation was used to generate a transgenic line under the control of the murine PrP promoter by Lewis *et al* (Lewis *et al.*, 2000). Mutant tau was expressed at levels comparable to endogenous tau levels. The mice developed motor and behavioral deficits, initially presenting with hindlimb dysfunction starting at 4.5 months for homozygous transgenic animals. These P301L mutant-expressing mice developed NFTs composed predominantly of straight tau filaments mainly in the spinal cord and brainstem. Pretangles were distributed in the cortex and hippocampus. The NFTs consist of hyperphosphorylated tau and are positive with silver staining. In addition the animals showed almost 50% neuronal loss in the spinal cord (Lewis *et al.*, 2000).

A second P301L transgenic mouse was developed by Götz *et al* expressing the longest tau isoform (4R2N) under the control of the mouse Thy-1 promoter (Götz *et al.*, 2001a). Pretangles and NFTs were identified in the cortex, brain stem and spinal cord of 8-months old animals. Tau filaments with straight and twisted ribbon morphologies were observed, and additionally activated astrocytes and neuronal apoptosis were demonstrated (Götz *et al.*, 2001a).

In the following two years, several other groups reported the production of transgenic mice carrying different *tau* mutations (G272V, V337M, P301S and R406W), using various promoters and tau cDNA constructs (Allen *et al.*, 2002; Götz *et al.*, 2001c; Lim *et al.*, 2001; Tanemura *et al.*, 2001; Tatebayashi *et al.*, 2002). An overview can be found in table 1. Although the expression levels of the transgenic lines varied greatly, between 0.10 and 15 times endogenous tau levels, in all mice NFTs could be detected, eventually combined with neuronal loss. The G272V mutation was expressed in mouse brains by combining a prion protein promoter-driven expression system with an autoregulatory transactivator loop, resulting in high expression in a subset of neurons and oligodendrocytes (Götz *et al.*, 2001c). V337M mutant mice showed NFTs in hippocampus and neocortex, and showed progressive behavioral changes (Tanemura *et al.*, 2001). The R406W mutation showed NFTs with similar characteristics as AD and FTDP-17 patients and associative memory deficits (Tatebayashi *et al.*, 2002). The P301S transgenic mice, differing from the ones mentioned above in the absence of the two aminoterminal exons, showed a severe neurological phenotype with abundant NFTs combined with 50% neuronal loss (Allen *et al.*, 2002). In addition, Lim *et al* reported mice expressing human tau with triple FTDP-17 mutations (G272V, P301L and R406W) developing tau filaments preferentially in the forebrain (Lim *et al.*, 2001).

All these above described animals highlight the relevance of tau mutations in disease progression. While some of these mice expressed considerably lower levels of tau, they did develop NFTs in cells affected by the disease in humans, and this did not occur in wild type tau transgenic mice. Despite this observation, large differences in phenotype between model

animals were observed, as listed in table 1. The choice of different constructs, *tau* mutations and promoters does not make it easy to compare the different mouse lines. In chapter 6 a mouse model generated and characterized in our laboratory is described. Transgenic mice, carrying the G272V and ΔK280 *tau* mutations in the longest tau isoforms, under the control of two different brain specific promoters were established. Although the expression levels were comparable to those described in the literature, the pathology was limited to tau hyperphosphorylation and the presence of an age-dependent insoluble fraction. Interestingly, the pathology was localized in the hippocampal area and the frontal and temporal cortex, closely resembling the location of pathology in patients with FTDP-17.

In 2001 an exciting development in linking Aβ pathology with tau pathology occurred; two groups independently provided convincing evidence for a causal connection between NFTs and plaques (Götz *et al.*, 2001b; Lewis *et al.*, 2001). A transgenic mouse overexpressing both mutant APP and mutant tau showed both plaques and tangles. This is not surprising taking into account the pathology of both parental lines, but of interest is that the double mutant had more tangles than tau mutant mice alone and tangles appeared in areas that were unaffected in the single-mutant tau transgenic mice (Lewis *et al.*, 2001). In a parallel experiment, another group showed that injection of fibrillar Aß into the hippocampus of mutant tau transgenic mice deteriorated tangle pathology in regions affected in AD (Götz *et al.*, 2001b).

Two groups either at the RNA or at protein level studied a global analysis of the influence of tau neuropathology at the molecular level. A gene expression profile was determined in tau mutant mice carrying the P301L mutation (Lewis *et al.*, 2000), showing thirty-four genes with consistent changed expression levels (Ho *et al.*, 2001). A proteomic analysis of tau 4R2N wild type transgenic mice (Spittaels *et al.*, 1999), using two dimensional gel electrophoresis, showed over thirty proteins differing in integrated intensity (Tilleman *et al.*, 2002). Both studies found several changes of gene expression in apoptotic and neuronal death pathways and the inhibition of internal transport and provide new clues for unraveling the mechanism of tau neuropathology (Ho *et al.*, 2001; Tilleman *et al.*, 2002). In this respect, a recent study of lithium treatment in a transgenic tau model with triple FTDP-17 mutations (G272V, P301L and R406W) showed that therapeutic options are started to be studied (Lim *et al.*, 2001; Perez *et al.*, 2003) It was shown that chronic lithium treatment results in reduction of aggregates, presumably acting through the inhibition of glycogen synthase kinase 3.

In conclusion, most mutated tau transgenic mouse models showed that tau pathology and subsequent neurodegeneration could be induced. These mice can be used in further studies regarding the aggregation process and neurodegenerative mechanisms. However, they do not model specifically FTDP-17, because they do not exhibit the selective pattern of tau pathology, but instead show widespread brain and spinal cord pathology.

Table 1: Schematic overview of tau transgenic mouse models

Tau transgene	Promoter	Mutation	Strain	Expression level	Pathology	NFTs or neuronal loss	Reference
4R2N	m-Thy-1	wildtype	C57Bl6	0.10%	Tau hyperphosphorylation	No	(Götz *et al.*, 1995)
3R0N	HMG-CoA*	wildtype	C57Bl6	0.14%	Tau hyperphosphorylation	No	(Brion *et al.*, 1999)
3R0N	PrP	wildtype	C57Bl6/ DBA	10x endogenous levels	Axonal degeneration; at later age tau inclusions	limited NFTs	(Ishihara *et al.*, 1999; Ishihara *et al.*, 2001)
4R2N	m-Thy-1	wildtype	FVB/N	4x endogenous levels	Axonal degeneration, ballooned axons, mild motor deficits	No	(Spittaels *et al.*, 1999)
4R2N	m-Thy-1	wildtype	C57Bl6	5x endogenous levels	Axonal degeneration and spheroids	No	(Probst *et al.*, 2000)
Genomic tau	-	wildtype	C57Bl6/ DBA/SW	4x endogenous levels	All six isoforms expressed, high proportion of 3R tau	No	(Duff *et al.*, 2000)
4R0N	PrP	P301L	C57Bl6/ DBA/SW	Equivalent to endogenous	Progressive motor disturbances and massive tau pathology	NFTs and 50% neuronal loss	(Lewis *et al.*, 2000)
4R2N	m-Thy-1	P301L	C57Bl6/ DBA	0.70%	Tau filament formation	NFTs	(Götz *et al.*, 2001a)
4R2N	PrP-TA**	G272V	C57Bl6	4x endogenous levels	Oligodendroglial tau filaments	NFTs	(Götz *et al.*, 2001c)
4R2N	PDGF-β***	V337M	C57Bl6	0.10%	Filamentous tau aggregates	NFTs	(Tanemura *et al.*, 2001)
4R2N	m-Thy-1	G272V/ V337M/ R406W	C57Bl6	Equivalent to endogenous	Formation of tau filaments and lysosomal abnormalities	NFTs	(Lim *et al.*, 2001)
3R0N/3R1N/3R2N (KO)	α-tubulin	wildtype	C57Bl6/ DBA	5x endogenous levels	Glial tau pathology; resembling CBD astrocytic plaques	Glial filaments	(Higuchi *et al.*, 2002)
4R0N	m-Thy-1	P301S	C57Bl6	2x endogenous levels	Twisted ribbon tau filaments	NFTs and 50% neuronal loss	(Allen *et al.*, 2002)
4R2N	CaMK II	R406W	C57Bl6	15x endogenous levels	Tau filaments in forebrain, associative memory impaired	NFTs	(Tatebayashi *et al.*, 2002)

* HMG-CoA: 3-hydroxy-methyl-glutaryl CoA reductase

** TA: tetracycline-dependent transactivator (TA) system

*** PDGF: platelet derived growth factor

5.4 Cultured cell models

In parallel with the development of mouse model systems, cell culture systems have been used often to investigate tau phosporylation and to define tau-tubulin interactions. These studies were mostly performed in untransfected cells or cells transfected with other proteins known to be involved in neurodegeneration or tau phosphorylation (Ko *et al.*, 2002). For instance by incubation of primary neuronal cultures with different proteins or chemicals the posttranslational modifications of tau were studied. However, all this different treatments in several cell systems did not lead to the formation of aggregated tau, as many investigators would have hoped to achieve (Ko *et al.*, 2002).

Logically, new possibilities in inducing tau aggregation also in cellular models rose after mutations in the *tau* gene were identified in patients with FTDP-17. However, none of the transient or stable overexpression experiments of mutant tau to date, except for one, have demonstrated tau aggregation. Overproduction of mutant tau protein (G272V, ΔK280, P301L, V337M and R406W) in a variety of cell lines caused varying degrees of reduced microtubule binding, disorganized morphology and defects in assembly and stability. Human neuroblastoma or CHO (Chinese Hamster Ovary) cells transiently transfected with 3R0N or 4R0N wild type or mutant (P301L, V337M and R406W) tau isoforms were almost indistinguishable in their phenotype. Microtubule extension was reduced in cells transfected with mutated tau at various degrees (Dayanandan *et al.*, 1999). CHO cells stably transfected with 4R0N wild type or mutant (G272V, P301L, V337M and R406W) showed similar results (Matsumura *et al.*, 1999). COS cells were transiently transfected with three isoforms of tau (3R0N, 4R0N, 4R2N) either wildtype or V337M mutated. The V337M 4R0N isoform showed more disruption of microtubule networks than the other V337M isoforms (Arawaka *et al.*, 1999). Another study with transiently transfected COS cells, combined with several missense mutations and 3R and 4R isoforms, showed only mild effects on MT binding. 3R tau isoforms showed increased microtubule disruptions compared to 4R tau isoforms (Sahara *et al.*, 2000). Other studies described more dramatic changes in cytoskeleton integrity. Mutant (P301L and V337M) 4R0N tau fused to a green fluorescent protein analog (EGFP) was transiently expressed in HEK293 cells. Much of the EGFP-tau was associated with microtubules and co-localized with tubulin. After fractionation, more mutant tau (mainly P301L) was seen in the cytoplasm compared to wild type tau (Nagiec *et al.*, 2001). Fusion constructs that showed fluorescence at different wavelengths were used to detect wild type and mutant tau in living cells. Mutated tau (P301L, V337M and R406W) colocalized with microtubules when expressed alone, but appeared significantly dissociated from the microtubules and diffuse in the cytoplasm when coexpressed with wild type tau (Lu & Kosik, 2001). These *in vivo* data correlate with the reduced binding capacities of tau missense mutations *in vitro*, but suggest a more complex regulation and kinetic competition between wild type and mutant tau. Since mutations in FTDP-17 are heterozygous, both the wild type and mutant isoforms are present in the cell. The observed discrepancies between the individual studies are most likely due to the differences in the levels of expression and the methods used for quantification of tau expression and microtubule binding. Nevertheless, even if missense mutations in these cell models cause only a modest reduction in microtubule binding affinity, this could have large cumulative effects on affected neurons over the human life span.

Until now, only Vogelsberg-Ragaglia *et al* have demonstrated aggregation of mutant tau proteins in intact cells. Stably transfected CHO cells, with different tau mutations (ΔK280, P301L, V337M, R406W) were generated. It was shown that different mutant tau constructs produced distinct pathological phenotypes. Cells expressing the ΔK280 mutant, but not other mutations, formed insoluble amorphous and fibrillar tau aggregates. A triple tau mutant (VPR), containing V337M, P301L and R406W mutations also formed similar aggregates (Vogelsberg-Ragaglia *et al.*, 2000). The aggregates increased over time and could be detected by immunofluorescence and electronmicroscopy. Although they are not identical to the human PHFs or NFTs, this model would be extremely useful for screening experiments. In a cellular model, high-throughput screening of compounds intervening the aggregation mechanism or potential therapeutic drugs will be much more cost–effective than in animal models.

In 1997 it was shown for the first time that massive overexpression of human tau isoforms (3R0N and 4R2N) can lead to the formation of tau inclusions and degeneration of a subset of neurons *in vivo* (Hall *et al.*, 1997). A unique cellular model of tau filament formation was developed by using anterior bulbar cells (ABC) neurons of a lower vertebrate, the ammocoete sea lamprey, *Petromyzon marinus*. To study the cytopathological changes throughout the disease process, the ABC neurons were microinjected with human tau constructs and studied in detail on a single cell level (Hall *et al.*, 2000; Hall *et al.*, 1997). Because these neurons have been morphologically characterized in detail, this lamprey ABC system can be used for studies of the effect of exogenous proteins in identified mature neurons in normal in situ environment (Hall & Cohen, 1983; Hall & Cohen, 1988; Hall *et al.*, 1989).

Overexpression of human tau caused hyperphosphorylation at several different sites and incorporation into filaments, resembling in some respect the straight filaments seen in human tauopathies (Hall *et al.*, 2000; Hall *et al.*, 1997). Several degenerative changes in the cells were observed, including loss of microtubules and synapses, plasma membrane degeneration and eventually externalization of human tau deposits and cell death (Hall *et al.*, 2000; Hall *et al.*, 1997). Hyperphosphorylation in these cells is spatiotemporaly correlated in a sequence of events, highly resembling the human neurogedenerative process (Hall *et al.*, 2001). This model has shown to be very powerful in characterizing the cytoskeletal pathology *in vivo* and is highly suitable for testing potential therapeutic agents. A first example was published in 2002, were Hall *et al* showed an arrest in neurofibrillary degeneration after treatment with proprietary compounds that have been demonstrated to block tau filament formation *in vitro* (Hall *et al.*, 2002).

5.5 Other tau model systems

The classical fruit fly, *Drosophila Melanogaster* has also been used for modeling tau related neurodegeneration. *Drosophila* is extensively characterized, relatively easy to manipulate and multiply and can be used in forward genetic screens to identify modifier genes of a pathogenic phenotype (de Silva & Farrer, 2002). Expression of transgenic human *tau* in *Drosophila* resulted in neuronal death, but without the formation of tangles (Wittmann *et al.*, 2001). Both wildtype (4R2N) and R406W mutated tau transgenic flies displayed

features of tau related neurodegeneration: decreased life span and progressive late onset neurodegeneration, which was more severe in the flies expressing mutant tau compared to wildtype tau (Wittmann *et al.*, 2001). This model suggests that tau overexpression alone can cause neuronal death, without the need for the formation of fibrillary aggregates. Coexpression of tau and the *Drosophila* homologue of GSK-3β, *shaggy*, showed severe enhanced neurodegeneration and NFT pathology (Jackson *et al.*, 2002).

The first modifier screen was published recently, showing that kinases and phosphatases are enhancers of tau pathology in *Drosophila* (Shulman & Feany, 2003). Interestingly, by comparing modifiying screens for several neurodegenerative disorders it was found that most tau modifiers do not play a role in the toxicity of neurodegenerative diseases like polyglutamine disorders and Parkinson's disease. Modifiers of other neurodegenerative disorders, including heat-shock proteins, chaperones and ubiquitin pathway components failed to modify tau toxicity (Shulman & Feany, 2003).

Recently, two new lower vertebrate model systems were described for modelling tau related neurodegeneration, by inducing human wildtype and mutant proteins. In *Danio Rerio* (zebrafish) neurons the functional consequences and trafficking patterns of human tau overexpression can be studied. The individual events and the early mechanism of neurodegeneration can be dissected more easily in these animals. Overexpression of wildtype (3R0N and 4R0N) showed a cytoskeletal disruption closely resembling the NFTs in human disease (Tomasiewicz *et al.*, 2002). This efficient vertebrate modelsystem has several potential lines of research in studying the individual embryos, groups of neurons and in gene arrays. Further characterization of this new tau transgenic model has not been described yet.

Furthermore, a *Caenorhabditis elegans* (worm) model was recently described, in which wild type and mutated (P301L and V337M) human tau (4R1N isoform) were overexpressed (Kraemer *et al.*, 2003). Neuronal expression of human tau caused progressive uncoordinated locomotion, which is characteristic of nervous system defects in worms. Subsequently, accumulation of insoluble tau and hyperphosphorylation could be detected, more pronounced in the mutated than the wildtype tau transgenic worms (Kraemer *et al.*, 2003).

Also this model (as all the previous described models for tau) is suitable for dissecting the sequence of events ultimately leading to neurodegeneration, focusing on the onset of the disease symptoms, and to identify genes that modify the tau-induced phenotype. However, until now all studies described only the events that are already known in humans, forming the end stages of the disease (eg. neuronal loss, hyperphosphorylation and insoluble tau filaments). Future studies should prove the usefulness of simple or more complex model systems in understanding better the human disease process, and in elucidating new molecular pathways involved.

5.6 Tau interactions with other proteins

Besides the expression of tau in model organisms and studying the obtained phenotypes, it is important to study the function of the tau protein in molecular and cellular pathways. Elucidating new interactions with other proteins in the cell will gain new insights in the function of tau both in the normal and pathological situation. Tau is a soluble protein that not only binds to microtubules, but also promotes MT (microtubule) assembly, stabilizes MTs and regulates trafficking along MTs (Cleveland *et al.*, 1977; Weingarten *et al.*, 1975). Besides tubulin, tau proteins can bind to many other proteins such as spectrin (Carlier *et al.*, 1984) or protein phosphatase 1 (PP1) (Liao *et al.*, 1998; Sontag *et al.*, 1999). Other tau interacting proteins have been reported to bind to the tau molecule through its microtubule-binding region. They include PP1, cdk5 (Sobue *et al.*, 2000), presenilin 1 (Takashima *et al.*, 1998b) and α-synuclein (Jensen *et al.*, 1999). Presenilin 1 and α-synuclein are interesting, because mutations in both proteins are associated with neurodegenerative disorders. Both proteins might modulate the phosphorylation state of soluble axonal tau (Jensen *et al.*, 1999; Takashima *et al.*, 1998a; Takashima *et al.*, 1998b).

A number of proteins interacting with the tau molecule through its proline-rich region have also been reported. Among these are phospholipase C-8 (Hwang *et al.*, 1996); a protein with a SH3 domain that could bind to the PPXXP tau motif (Jenkins & Johnson, 1998); fyn, a tyrosine kinase also containing a SH3 domain (Lee *et al.*, 1998); and actin (Correas *et al.*, 1990). Recent studies suggest that tau facilitates the process outgrowth of neurons and oligodendrocytes via its interactions with the nonreceptor tyrosine kinase, fyn. Tau has been shown to interact with components of the neural plasma membrane through its aminoterminal non-microtubule binding domain, which protrudes from the microtubule surface when tau interacts with microtubules (Brandt *et al.*, 1995). This suggests a role for tau as a linker protein between microtubules and the membrane skeleton. Binding between the proline-rich sequence in the amino-terminal part of tau proteins and the SH3 domains or src family non-receptor tyrosine kinases, such as fyn, was observed by Lee *et al* (Lee *et al.*, 1998). They described the co-localization of tau and fyn just beneath the plasma membrane, and an association between the tau-fyn complexes and the actin cytoskeleton. Recently it was shown in oligodendrocytes that besides tau, fyn also binds to tubulin via its SH2 domain, thereby promoting process outgrowth and wrapping of axons. At the same time, the fyn protein is upregulated in a subset of neurons in AD brain that contain hyperphosphorylated tau, supporting the involvement of the fyn protein in neurodegenerative processes (Shirazi & Wood, 1993). Furthermore, fyn KO mice displayed specific neurological deficits (Grant *et al.*, 1992) and in β-amyloid treated cells fyn is upregulated (Williamson *et al.*, 2002).

Another important tau interacting protein is prolyl isomerase 1 (Pin-1). Pin-1 is a chaperone protein that binds to phosphoproteins containing phospho-serine or threonine followed by proline, it regulates entry and progression through mitosis, by interacting with specific phosphoproteins (Lu *et al.*, 1996). Thus, tau was a likely substrate that could interact with Pin-1 via its proline-rich region. Indeed, in 1999 Lu *et al* showed an interaction between tau and Pin-1. Only one phosphorylation site (threonine 231), located in the proline-rich region and necessary for full activity of tau, is required for the interaction (Lu *et al.*, 1999).

Furthermore, it was shown that Pin-1 can be co-purified with PHFs, resulting in the depletion of Pin-1 in the brains of AD patients. Pin-1 can restore the ability of phosphorylated tau to bind microtubules and promote microtubule assembly *in vitro* (Goedert, 1999a; Lu *et al.*, 1999). As depletion of Pin-1 induces mitotic arrest and apoptotic cell death, sequestration of Pin-1 into PHFs may contribute to neuronal death. Disruption of the Pin-1 gene in mice showed no abnormalities in early development and early life, although cell-proliferative abnormalities were observed, including reduced bodyweight and retinal and testicular atrophies (Fujimori *et al.*, 1999; Liou *et al.*, 2002). Recently, a following study showed that Pin-1 KO mice developed neuronal degeneration in an age-dependent manner in some brain regions, characterized by motor and behavioral deficits, tau hyperphosphorylation, tau filament formation and neuronal loss (Liou *et al.*, 2003). Furthermore, they showed that Pin-1 expression is inversely correlated with predicted neuronal vulnerability and degeneration in AD brains (Liou *et al.*, 2003). Combining these recent results, Pin-1 might very well play an important role in protecting against neurodegeneration and this will open new opportunities in insight into pathogenesis and treatment.

One study applying the yeast two-hybrid system revealed a putative new class of tau interacting proteins. Hoenicka *et al* identified in a two-hybrid screen with the tau protein two positive clones, both consisting of an Alu-derived amino acid sequence. *In vitro* experiments showed that these Alu-derived peptides interact with tau and seems to modulate its phosphorylation (Hoenicka *et al.*, 2002). Since Alu-derived sequences are very common, and in general positive clones obtained from two hybrid screens should be confirmed with other *in vivo* experiments, the assumption of a new group of tau interactors is possibly a step to far. A real interacting protein, containing and interacting via its Alu-derived amino acid sequence, should first be described and analyzed. In this case, the natural relevance of interacting proteins found with the yeast two-hybrid method is questionable. A yeast two-hybrid screen with the tau protein was also performed in our laboratory. The results will be discussed in more detail in Chapter 8.

5.7 Degradation of tau protein

The link between neurodegeneration and disturbed degradation of a specific (group of) protein has been described for several neurogedenerative disorders. Several findings for tau described below and also in chapter 7, point in the same direction. By developing specific polyclonal antibodies, the composition of tau aggregates from the brains of FTDP-17 carrying a common tau mutation, P301L, were characterized in detail (Rizzu *et al.*, 2000). They showed that, although both mutated and wildtype tau proteins were present in the insoluble aggregates, the main component of these deposits was the mutated tau protein. In the soluble fraction on the other hand, a strong reduction in the level of mutated tau protein was observed. Furthermore, in the soluble fraction two bands, smaller than the normal and mutated full-length proteins, were observed. These two bands stained strongly with the antibody specific for the point mutation and only weak with the antibody recognizing normal 4R tau (Rizzu *et al.*, 2000). These results suggest an alteration of the proteolytic processing of the mutated P301L protein; the observed bands may be intermediate degradation fragments

generated during the normal proteolysis of the protein. The accumulation of these bands suggests that this normal processing is impaired for the mutant protein.
In chapter 7 experiments are described following this hypothesis, using the transgenic mouse model of Lewis *et al* (Lewis *et al.*, 2000). In this mouse model, two bands, running below the full length tau band on an SDS PAGE gel and at the same level as the two bands found in the experiments with human material were observed. Characterization of these bands showed that they are age-independent and inducible by the calpain degradation system.

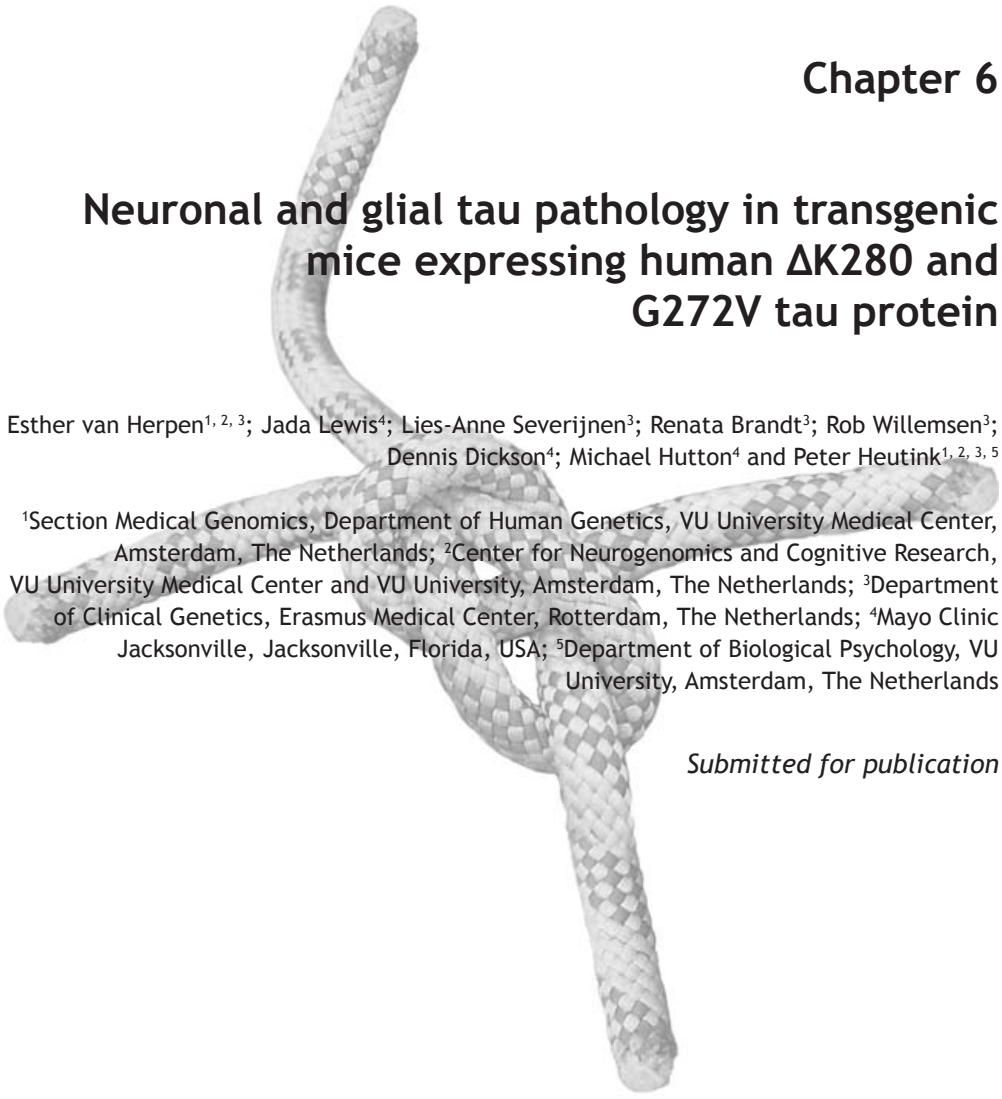
The protein degradation systems that are responsible for tau metabolism *in vivo* are not well characterized, although tau has previously been shown to be processed by various proteases *in vitro*, including caspases, calpains, cathepsins and trombin (David *et al.*, 2002). Since inappropriate apoptotic cell death is known to be involved in several neurodegenerative disorders (Johnson *et al.*, 1997), increasing effort has been put in experiments related to degradation pathways. *In vivo* experiments in apoptotic neuronal cells showed tau to be susceptible to both calpain and caspase degradation, resulting in the generation of multiple fragments. The appearance of these fragments is inhibited by both calpain and caspase inhibitors, suggesting that tau is a substrate for these proteases during apoptosis (Canu *et al.*, 1998). Furthermore, a tau fragment including the microtubule binding domains and the proline-rich region was found to induce apoptosis when expressed in COS cells (Fasulo *et al.*, 1998). The transition point, beyond which the tau fragments lose their apoptosis-inducing ability, is located around one of the putative caspase sites in the C-terminal part of the protein (Fasulo *et al.*, 2000). The C-terminus of tau inhibits filament formation; removal of as few as 12 amino acids from its C-terminus accelerated tau filament formation *in vitro* (Chung *et al.*, 2001). Proteolytic cleavage of tau at Asp421, by multiple caspases, generated a gain-of-function phenotype: enhanced filament formation *in vitro*.

On the other hand, in experiments using stably transfected neuroblastoma cells a proteosome inhibitor, lactacystin, could block tau degradation. However, neither in this nor in other studies ubiquitylation of tau could be demonstrated. *In vitro* experiments with recombinant tau proteins showed that ubiquitin-independent pathways could be responsible for the proteosomal degredation of protein (David *et al.*, 2002). One study has been performed comparing the wild type and some FTDP-17 mutated tau proteins. It was reported that mutants V337M and R406W were less susceptible than mutant P301L or corresponding wildtype tau to degradation by calpain I, using recombinant 4R and 3R proteins (Yen *et al.*, 1999). Moreover, tau can also be processed by the lysosomal degradation system, as was shown in experiments with lysosomal proteases in cultured hippocampal slices. Cathepsin D inhibitors blocked the formation of hyperphosphorylated tau fragments, thereby hypothesizing that partial lysosomal dysfunction links to the formation of tau filaments (Bi *et al.*, 2000).

Recently, an inverse relationship has been described between aggregated tau proteins and the levels of heat shock proteins (HSP) in AD brains. Increased levels of HSP promote tau solubility and tau binding to microtubules and cause reduced tau phosphorylation (Dou *et al.*, 2003). These results suggest that upregulation of molecular chaperones can suppress the formation of NFTs by partitioning tau into a productive folding pathway. P25, which is a truncated form of p35 produced by calpain digestion and an activator of cdk5, accumulates

in neurons of AD patients. Binding of p25 to cdk5 constitutively activates cdk5 and alters its substrate specificity. Furthermore, the p25-cdk5 complex hyperphosphorylates tau (Patrick *et al.*, 1999). Transgenic mice overexpressing human p25, an activator of the kinase cdk5 developed disturbances in the cytoskeletal architecture, hyperphosphorylated tau as well as behavioral changes. However, there was no accumulation of insoluble tau in these mice (Ahlijanian *et al.*, 2000).

Regarding the precise degradation pathway of tau and possible differences between normal and mutated tau, the previous described experiments are not fully conclusive. Further detailed, *in vivo* experiments should elucidate the underlying important, possible pathogenic mechanisms.



Chapter 6

Neuronal and glial tau pathology in transgenic mice expressing human ΔK280 and G272V tau protein

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Abstract

The identification of *tau* mutations in patients with frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) showed that tau dysfunction alone can be sufficient to cause neurodegeneration. Furthermore, it created options to generate mouse models of tauopathy by expressing transgenes with pathogenic mutations. We established and characterized transgenic mouse lines expressing the $MAPT^{\Delta K280}$ and $MAPT^{G272V}$, under the control of two different brain specific promoters (NSE and mThy-1). Expression levels in the individual lines varied from two to threefold the endogenous mouse tau levels. Age-dependent tau hyperphosporylation was observed in the hippocampal region, the mediotemporal cortex and the pons, partly mimicking the pathology found in human patients with FTDP-17. Sarkosyl insoluble tau was detected only in aged animals. However, no aggregates or filaments were detected by immunohistochemistry, unlike in several published studies in tau transgenic mice. This mouse model resembles the pathology of patients with FTDP-17, however the phenotype is much milder. This model system can be used to study the initial stages of tau pathogenesis and to identify modifying factors, either genetic or environmental.

Introduction

Many neurodegenerative disorders are characterized by the formation of insoluble protein aggregates and the degeneration of specific populations of neurons (Heutink, 2000; Lee *et al.*, 2001; Rosso & van Swieten, 2002). Several neurodegenerative disorders, such as Alzheimer's disease (AD), frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), progressive supranuclear palsy (PSP) and Pick's disease (PiD), are characterized by the presence of neurofibrillary inclusions composed of hyperphosphorylated microtubule associated protein tau (Heutink, 2000; Lee *et al.*, 2001; Rosso & van Swieten, 2002). Tau pathology occur in either the presence or absence of MAPT (*tau*) gene mutations (Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998). The tau protein functions in tubulin polymerization, stabilizing microtubules and maintaining neuronal integrity (Heutink, 2000; Lee *et al.*, 2001; Rosso & van Swieten, 2002). In the adult human brain six tau isoforms ranging from 352 to 441 amino acids are produced as a result of alternative mRNA splicing of exons 2, 3 and 10.(Andreadis *et al.*, 1992) The interaction between tau and tubulin is mediated by three or four imperfect repeats encoded by exons 9-12. The alternative splicing of exon 10 results in isoforms with three (3R) or four repeat (4R) domains (Andreadis *et al.*, 1992; Lee *et al.*, 1989). In adult human brain similar levels of 3R and 4R tau are expressed (Lee *et al.*, 1989).

MAPT gene mutations associated with FTDP-17 include missense-, deletion- or silent mutations in the coding region or mutations located in the intron following exon 10 (Heutink, 2000; Lee *et al.*, 2001; Rosso & van Swieten, 2002). Functionally, some of these mutations lead to a reduced ability to bind to microtubules and to increased tau aggregation while others disrupt exon 10 splicing and thus change the ratio of 3R to 4R tau isoforms (Hasegawa *et al.*, 1998; Hutton *et al.*, 1998; Spillantini *et al.*, 1998; Yoshida *et al.*, 2002). One mutation, ΔK280, located in the interrepeat region between repeat 1 and 2, is of particular interest.

This region is known to be important in microtubule binding and it was shown long before the identification of tau mutations in FTDP-17 that the binding activity of this region is largely dependent on this single amino acid (Goode & Feinstein, 1994). Interestingly, by using *in vitro* splice assays, it was shown that the ΔK280 mutation dramatically reduces exon 10 splicing in and is therefore predicted to increase the relative proportion of 3R tau. This also has the effect of reducing the expression of 4R isoforms with this mutation (D'Souza *et al.*, 1999; Rizzu *et al.*, 1999). Because only a single patient has been described with this mutation and no post-mortem material is available, the effect of this mutation *in vivo* is still unknown.

Several transgenic mice, expressing wild type tau DNA constructs, have been generated to model and study human tauopathy. Minimal tau aggregates were found in these animals, although tau hyperphosphorylation, insoluble tau, axonopathy and motor deficits could be detected, mainly in aged mice (Götz *et al.*, 1995; Ishihara *et al.*, 1999; Ishihara *et al.*, 2001; Lee *et al.*, 2001). After the identification of tau mutations, several groups generated mice that express human tau cDNA's containing FTDP-17 associated mutations. The pathology described in these animals includes neurofibrillary tangles (NFTs), sometimes combined with neuronal loss (Allen *et al.*, 2002; Götz *et al.*, 2001a; Götz *et al.*, 2001b; Lewis *et al.*, 2000; Lim *et al.*, 2001; Tanemura *et al.*, 2001; Tatebayashi *et al.*, 2002). Although these models show tau-induced neuropathology and neurodegeneration, distribution of pathology in these models is different from that of human patients with FTDP-17. Because different regions of the brain and spinal cord are affected.As a result the animals have mostly been observed to have motor function deficits, which are not generally associated with FTDP-17.

In an attempt to model FTD in mice, we used two promoters with a general neuronal expression pattern (Forss-Petter *et al.*, 1990; Spanopoulou *et al.*, 1989). Here, we describe the generation and characterization of three transgenic mouse lines, two of them expressing a $MAPT^{\Delta K280}$ transgene (under the control of the mouse Thy-1 (mThy-1) and neuron-specific enolase (NSE) promoter) and another line expressing $MAPT^{G272V}$ (under the control of the NSE promoter). The brains of the transgenic animals developed sarkosyl insoluble tau protein species in an age-dependent fashion. In addition, accumulation of hyperphosphorylated tau in pre-tangle lesions could be detected in aged animals up to 24 months of age. However, no NFTs or other filamentous structures could be detected.

Materials and Methods

Transgenic constructs and mice

Tau cDNAs were kindly provided by A. Andreadis. Two subclones were combined into exons 1-5, 7, 9-13, intron 13 and exon 14. Site-directed mutagenesis was carried out to introduce the G272V and ΔK280 mutations in the tau constructs according to the manufacturer's (Stratagene) recommendations. The tau cDNA was subcloned into pBluescript vector, followed by the introduction of the promoter sequences. The neuron-specific enolase (NSE) promoter and the murine Thy-1 (mThy1) promoter (kindly provided by Prof. F. Grosveld, Erasmus MC, Rotterdam) were subcloned into the EcoRI and EcoRI/SalI sites respectively. Constructs were linearized and vector sequences were removed by digesting with KpnI followed by gel

purification (QiaQuick gel purification kit). All constructs were sequenced using the BigDye cycle sequencing kit (PE biosystems, Foster City, CA) on an ABI 377 automated sequencer prior to injection. A concentration of 0.3 ng/µl was microinjected into pronuclei of FVB/N embryos.

DNA analysis

Founders and other transgenic offspring were screened by PCR, using for all lines a specific tau primer set in exon 14 (Forward: TGACCTTGATGTCTTGAGAGC and Reversed: GAATTCGGGACATTGTGACG). A primerset in the Fragile X mental retardation gene 1 (FMR1) was used as internal control PCR. Furthermore, specific PCR's for the promoter sequences were established and regularly performed.

Antibodies

A panel of anti-tau antibodies was used: mouse monoclonal H-7, T-14, AT8, AT180, PHF and MC-1. H-7 (Innogenetics, Belgium) is a phosphorylation independent anti-tau antibody, recognizing both human and murine tau isoforms (Mercken *et al.*, 1992). T-14 (Zymed, USA), another phosphorylation-independent antibody, recognizes human tau isoforms, but does not recognize murine tau (Kosik *et al.*, 1988). AT8, AT180 (both obtained from Innogenetics, Belgium) and PHF (a gift from Dr. P. Davies, Albert Einstein College of Medicine, Bronx, USA) are phosphorylation dependent antibodies, which recognize both human and murine tau phosphorylated at specific sites (S202 and T205, T231, S396 and S404 respectively (Buee-Scherrer *et al.*, 1996; Goedert *et al.*, 1994; Goedert *et al.*, 1995; Otvos *et al.*, 1994). MC-1 (a gift from Dr. P. Davies) is a conformation-dependent anti-tau antibody and recognizes amino acids 5-15 and 312-322 of tau (Jicha *et al.*, 1999; Jicha *et al.*, 1997). Antibodies directed against the glial fibrillary acidic protein (GFAP; obtained from DAKO, Denmark) were used to detect activation of astrocytes.

Histology and immunohistochemistry

For histological analysis, brain and spinal cord were obtained from transgenic mice and wildtype littermates. Animals were perfused transcardially with buffered 3% paraformaldehyde and the tissues were postfixed for two hours. Adjacent saggital sections (5 µm) were cut and stained with haematoxylin, eosin (H&E) and modified Gallyas silver stain (Gallyas, 1971) to characterize the neuropathology in these mice, or used for immunohistochemistry with the antibodies described above. After endogenous peroxidase inhibition, the sections were subjected to antigen retrieval using a microwave oven for 13 minutes in 0.01M sodium citrate. The sections were incubated overnight at 4°C with the primary antibodies, followed by the secondary antibodies and visualized by DAB staining. As primary antibodies, H-7 (1:500), T14 (1:150), AT8 (1:50), AT180 (1:100), PHF (1:100), MC-1 (1:150) and GFAP (1:500) were used. For the T14 antibody, an alternative antigen retrieval protocol was used, by 8 minutes 90˚C heating in 0.1M citric acid. For AT8 we used an alternative staining procedure suitable for the M.O.M. immunodetection kit (Vector Labs, Burlingame, CA). Tau P301L transgenic mice JNPL3 served as positive control in these experiments (Lewis *et al.*, 2000).

Extraction of sarkosyl insoluble tau and immunoblotting

Whole brain homogenates were obtained by homogenization in Hepes buffer (10 mM Hepes,

0.05% Triton and 0.05% Tween, 5 mM $MgCl_2$ and 0.3 M KCl) followed by 20 minutes centrifugation at 10.000 g. From the supernatants the protein concentrations were determined by using the BCA protein assay (Pierce, Rockford, IL) and used for immunoblot analysis. Sarkosyl-insoluble tau was extracted from whole brains of the transgenic mice, as described for cerebral cortex from AD and FTD brains (Goedert *et al.*, 1992b). The ultracentrifugation at 32.500 rpm was extended to 1 hour and 30 minutes. The sarkosyl insoluble material was analyzed by immunoblotting and electron microscopy. SDS-PAGE and transfer to nitrocellulose membranes were performed as described previously (Goedert *et al.*, 1992b; Rizzu *et al.*, 1999). The sarkosyl-soluble material was concentrated by using Centricon devices (Millipore, Bedford, MA) and protein concentrations were determined as described above. For immunoblotting experiments antibodies H-7 (1:2000), T14 (1:2000), AT8 (1:400), AT180 (1:1000), MC-1 (1: 1000) and PHF (1:1000) were used. Tau P301L transgenic mice JNPL3 served as positive control in these experiments (Lewis *et al.*, 2000).

Electron microscopy

Aliquots of sarkosyl-insoluble dispersed filament preparations were processed for electronmicroscopy as decribed (Goedert *et al.*, 1992b). Sarkosyl-insoluble material extracted from AD brain served as positive control.

Results

Three different constructs (NSE-MAPT^{G272V}, NSE-MAPT$^{\Delta K280}$ and (mThy1-MAPT$^{\Delta K280}$) all expressing the longest tau isoform (4R2N) including exon 10 and the amino terminal exons 2 and 3, were used to produce transgenic mice. From each of the lines, 3-5 founder animals were obtained. The individual lines were checked for transmission of the transgene in a Mendelian manner by PCR and for human tau protein expression in the brains of the mice by Western blotting. Subsequently, for each of the constructs, one founder was selected to establish a line. Experiments were performed with lines V2 (NSE-MAPT^{G272V}), K1 (NSE- MAPT$^{\Delta K280}$) and KT1 (mThy1- MAPT$^{\Delta K280}$). Heterozygous animals for each line were maintained on a FVB/N background and aged up to 24 months of age.

The transgene expression level in the three different lines was determined by Western blotting using total tau antibodies H-7 and T14 (human specific) (Figure 1A). Transgenic tau migrating with apparent molecular mass of 66 kDa could be detected in brains of transgenic mice. Levels of human tau were approximately two-fold (V2, K1) and three-fold (KT1) higher compared to the levels of endogenous mouse tau. To determine the distribution of human mutated tau in the brain, mice from each line were analyzed by immunohistochemistry using the phosphorylation independent anti-tau antibodies H-7 and T14. Sagittal sections of the KT1 and V2 transgenic lines showed numerous H-7 positive neurons in the cortex, hippocampus and dentate gyrus and the brain stem. (Figure 1B-G) Expression levels of human tau were highest in cortex, hippocampus (Figure 1D/E), fornix fimbriae and cerebellum, lower levels were observed in the brain stem (Figure 1F/G) and spinal cord, and human tau was not detectable in the olfactory bulb. No obvious qualitative differences were observed in expression patterns between mice generated with the NSE or mThy1 promoter, which are

both known to direct a neuronal expression pattern (Forss-Petter *et al.*, 1990; Spanopoulou *et al.*, 1989) (Figure 1B/C). However, higher expression was observed in the hippocampus and cerebellum for the KT1 (mThy1-MAPT$^{\Delta K280}$) line. Furthermore, cells with high levels of expressed tau showed evidence of granular accumulation (Figure 1D).

In order to determine the pathological changes and the phosphorylation status of tau in aged transgenic animals (up to 24 months), we used several phosphorylation-dependent anti-tau antibodies. By immunohistochemical analysis, anti-tau antibody AT8 stained numerous glial cells in the fornix fimbriae, the brain stem (mesencephalon and pons) and parts of the white matter of the cortex signifying early changes in tau phosphorylation (Figure 2A/C). Furthermore, neuronal staining was observed in the endplate of the hippocampus (Figure 2E/F), pons, (Figure 2G/H) and mediotemporal lobe (Figure 2A/C). In the non-transgenic control animals, due to normal aging effects, the same glial staining pattern could be detected, although much weaker than in the transgenic animals (Figure 2D). The neuronal staining was not found in the non-transgenic control animals. To detect activated astrocytes, we used an antibody against glial acidic fibrillary protein (GFAP). This antibody revealed numerous activated astrocytes and glial cells in the fornix fimbriae and the brain stem (Figure 2B). Much smaller numbers of activated astrocytes were observed in the same regions in non-transgenic control animals. Staining with the conformation-dependent anti-tau antibody MC-1 and the phosphorylation-dependent antibodies AT180 and PHF did not show any positive results. Gallyas silver stainings were also negative (data not shown).

To examine the accumulation of pathological tau species biochemically, sarkosyl soluble and insoluble tau proteins were extracted from transgenic mouse brains aged 12, 18 and 24 months. Western blot analysis and with antibody H-7 detected sarkosyl insoluble tau species migrating at 66 Kda, that increased with the age of the mice (Figure 3A). In addition, Western blot analysis of soluble tau extracts showed that human tau proteins were immunoreactive with antibodies AT180 and MC-1, consistent with a pathological process (Figure 3B). Antibodies AT8 and PHF did not gave specific signals above background staining (data not shown).

Electron microscopy on protein extracts from 24-months old transgenic mice did not reveal any evidence of tau filaments. No signs of axonopathy and neuronal loss were observed in the individual transgenic lines and there were no indications for gender differences. Several behavioural and neurological experiments (open field, tail lift, vertical and horizontal pole, footprint, hanging wire, dominance, rotarod and conditioned taste aversion) were carried out in these animals up to the age of 18 months (Crawley & Paylor, 1997), however no obvious differences were observed between transgenic animals and wildtype littermates (data not shown).

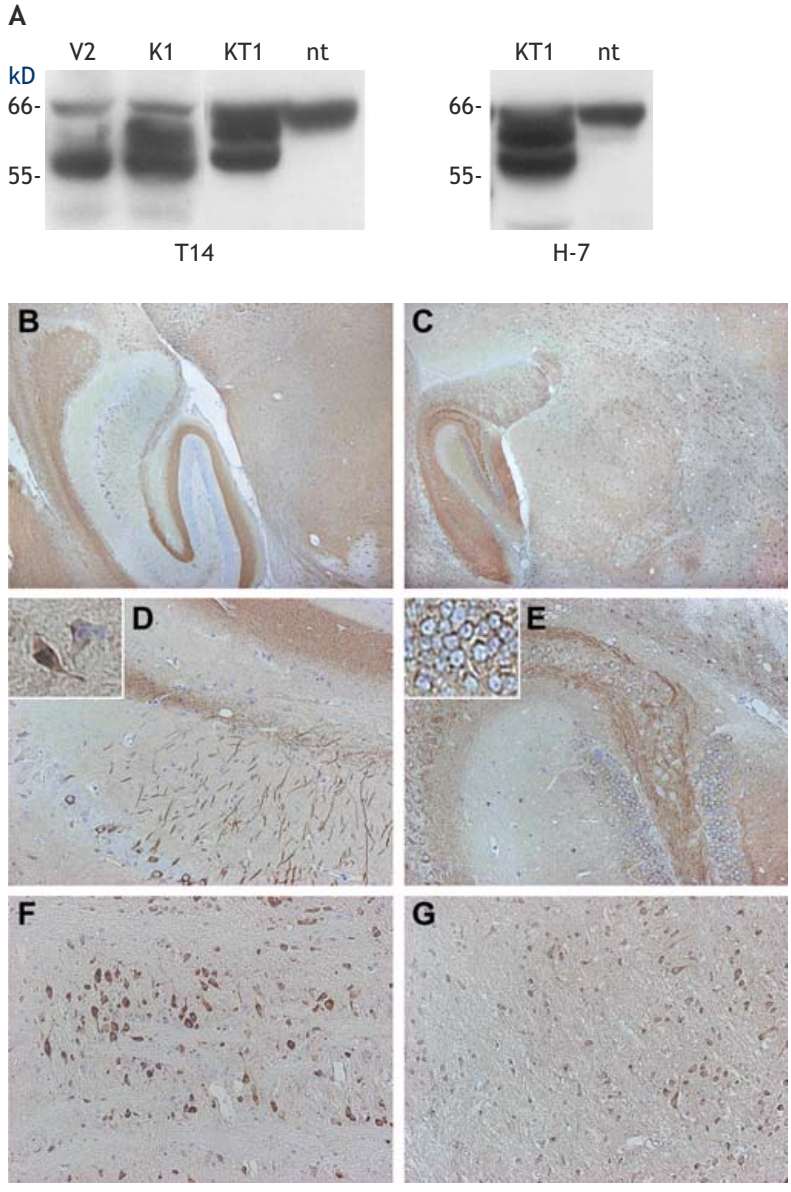

Figure 1. Immunoblot and immunohistochemical analysis in brains from mice of V2, K1 and KT1 lines. (A) Whole brain homogenates of 3-months old mice of lines V2, K1, KT1 and a non- transgenic (=nt) control mouse were analyzed by immunoblotting using antibodies T14 and H-7 (only line KT1). T14 recognizes both human and murine tau, while the H-7 antibody is human-tau specific. (B-G) Sagittal sections of 12-months old mice from lines KT1 (B,D,F) and V2 (C,E,G) showed immunoreactivity with anti-tau antibody H-7. (B,C) The hippocampal area shows a high expression of human tau. (D,E) Detailed pictures of the hippocampus show expression pattern and levels. (F,G) In the pons, a large proportion of the cells are H-7 immunoreactive. The magnifications are 40X in B-C and 200X in D-G.

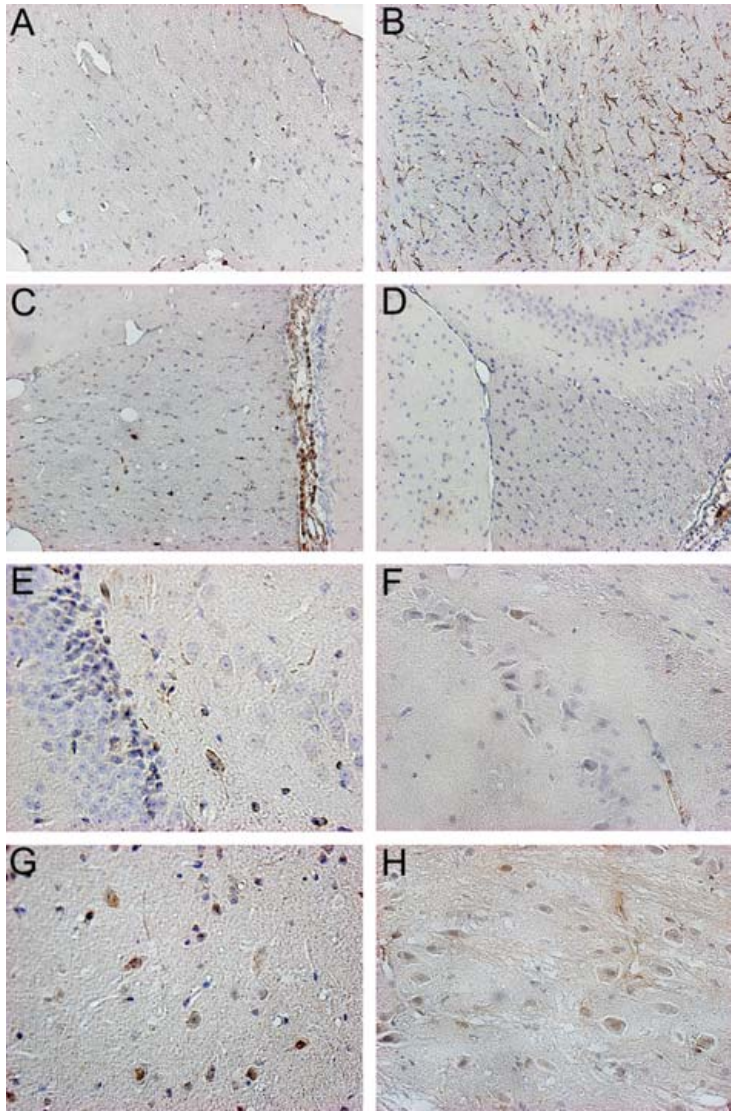

Figure 2. Microscopic neuropathology in aged mice of the individual transgenic lines (V2, K1 and KT1) (A,B,C,D) The fornix fimbriae from transgenic line KT1 showed immunoreactivity with the phosphorylation-dependent anti-tau antibody AT8 (A) and the glial acidic fibrillary protein (GFAP) antibody (B). In transgenic line V2, immunoreactivity with anti-tau antibody AT8 was observed (C), but not in non-transgenic mice (D). The antibody AT8 was also used to stain the endplate of the hippocampus and the region of the pons in KT1 (E and G respectively) and V2 (F and H respectively) transgenic animals. All animals were aged up to 24 months. The magnifications are 200X in A-D and 400X in E-H.

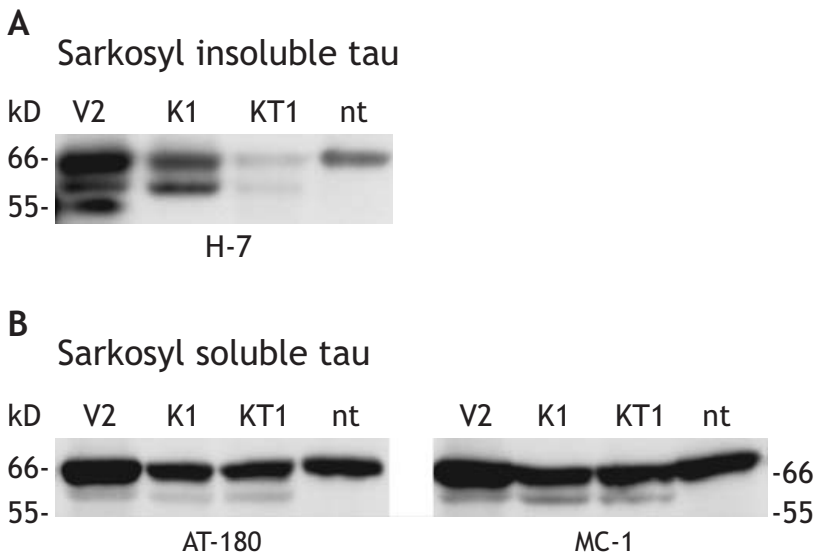

Figure 3. Immunoblotting of sarkosyl insoluble and soluble tau derived from the brains of the V2, K1 and KT1 transgenic lines
(A) Sarkosyl-insoluble material from brains of K1, KT1, V2 and non-transgenic (nt) mice were examined with the human-tau specific H-7 antibody. (B) Immunoreactivity of sarkosyl-soluble tau, isolated from brains of K1, KT1, V2 and nt mice was observed with the phosphorylation-dependent anti-tau antibody AT180 and the confirmation-dependent anti-tau antibody MC-1. All animals were aged up to 24 months.

Discussion

In order to study the functional effects of human *tau* mutations and to elucidate the molecular and cellular pathogenic mechanisms, transgenic mouse models can be very useful tools. Here we describe the generation and characterization of three transgenic mouse lines (V2, K1 and KT1) expressing the longest human tau isoform with different mutations ($MAPT^{\Delta K280}$ and $MAPT^{G272V}$) and under the control of two different neuronal-specific promoters (NSE and mThy-1). By light microscopy, expression of human tau was shown in neuronal cells. General distribution throughout the brain was observed in V2 and K1 lines, whereas the KT1 line also showed elevated expression levels in hippocampus and cerebellum. Furthermore, in the V2 and K1 lines (NSE promoter), human tau is only expressed in fully differentiated neurons, starting from postnatal day 1, while in the KT1 line (mThy-1 promoter) tau expression starts in the embryological phase.

Limited accumulation of hyperphosphorylated tau was detected by AT-8 staining and this was restricted to specific areas of the brain (fornix fimbriae, brain stem and white matter of the cortex). Anti-tau antibody AT8 detected immunoreactive neurons, demonstrating the presence of early changes in tau phosphorylation; however, other conformation- and phosphorylation-dependent anti-tau antibodies showed no specific staining (MC1, AT180 and PHF) suggesting that mature tau pathology had not fully developed. No filaments could

be detected by electronmicroscopy. Biochemically, these mice showed an age-dependent increase in sarkosyl-insoluble tau protein, consistent with a pathological accumulation of tau. Compared to the JNPL3 tau transgenic mice, that show sarkosyl-insoluble tau as early as three months of age (Lewis *et al.*, 2000), the sarkosyl insoluble fraction in our aged mice was only detectable from 1-year-old mice onwards and did not show a shift in molecular weight. The sarkosyl-soluble tau protein was immunoreactive with AT180 and MC-1, respectively a phosphorylation- and conformation-dependent anti-tau antibody. Staining with AT8 and PHF showed no specific signal. The immunohistochemistry and the biochemical experiments showed tau immunoreactivity with different antibodies. This observation may well reflect relative differences in the detection limits of the individual antibodies on Western blotting and immunohistochemistry given that the level of accumulated hyperphosphorylated tau was low in each of these mouse lines. Another possibility could be that hyperphosporylated tau accumulated in the insoluble fraction, instead of in the soluble fraction, which was examined.

Although the observed pathology in these mice is very limited in general, the pattern of the pathological changes, as detected with light microscopy, is partly comparable with the situation in human patients with FTDP-17, especially in the mediotemporal lobe and endplate of the hippocampus. In FTDP-17 the initial pathological changes are mainly located in the frontal and/or temporal cortex and the hippocampal area. Several other mouse models described in the literature showed high expression of the mutated human tau protein and also pathological changes in spinal cord and cerebellum. The neuropathology, motor and behavioural problems occurring in these previous models do not resemble FTDP-17, but do overlap with a PSP-like disorder (Allen *et al.*, 2002; Götz *et al.*, 2001b; Lewis *et al.*, 2000).

Examination of motor and neurological functions in the mice showed no abnormalities. However, experimental paradigms testing memory and attention, which might be expected to be disrupted given the nature of the pathology in this were not performed. In contrast to other previously described transgenic mice, where an obvious motor phenotype prevents simple analysis of cognitive function, this mice are suitable for further testing and detailed behavioral characterization. In an attempt to explain the lack of severe pathology in our mice, compared to those described in literature, the transgenic mice were backcrossed onto different genetic backgrounds. However, the change to another genetic background (C57Bl6, 129SV, CBA, Balb/c) seems to show no different result in either behavioural or immunohistochemical and biochemical experiments (data not shown).

The transgenic mouse models for mutant human tau described in the literature until now all differ in the use of genetic background or strain, promoter, tau isoform, mutation or exact construct and are maintained in different laboratories under different conditions (see Table 1). There appears to be a tendency that tau isoforms including the two amino-terminal exons 2 and 3 (2N) have a reduced amount of pathology compared to the isoforms that lack those exons (0N). Therefore, it is possible that higher expression levels are required to form pathological changes with this isoform. Furthermore, the choice of promoter is important, the best example being the PrP-P301L mice with a severe motor phenotype (Lewis *et al.*, 2000). It is possible that with the PrP promoter very high levels of expression in specific

but limited neuronal populations (e.g. motor neurons) results in the observed pathological changes. This may explain why overall levels of expression reported in tau transgenic mice using the MoPrP promoter are similar to, or lower than the lines described here, generated with the NSE and mThy1 promoters. Another difference between the individual mouse models is the presence or absence of the intron sequence between exons 13 and 14 in the construct. This intron sequence is present in the mRNA and known to be involved in the stabilization of the mRNA and the targeting of tau into the axonal terminals. To rule out the effects of differences between individual laboratories, it would be helpful to re-evaluate and compare all transgenic lines in one laboratory.

Table 1: Overview of different transgenic mice from the literature, all carrying the mutated human tau protein

Tau isoform	*Tau* mutation	Presence of intron 13 and/or exon 14	Promoter	Genetic mouse strain	Reference
4R2N	ΔK280; G272V	present	mThy-1 / NSE	FBV/N	This manuscript
4R0N	P301L	present	PrP	C57Bl6/DBA/SW	(Lewis *et al.*, 2000)
4R2N	P301L	not present	mThy-1	C57Bl6/DBA	(Götz *et al.*, 2001a)
4R2N	G272V	not present	PrP-TA*	C57BL6	(Götz *et al.*, 2001b)
4R2N	V337M	not present	PDGF-β**	C57BL6	(Tanemura *et al.*, 2001)
4R2N	G272V/V337M/ R406W	?	mThy-1	C57BL6	(Lim *et al.*, 2001)
4R0N	P301S	not present	mThy-1	C57BL6	(Allen *et al.*, 2002)
4R0N	R406W	not present	CaMK II	C57BL6	(Tatebayashi *et al.*, 2002)

* TA: tetracyclin-dependent transactivator (TA) system
**PGDF: platelet derived growth factor

Intriguingly, the effects of the ΔK280 mutation can be explained by two possible mechanisms. A large effect on splicing in favor of the 3R isoforms is observed, but also the effect of the mutated protein on microtubule binding is striking. Because of the lack of patient material, model systems are a very important resource to elucidate the molecular pathway of this mutation. The only identified patient with the ΔK280 mutation had no parkinsonian symptoms, which are often found in patients with mutations affecting the ratio of tau 3R and 4R isoforms, suggesting that this mutation fits in the group of missense mutations in exon 10. Furthermore, until now, the only cellular model with detectable tau aggregates by stable overexpression of a single pathogenic mutation is with the ΔK280 mutation (Vogelsberg-Ragaglia *et al.*, 2000), indicating the large effect of the mutated protein. In the two different MAPT$^{\Delta K280}$ mouse lines presented here, overexpressing the same tau isoform as in the cellular model system, only small amounts of pathology could be detected. However, when compared with the V2 line (expressing G272V tau) and several other mouse lines generated at the same time (data not shown), the amount of pathology is similar. As a result we cannot yet determine if expression

of ΔK280 mutant tau in vivo is sufficient to result in a robust tauopathy as is predicted by the effects of this mutation on tau microtubule binding.

Although transgenic mouse studies of tauopathy generally have major limitations because of the use of a specific heterologous promoter, the requirement of overexpression and the expression of only a single or subset of tau isoforms, they can provide invaluable information regarding the pathophysiological mechanisms of neurodegeneration. Several of the tau transgenic mouse models described to date show that robust tau pathology and related neurodegeneration can be induced (Allen *et al.*, 2002; Götz *et al.*, 2001b; Lewis *et al.*, 2000). These mice can be used in further studies regarding the tau aggregation process and neurodegenerative mechanisms. The mouse model we have generated shows expression of human mutated tau protein throughout the brain, due to the choice of the brain specific promoters. The pathology is partly restricted to areas of the brain that are also affected in humans with FTDP-17, like the hippocampus and the mediotemporal cortex. The limited amount of neuropathology in the transgenic mice, compared to the human disease, may be explained by different neuronal vulnerability in mice and humans, or by the expression of a single isoform of human mutated tau on a wild-type mouse tau background. Additional modifying factors, either genetic or environmental, which are absent in the mice but present in the FTDP-17 patients, might also explain this difference.

In conclusion, this mouse model can be used in the future to study in detail the initial stages of tau pathogenesis in FTDP-17 and to search for modifying factors. Furthermore, this mouse model is suitable for detailed characterization of memory or attention deficits by behavioural experiments such as the Morris water maze.

Acknowledgements

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Chapter 7

Accumulation of proteolytic tau fragments in transgenic mice are inducible in vitro by calpain

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In preparation

Abstract

Mutations in the microtubule-associated protein tau (MAPT) gene are associated with frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). Here we describe the characterization of accumulating proteolytic tau fragments observed in transgenic P301L mutated tau mice. The bands are comparable in size with fragments observed in FTDP-17 post-mortem brain material with the same P301L mutation, and are not dependent on the age or progression of disease symptoms in the mice. The proteolytic fragments are composed of a large part of the tau protein, only lacking the carboxyterminus. Furthermore, we demonstrate that *in vitro* activation of endogenous mouse calpain by incubation with Ca^{2+} results in an increased production of the proteolytic fragments. This production is inhibited when specific calpain inhibitors are added to the reaction. Alteration of the proteolytic processing could play an important role in the pathogenesis of not only FTDP-17, but also other neurodegenerative disorders.

Introduction

Tauopathies, a group of neurodegenerative disorders, are characterized by filamentous tau aggregates (Ingram & Spillantini, 2002; Rosso & van Swieten, 2002). The finding of *tau* gene mutations in frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) demonstrated that tau dysfunction can directly result in neurodegeneration (Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998). The tau protein functions in regulating the dynamic stability of the neuronal cytoskeleton, axonal outgrowth and transport, through its ability to bind to tubulin and promote microtubule assembly. In the adult human brain six tau isoforms, ranging from 352 to 441 amino acids are produced as a result of alternative mRNA splicing of exons 2, 3 and 10 (Ingram & Spillantini, 2002; Rosso & van Swieten, 2002). The interaction between tau and microtubules is mediated by three or four imperfect repeats in the carboxy terminal part of the protein. The alternative splicing of exon 10 results in isoforms with three of four of these microtubule-binding domains (Lee *et al.*, 1989).

Mutations identified in the tau gene are localized both in coding and non-coding sequences of the gene. The intronic mutations are located in the intron following exon 10, and influence the alternative splicing of this exon. Functionally, this leads to a change in the ratio of three and four repeat tau isoforms (Ingram & Spillantini, 2002; Rosso & van Swieten, 2002). The coding mutations (missense and small deletions of one amino acid) are located in the microtubule repeat domain and likely to influence the binding to tubulin. *In vitro* experiments showed that the missense mutations in the *tau* gene reduce the ability of the tau protein to bind to the microtubuli, and some also enhance the heparin-induced assembly of tau into filaments (Ingram & Spillantini, 2002; Rosso & van Swieten, 2002). By ongoing identification and characterization of novel *tau* gene mutations in patients, patient material was extensively studied by immunohistochemical and biochemical methods, and *in vitro* microtubule binding and filament formation assays were performed (Grover *et al.*, 2003; Rosso *et al.*, 2002; Van Herpen *et al.*, 2003). Strikingly, the effect of the *in vitro* studies did not always correlate with the clinical and pathological observations (Van Herpen *et al.*, 2003).

This raised the question which other mechanisms could be involved in the neurodegenerative process in patients. Studies with a P301L mutation-specific antibody have shown that tau deposits in P301L brains consist mainly of mutant P301L protein, and that mutant tau is dramatically depleted in the cytoplasmic fraction (Rizzu *et al.*, 2000). Furthermore, an increase in tau-immunoreactive cleavage products was observed in the cytoplasmic fraction with the P301L specific antibody, suggesting that an inappropriate digestion of the mutated protein plays an important role in the pathogenesis of FTDP-17 (Rizzu *et al.*, 2000). In addition, it was recently shown that different amino-terminally cleaved tau fragments are observed in the sarkosyl-insoluble brain extracts of patients with two different tauopathies, progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) (Arai *et al.*, 2004).

Interestingly, similar tau-immunoreactive cleavage products were observed in the cytoplasmic fraction of the P301L transgenic mice (JNPL3) described by Lewis *et al* (Lewis *et al.*, 2000). These mice express the most common human P301L *tau* mutation, under the control of the mouse prion protein promoter (MoPrP). The transgenic animals developed neurofibrillary tangles (NFT's) and Pick body-like lesions in many regions of the brain, starting at the age of 4.5 months for homozygous animals and at the age of 6.5 months for heterozygous animals (Lewis *et al.*, 2000).

Here, we describe the detailed characterization of these tau-immunoreactive bands in the transgenic P301L tau mice. We show that the size of these bands (35 and 40 kDa) is comparable with those found in human patients. Furthermore, we demonstrate that the cleavage bands are present as early as in newborn animals, and do not increase with the age of the mice or the development of pathological symptoms. Furthermore, we demonstrate that *in vitro*, the intensity of the cleavage products can be increased by activating calpain and inhibited by specific calpain inhibitors.

Materials and Methods

Transgenic mice

JNPL3 (P301L) mice generated by Lewis et al. were used for all experiments. The transgenic tau construct consist of tau cDNA exons 1, 4-5, 7, 9-13, intron 13 and exon 14 (4R0N) under the control of the MoPrP promoter as described previously (Lewis *et al.*, 2000). The animals were intercrossed with FVB/N to establish lines in our laboratory. All experiments were performed on heterozygous transgenic animals from the F2 and F3 generation.

Antibodies

Phosphorylation-independent anti-tau antibodies Tau14 (Zymed, San Fransisco, USA; amino acids 83-120 (in the numbering of the 441 amino acid isoform of human tau), used in a dilution of 1:2000 for immunoblotting) (Kosik *et al.*, 1988), H-7 (Innogenetics, Belgium; amino acids 159-163, 1:2000) and T46 (Zymed, San Fransisco, USA; amino acids 404-441) were used to detect human tau.(Mercken *et al.*, 1992) BR133 (directed against the aminoterminus of tau; 1:1000) and BR134 (directed against the carboxy-terminus of tau; 1:2000) (a gift from Dr. M. Goedert, Medical Research Council, Cambridge, UK) recognize both human and mouse tau and are phosphorylation-independent (Goedert *et al.*, 1989a). Polyclonal

antibodies tau-P301L and tau-P301 (directed against the P301L mutation and its normal counterpart; amino acids 291-305; 1:1000) are phosphorylation-independent (Rizzu *et al.*, 2000). Phosphoryalation-dependent anti-tau antibodies AT8 (Innogenetics, 1:100) and PHF1 (a gift from Dr. P. Davies, Albert Einstein College of Medicine, Bronx, USA, 1:1000) were used to detect tau phosphorylated at sites S202/T205 and S396/S404, respectively (Buee-Scherrer *et al.*, 1996; Goedert *et al.*, 1994).

Gel electrophoresis and immunoblotting

Homogenates of whole brains from transgenic mice (aged up to 12 months) and non-transgenic littermates were prepared by lysing and homogenizing one hemisphere with an automated homogenizer in Hepes-buffer (10 mM Hepes, 0.45% Triton-X-100, 0.05% Tween 20, 5 mM $MgCL_2$ and 300 mM KCl). The homogenized extracts were centrifuged immediately at 13.000 g for 20 minutes and the supernatants were directly used for SDS-PAGE. Sarkosyl insoluble and soluble tau was extracted from human brain and dephosphorylated as previously described (Goedert *et al.*, 1992b). Samples were run on 12% SDS-PAGE and eletrophoretically transferred to a nitrocellulose membrane (Scheicher & Schuell, Dassel, Germany). Residual protein-binding sites were blocked by incubation in 5% milk powder in PBS containing 0.1% Tween 20 for 1 h at room temperature. The first and secondary antibodies were also incubated for 1 hr at room temperature in 5% milk powder in PBS containing 0.1% Tween 20. After incubation with the appropriate secondary antibody, conjugated with horseradish peroxidase, the reaction products were visualized by using the ECL kit (Amersham Pharmacia Biotech).

Calpain induction of brain homogenates

Fresh mouse brain lysates (without adding any protease inhibitors to the homogenization buffer) were used to activate endogenous mouse calpain activity by using Ca^{2+} as described previously (Lee *et al.*, 2000). Ca^{2+} was added in different end concentrations (1 mM – 1M) to the mouse brain homogenates and incubated for 1 hr at 4 °C. Protease Inhibitor Cocktail (PRIC) tablets (Roche, Germany; end concentration according to specification sheet), calpeptin (end concentration 2µM) and calpain inhibitor II (end concentration 5µM) (both from Calbiochem, USA) were used in separate experiments to specifically inhibit the Ca^{2+}-dependent calpain activity.

Results

Initially, two bands, immunoreactive with anti-tau antibodies and running below the full-length tau band in the transgenic JNPL3 mice were observed. The estimated size of the bands was 35 and 40 kDa, with the full-length tau band (4R0N) running around 50 kDa. Following the observation in human brain (Rizzu *et al.*, 2000), we hypothesized that due to impaired degradation of mutated tau protein these proteolytic fragments accumulated. We compared the sizes of the observed bands in the transgenic mice with the bands observed in studies with brain material of FTDP-17 patients carrying the P301L mutation (Rizzu *et al.*, 2000). Because of the observed similarity between the bands detected in the mice and the accumulating bands in FTDP-17 patients, we started a detailed characterization.

Dephosphorylated sarkosyl-soluble tau from the frontal cortex and whole brain homogenates from transgenic JNPL3 stained with the H-7 anti-tau antibody showed comparable heights for

the bands running below full-length tau (Figure 1A). After dephosphorylation, we observed no difference in the running pattern of the bands, suggesting that the proteolytic fragments are not hyperphosphorylated (data not shown). Furthermore, the proteolytic fragments stained only with the P301L antibody, and not with the P301 antibody, indicating that the fragments are composed only of mutated human tau protein, and no or limited amounts of endogenous mouse tau is included in the fragments (data not shown).

In animals of different ages (from 0 to 12 months) we studied the presence and intensity of the two proteolytic fragments. We observed that the fragments were already present in embryonic brain (E19), and the levels of intensity did not increase with the age of the animals. The Mo-PrP promoter is turned on at embryonic day 11 (Baybutt & Manson, 1997), but we did not examine younger embryos. In non-transgenic animals no proteolytic tau fragments could be detected (Figure 1B). In heterozygous animals, the first behavioral and motor problems start around 6.5 months, and the first pathological signs are observed around 4 months of age. It seemed that the occurrence of these bands did not correlate with the pathological or behavioral symptoms in the mice.

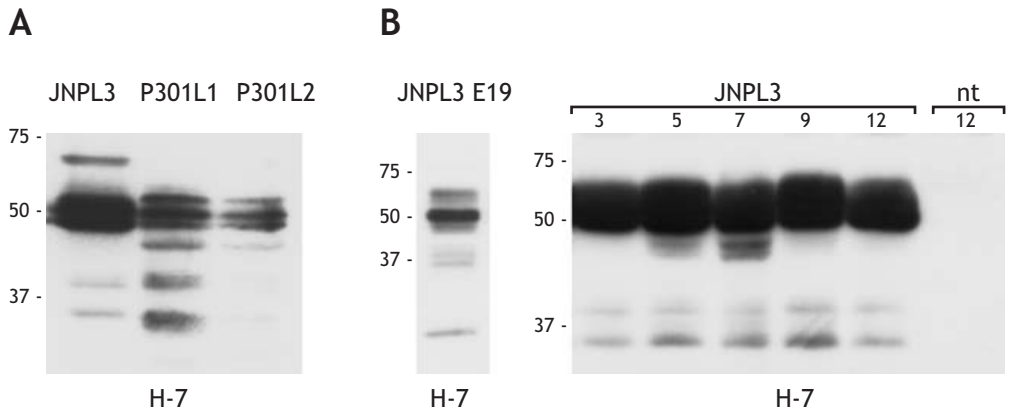
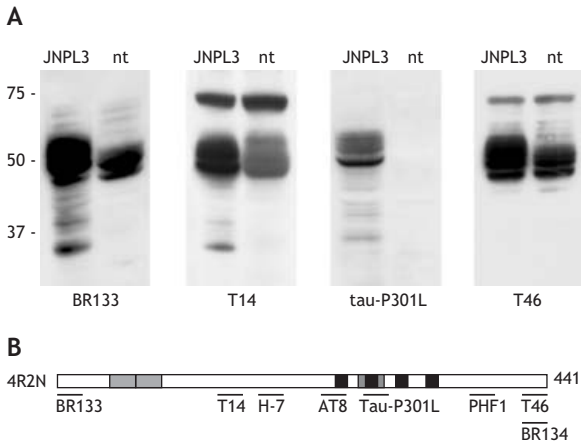


Figure 1. Immunoblot of proteolytic fragments in isolated brain material of FTDp17 patients and JNPL3 mice. (A) Immunoblot of whole brain homogenate of a 4-months old JNPL3 mouse and sarkosyl-soluble tau from the frontal cortex of two FTDP-17 patients carrying the P301L mutation (P301L1 and P301L2), using the phosphorylation-independent anti-tau antibody H-7. (B) Immunoblot of whole brain homogenates of JNPL3 mice of different ages, embryonic day 19, and 3, 5, 7, 9 and 12 months old, together with a 12-months old non-transgenic (nt) littermate. The phosphorylation-independent anti-tau antibody H-7 was used for immunodetection.

Several different tau antibodies, phosphorylation dependent and independent, were used to define the borders of the proteolytic fragments. The fragments were immunoreactive with almost all tested antibodies, except for the C-terminal and BR134 antibodies, recognizing the carboxy-terminus of the protein (Figure 2A,C). The sizes of the fragments and the observed immunoreactivity in the biochemical experiments do not correlate. According to the scheme presented in figure 2B, the part of tau that is present in the fragments suggests that these bands should be a mixture of several different fragments. This is in accordance with the immunoblotting results, showing not always a single straight band.



C

antibody	epitope (amino acids)	immunoreactive
BR133	1-16	+
T14	141-149	+
H-7	159-163	+
AT8	Ser 202 + Thr 205	+
Tau-P301L	291-305	+
PHF1	Ser 396 (+ Thr 404)	+
T46	404-441	-
BR134	428-441	-

Figure 2. Biochemical characterization of the proteolytic tau fragments in JNPL3 mice.
(A) Immunoblots of 4-months old JNPL3 and non-transgenic (nt) littermates with different anti-tau antibodies. The proteolytic tau fragments are detected with BR133, T14 and tau-P301L antibodies,but not with the antibody directed to the C-terminus. (B) Schematic overview of the longest tau isoform of 441 amino acids (exon 2, 3 and 10 are indicated with gray boxes, and the microtubule binding repeats with black boxes). The epitopes of all anti-tau antibodies tested are indicated in the scheme. (C) Overview of antibodies, epitopes and results of biochemical analysis of the proteolytic tau fragments in the JNPL3 mice.

To investigate the role of calpain in the production of the proteolytic fragments, we activated endogenous mouse calpain *in vitro*. Incubation of fresh mouse brain homogenates with various concentrations of Ca^{2+} showed increasing amounts of the proteolytic fragments compared to untreated samples. The amount of fragment increased in ratio to full-length tau and also the number of bands increased (Figure 3). Incubation with excessive concentration

of Ca^{2+} leads to the complete degradation of the tau protein. By addition of PRIC (containing several calpain inhibitors) or specific calpain inhibitors (calpeptin and calpain inhibitor II) to the mixture of fresh mouse brain homogenates and Ca^{2+}, the production of the proteolytic fragments is partially blocked (approximately 70%) (Figure 3).

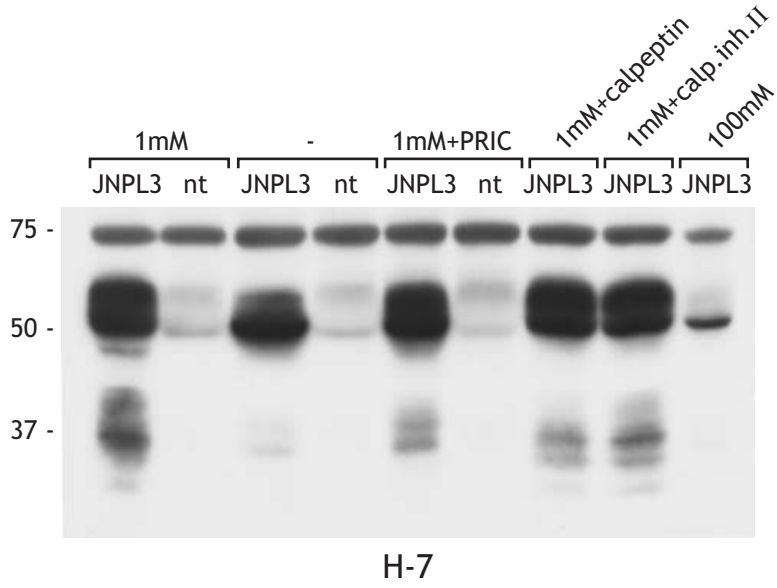


Figure 3. Ca^{2+} is responsible for increased production of the proteolytic tau fragments and specific calpain inhibitors inhibit this production
Incubation of fresh whole brain homogenates of 4-months old JNPL3 mice and non-transgenic littermates (nt) with 1 mM Ca^{2+} for 1 hour shows an increased production of the proteolytic tau fragments compared to the untreated homogenates. Combined incubation with 1 mM Ca^{2+} and PRIC (protease inhibitor cocktail), 2µM calpeptin or 5µM calpain inhibitor II showed a decline in production of the fragments. Incubation with 100 mM Ca^{2+} showed enhanced tau degradation. The phosphorylation-independent anti-tau antibody H-7 was used for immunodetection.

Discussion

Here we describe that the proteolytic fragments observed in the sarkosyl soluble protein fraction of brain material with FTDP-17 patients carrying the P301L mutation are also present in tau transgenic mice (JNPL3), expressing P301L mutated tau under the control of the mouse PrP promoter (Lewis *et al.*, 2000). In patient material it was shown that these proteolytic fragments are always present, since they could also be detected with the antibody directed against the wild-type tau, but accumulate when composed of mutant tau (Rizzu *et al.*, 2000). The JNPL3 mice allowed us to study the origin of these proteolytic fragments and can give insight which degradation system is impaired in patients. Although there are differences between the human sample (all six tau isoforms and a mixture of mutated and normal tau present) and the transgenic mouse line (only the 4R0N mutated tau isoform present), the proteolytic fragments have comparable molecular weights. Compared to full-length tau,

more proteolytic fragments seemed to be present in the transgenic mice than in the human patient material, and also more fragments with intermediate sizes were observed.

Furthermore, we showed that the formation of the proteolytic fragments is independent of the behavioral or pathological changes in the transgenic mice, but were already present in late stage embryonic brain and did not increase with the age of the mice. It is currently unknown if the early presence of these fragments can be explained by the overexpression of mutated tau protein in this mouse model, or is actually representing the human situation. In addition however, we were not able to show the presence of these proteolytic fragments in other transgenic mice lines carrying the same P301L mutation, but with a much milder phenotype. It would be interesting, although difficult to realize, to examine the presence of these proteolytic fragments before disease onset in FTDP-17 patients.

Since these fragments are preferentially formed from mutated tau protein (Rizzu *et al.*, 2000), the degradation of mutant tau protein is probably impaired before the onset of the neurodegeneration and could be an important feature in the onset of FTDP-17. The intermediate fragments that are formed and accumulate in the cells, can cause a dysfunction in different ways. The fragments themselves can be toxic for the cells, as suggested by the experiments of Fasulo *et al*. who showed that a tau fragment, including the proline-rich middle region and the microtubule binding domains (amino acids 151-391), induced apoptosis when overexpressed in COS cells. Essential to obtain this effect is the absence of the carboxyterminal tail of the tau protein in the construct (Fasulo *et al.*, 2000). This is in accordance with our observations that the carboxyterminal part of the tau protein is missing in the proteolytic fragments as shown in figure 2. The fragments could also act as a seed for the formation of aggregates in the cell. A consequence of this hypothesis is that although the first fibrils are formed during early life, the clinical features of the disease appear only after decades of slow accumulation, making this hypothesis less likely. Recently it has been observed that different amino terminally cleaved tau fragments are present in the sarkosyl-insoluble fraction in patients with PSP and CBD (Arai *et al.*, 2004). However, the fragments described in human FTDP-17 patients with the tau P301L mutation and the proteolytic fragments described here are mainly observed in the sarkosyl-soluble, cytoplasmic fraction and not detectable in the sarkosyl-insoluble fraction (Rizzu *et al.*, 2000).

The scheme presented in figure 2B shows that a large part of tau is present in the proteolytic bands. The exact composition of the proteolytic fragments could not be determined from the immunostainings with the panel of anti-tau antibodies, and probably composes of mixture of tau fragments. Up until now, we failed to obtain the exact composition of the bands by immunoprecipitating experiments and subsequent Mass Spectrometry analysis due to technical difficulties.

Since inappropriate apoptotic cell death is known to be involved in several neurodegenerative disorders (Johnson *et al.*, 1997), increasing effort has been put in experiments related to degradation pathways. *In vivo* experiments in apoptotic neuronal cells showed tau to be susceptible to both calpain and caspase degradation, resulting in the generation of multiple fragments. The appearance of these fragments is inhibited by both calpain and caspase

inhibitors, suggesting that tau is a substrate for these proteases during apoptosis (Canu *et al.*, 1998). The protein degradation systems that can be responsible for tau metabolism are widespread and include caspases, calpains, the proteasome and the lysosomal system (David *et al.*, 2002). One study compared the degradation by calpain in wild type and some FTDP-17 mutated tau proteins. It has been reported that using *in vitro* studies, mutant V337M and R406W tau proteins were less susceptible than mutant P301L or wild type tau to degradation by calpain (Yen *et al.*, 1999). Interestingly, in this experiment no effect was found for the P301L mutation, while the proteolytic fragments described here are found in animals and patients with this particular mutation.

We demonstrated that in fresh mouse brain homogenates, 1 mM of Ca^{2+} efficiently increased the formation of these proteolytic fragments. To identify the protease activated by Ca^{2+}, we tested specific calpain inhibitors, which showed a partial reduction in the formation of the proteolytic fragments, indicating that calpain could be responsible for the formation of these bands. However, these experiments are performed in an *in vitro* system, and should also be proved in an *in vivo* system, such as cultures of primary neuronal cells from these transgenic mice.

In conclusion, we characterized the proteolytic fragments observed in transgenic mutated P301L tau mice, and showed that the sizes corresponded to the bands found in human patients. *In vitro*, endogenous calpain incubation can produce these bands. In future experiments, the exact amino acid composition can be obtained and the possible effects of these fragments can be studied in transfected cells. Alteration of the proteolytic processing probably plays an important role in the pathogenesis of not only FTDP-17, but also other neurodegenerative disorders.

Acknowledgements

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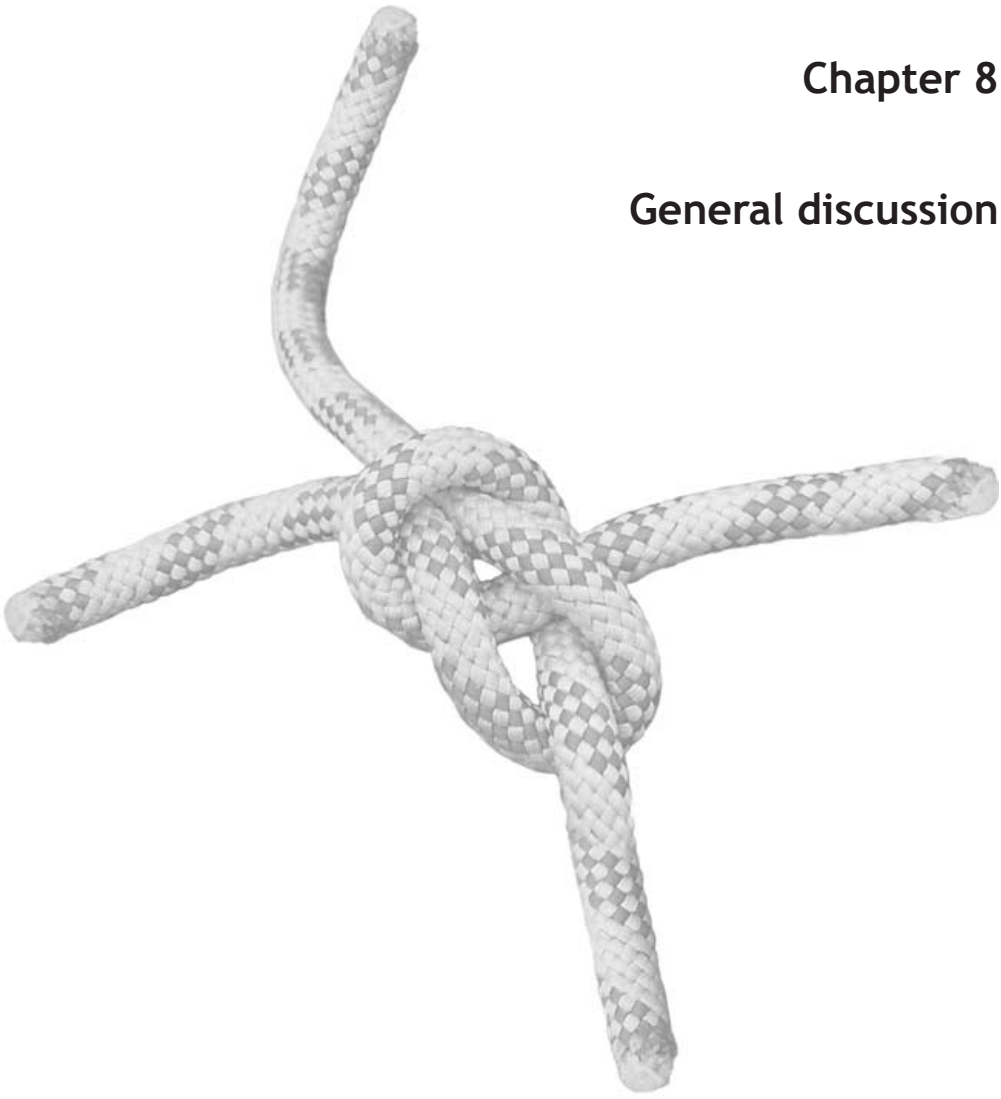


Chapter 8

General discussion

General discussion

The research in the field of frontotemporal dementia (FTD) has increased enormously in the last decade. First, after the definition of the clinical and neuropathological criteria by the Lund and Manchester groups in 1994, and even more after the identification of the first mutations in the tau gene in 1998, FTD stepped out of the shadow of the much more common Alzheimer's disease (Lund and Manchester Groups, 1994; Hutton et al., 1998). On a consensus conference in 1996 it was determined, based on clinical similarities between several FTD families with linkage to chromosome 17q21-22, to call this new entity frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Foster et al., 1997). The clinical description was almost identical to the original description of FTD by the Lund and Manchester groups in 1994, apart from the autosomal dominant inheritance pattern. The research described in this thesis, focused on FTDP-17, but parts of it can be extended to FTD in general.

8.1 Tau on the MAP

More than 25 years have passed since the microtubule associated protein (MAP) tau was first purified as a potent inducer of tubulin assembly into microtubules (Cleveland *et al.*, 1977). Although a lot of research has been performed on tau function and dysfunction since that time, tau has been slow to reveal its secrets. Aggregated tau protein is the hallmark of not only FTDP-17, but also of several other neurodegenerative disorders, known as tauopathies and distinguishable by the topographic distribution and the specific cell type of the inclusions (Chapter 1) (Lee *et al.*, 2001). The discovery of mutations in the *tau* gene in patients with FTDP-17 demonstrates that tau dysfunction alone is sufficient to result in a neurodegenerative disorder. Single mutations within the *tau* gene can give rise to a wide spectrum of clinical presentations and neuropathology, both within and between families, demonstrating that *tau* mutations are pleiotropic (Rizzu *et al.*, 1999; van Swieten *et al.*, 1999). Furthermore, the clinical and pathological heterogeneity associated with *tau* mutations has been expanded beyond FTDP-17 and now includes PSP, CBD and PiD (Rosso *et al.*, 2002; Van Herpen *et al.*, 2003).

Tau mutations account for only 9 to 40% of all cases of familial FTD. Furthermore, only about 40% of FTD patients have a positive family history of dementia, making the remaining 60% sporadic cases (Rosso *et al.*, 2003b). Further calculation suggests that mutations in the *tau* gene are the direct cause of FTD 4-16% of the total FTD population (familial and sporadic cases together). In the Dutch FTD population of 245 patients, in 32% of patients with a positive family history a *tau* mutation could be detected. Other studies regarding *tau* mutation analysis are summarized in table 1. These results justify screening for *tau* mutations in familial FTD cases, as in FTD patients in which autopsy revealed predominant tau-positive neuropathology without the presence of widespread β-amyloid or α-synuclein pathology.

Table 1: Overview of all studies estimating the contribution of tau mutations in FTD

Study	Patient groups	Frequencies of *tau* mutations
Houlden *et al.*, 1999	Stringently diagnosed Lund/Manchester criteria (22 patients) Clinicobasal referral series (82 patients) Community based dementia series (>400 patients)	13.6% 3.6% < 0.2%
Rizzu *et al.*, 1999	FTD (90 patients) FTD + Positive family history (37 patients)	17.8% 43%
Poorkaj *et al.*, 2001	Non AD/nonvascular dementia (101 patients) overall Familial cases	5.9% 10.5%
Sobrido *et al.*, 2003	FTD (Lund/Manchester criteria) (48 patients) familial cases	11%
Binetti *et al.*, 2003	38 Italian FTD patients (38 patients) familial cases	7.6%
Rosso *et al.*, 2003a	FTD (Lund/Manchester criteria) (245 patients) FTD + Positive family history (105 patients)	14% 32%

Although normally *tau* mutations are associated with an autosomal dominant pattern of inheritance (Heutink, 2000; Rizzu *et al.*, 1999), two recent studies described an autosomal recessive mode of inheritance leading to a comparable neuropathological disorder (Nicholl *et al.*, 2003; Pastor *et al.*, 2001). Genetic heterogeneity still exists in FTDP-17 families, because a number of these families (including HFTDIII) show neither tau mutations nor tau neuropathology (Froelich *et al.*, 1997; Lendon *et al.*, 1998; Rosso *et al.*, 2001). It cannot be excluded that mutations in the *tau* gene have remained undetected, for example in regulatory elements in the promoter region, which may lead to neurodegeneration through a different mechanism. In my opinion, since these patients lack any tau pathology it is more likely that the disease in these families is caused by mutations in other genes. Considering the strong linkage data, candidate genes have to be located in the same region as the tau gene (Rademakers *et al.*, 2002; Rosso *et al.*, 2001). In the past few years several genes have been sequenced but until this point all with negative results. Although the gene for glial fibrillary acidic protein (GFAP) was sequenced in FTDP-17 patients in 1998 and no mutations were detected (Isaacs *et al.*, 1998), it is still an interesting candidate gene. Recently, new data regarding specific brain GFAP isoforms were revealed (Condorelli *et al.*, 1999; Nielsen *et al.*, 2002), and patients in HFTDIII show extraordinary pronounced immunostaining with anti-GFAP antibodies (Rosso *et al.*, 2001). In an attempt to isolate the main protein in the aggregates in patients of this family, GFAP was detected in the insoluble fraction (S. Rosso,

unpublished results). However, in general the presence of activated astrocytes is a non-specific phenomenon, just reflecting severe neuronal loss. Genetic heterogeneity is further emphasized by the identification of three additional loci (located on chromosomes 3 and two different loci on chromosome 9) by linkage analysis in FTD families with variable phenotypes but without tau pathology (Brown, 1998; Hosler *et al.*, 2000; Kovach *et al.*, 2001). One study however, showed the presence of tau pathology in the brains of patients with FTD linked to chromosome 3, suggesting a possible link between tau and the genetic defect present on chromosome 3 (Yancopoulou *et al.*, 2003).

8.2 Identification and implications of novel *tau* mutations

Currently, more than 30 different tau mutations have been identified in FTDP-17 patients and related disorders (Heutink, 2000; Lee *et al.*, 2001). Many papers have been published with a detailed description and characterization of the clinical, neuropathological and biochemical features of individual patients with different tau mutations. Continuing efforts to identify new mutations are important for both diagnostic and research purposes. A correct and early diagnosis is of great importance for the patient and the family in order to understand and accept the changes in behavior in the patient. Furthermore, a clinical prediction of the disease by presymptomatic DNA testing can then be offered to children and other family members of the patient. Despite the lack of therapeutic intervention available, at-risk individuals can consider predictive testing informative and helpful in making life-planning decisions and to seek relief from anxiety. However, from studies examining the impact of predictive testing in FTDP-17 and also in Huntington's disease it is known that only a small number (approximately 8%) of at-risk individuals to whom DNA testing was offered, requested genetic testing (Steinbart *et al.*, 2001; Van Deerlin *et al.*, 2003). Professional guidance is crucial in this process.

From a scientific point of view, the identification of new *tau* mutations can give more insight into the different mechanisms through which tau dysfunction can lead to neurodegeneration. The identification of novel *tau* mutations in the last few years has broadened the spectrum of neurodegenerative disorders in patients with specific *tau* mutations. A striking example is the recently described S352L mutation associated with apparent recessive inheritance and extensive tau neuropathology (Nicholl *et al.*, 2003). The presenting symptoms were respiratory hypoventilation, probably due to the involvement of medullary neurons. The heterozygous mutation carriers were healthy (Nicholl *et al.*, 2003). In this case not only the disease-spectrum was broadened, but also another mode of inheritance seemed to be involved.

The two novel mutations reported in chapters 3 (S320F) and 4 (L315R) of this thesis are the only two mutations found to date in exon 11 of the *tau* gene (Rosso *et al.*, 2002; Van Herpen *et al.*, 2003). The S320F mutation was found in a patient initially diagnosed with AD. Only after neuropathological examination showing a tauopathy without β-amyloid deposition, mutation analysis of the *tau* gene was performed. This example shows that other familial FTD cases with atypical presentation can be missed.

However, sequencing of the *tau* gene in a series of AD patients did not reveal any mutations, making the S320F patient probably an exception (Roks *et al.*, 1999).

The L315R mutation was identified during diagnostic *tau* sequencing in a patient with classical features of FTD. Both mutations in exon 11 are only 5 amino acids apart, but have a very different effect on the microtubule binding of the tau protein. The S320F mutation is located within the third microtubule-binding domain of the protein, and results in a 95% reduction in an *in vitro* microtubule-binding assay. The L315R mutation, located in the inter-repeat domain between microtubule binding repeats three and four, showed only a 25% reduction in microtubule assembly. An *in vitro* filament formation assay was only performed for the L315R mutated protein, but showed no significant difference between mutated and wild type tau protein.

Up until now pathogenic features of novel amino acid changes in the tau protein can only be determined by two previously mentioned *in vitro* assays, the microtubule binding assay and the filament formation assay, induced by heparin or other polyanions. The outcome of these assays differs for many mutations and the results described for the L315R mutation (chapter 4) showed that the correlation with the severity of the disease symptoms could be poor. Compared with other mutations the effect on microtubule binding of the L315R *tau* mutation is relatively small, in contradiction with the early onset of disease and the severe pathology observed in autopsied patients. The two described mutations in the fifth codon of exon 1 of the *tau* gene also showed a slight reduction in microtubule assembly (Hayashi *et al.*, 2002; Poorkaj *et al.*, 2002). Although it was recently suggested that the aminoterminal part of the tau protein is involved in tubulin polymerization (Gamblin *et al.*, 2003), the predictive value of this *in vitro* assay can be questioned. It is still unknown how the results of the *in vitro* measurements correlate with what happens *in vivo*. Furthermore, the determination of a specific amino acid change to a pathogenic mutation has large consequences. Increasing the knowledge of tau dysfunction and the molecular and cellular mechanisms involved in FTDP-17 would be helpful in the development of a better functional assay.

An interesting finding in the families with the L315R mutation, as described in chapter 4, is the nonpenetrance in an 82-years old mutation carrier with no signs of dementia. This reduced penetrance might explain the relatively small effect of the L315R mutation in the performed functional assays. Beforehand, incomplete penetrance was only suggested for three other *tau* mutations, but this was never established in a living subject (Janssen *et al.*, 2002; Murrell *et al.*, 1999; Pickering-Brown *et al.*, 2002; Rizzini *et al.*, 2000). The finding of incomplete penetrance underscores the importance of extensive evaluation of the family history of patients. The exact percentage of the incomplete penetrance is still unknown, as well as the eventual restriction to specific *tau* mutations. Until now it is a rare finding and more research is necessary to elucidate the matter. Follow-up examination, both clinically and neuropathologically, of the healthy mutation carrier would be extremely helpful. Further examination of the delicate matter of reduced penetrance requires good collaboration between clinicians, counselors and research laboratories. The question is whether this observation of nonpenetrance should lead to a different approach in the genetic counseling of patients and family. In the case of the L351R mutation the risk of developing a dementing

illness after the identification of a specific *tau* mutation can no longer be set at 100%. However, it is questionable whether this exceptional finding has to lead to a general change in counseling.
Notably, children of nonpenetrant individuals are not free of risk, as they may be penetrant and have a 50% change of inheriting the mutant allele.

Another remarkable observation in the brain of patients with the L315R mutation (see chapter 4) was that besides neuronal, also severe astrocytic tau pathology was detected. This astrocytic pathology, resembling tufted astrocytes observed in PSP, was unexpected because abundant tau inclusions in glial cells are normally associated with mutations that only affect 4R tau or that increase the relative amount of 4R tau (Heutink, 2000; Lee *et al.*, 2001; Rosso & van Swieten, 2002). Only for the L266V mutation in exon 9 of *tau* similar neuronal and astrocytic pathological findings have been described (Kobayashi *et al.*, 2003). There appeared to be an inverse relationship between the relative numbers of neuronal and glial tau inclusions, suggesting that the astrocytic inclusions may develop later than neuronal deposits or that they may have a longer survival time.

8.3 Mechanisms of neurodegeneration

Mutations in the *tau* gene or tau hyperphosphorylation were proposed to cause a functional loss of tau for interaction with microtubules. As a result, axonal microtubules would be destabilized and eventually depolymerize and fast axonal transport would be impaired, ultimately leading to degeneration of neurons. However, this 'loss of function' scenario seems unlikely to be the explanation for all pathological changes in FTDP-17 and other tauopathies, based on inactivation experiments of tau in cells and animals. First of all, experimental studies with *tau* knock-out mice did not reveal a severe phenotype and the mice lack major cytoskeletal abnormalities (Harada *et al.*, 1994; Ikegami *et al.*, 2000). These findings argue against an essential role of tau in the maintenance of axonal transport, although detailed examination of the knockout mouse showed that in cultured neurons lack of tau expression inhibited the formation of axons (Dawson *et al.*, 2001). In general, mutations in most dominant disorders result in a 'gain of function' of the mutated protein, and 'loss of function' due to a dominant mutation is a less likely explanation. Furthermore, the observation that mutations in the *tau* gene lead to a neurodegenerative disorder with an adult age at onset is more consistent with a slow accumulation of a toxic process over time.

Not only *tau* mutations, but also polymorphisms in *tau* (associated with an increased risk for the development of a tauopathy) can indirectly influence the dysfunction of the tau protein. Sporadic tauopathies, like PSP and CBD show a robust association with the H1/H1 haplotype, suggesting that variability in tau expression or tau splicing can contribute to the disease risk (Singleton *et al.*, 2004). Mutations are defined as rare base changes that often result in an amino acid change, or have otherwise been proven to have a pathogenic effect. Polymorphisms can be defined as more frequent observed changes in DNA sequences, usually not resulting in amino acid changes and often present in intronic or regulatory sequences.

The presence of more unbound tau protein in the cytoplasm of the cell can cause several problems by itself. Moreover, intronic *tau* mutations show that disturbance of the delicate balance of 3R and 4R tau isoforms, with different microtubule binding properties, can be enough to cause the disease pathology. Unbound cytoplasmatic tau is free to be hyperphosphorylated, and hyperphosphorylated tau is known to be more prone to form filamentous structures and aggregates. For a long time it was a common thought that abnormal phosphorylation of tau was responsible for its aggregation, but normal tau protein is phosphorylated in fetal and adult brain and does not aggregate or forms filamentous inclusions (Shahani & Brandt, 2002). Moreover, non-phosphorylated recombinant tau can form filamentous structures, when induced by heparin or other polyanions. This suggests that mechanisms other than phosphorylation are involved in tau aggregation. Hyperphosphorylation of tau may have a more indirect role by increasing the amount of free cytoplasmic tau protein in the cell. In studies with tau transgenic mice the formation of inclusions appeared to be dependent on the concentration and expression levels of tau, in favor of a 'toxic gain of function' scenario. This 'toxic gain of function' of mutated tau can be caused by a change of phosphorylation status of the unbound tau, aggregation of tau protein or deviated degradation and subsequently the formation of intermediate proteolytic fragments of mutated tau protein (see figure 1). These possibilities will be discussed later.

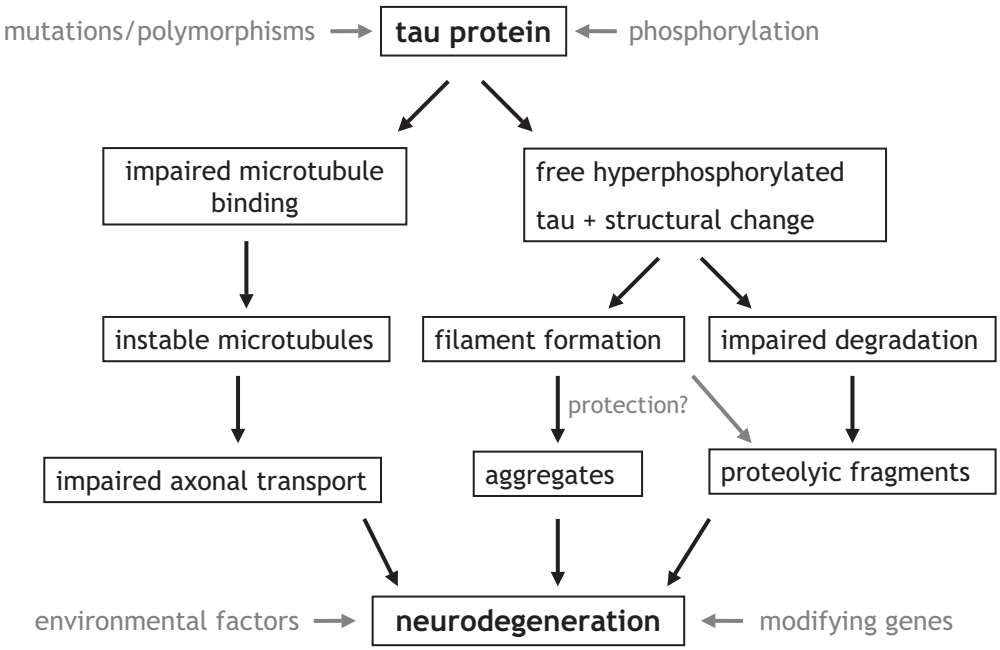


Figure 1: Schematic overview of putative mechanisms leading to neurodegeneration in FTDP-17 and other tauopathies.

8.4 Animal models for tau and FTDP-17

Developing cellular and animal models is very important to verify and analyze the role of tau protein in neurodegeneration and the functional interaction between tau and other disease-causing factors. In my opinion, a 'knock-in approach' will mimic the human situation in the best possible way, and in that way many disadvantages and the large variation of transgenic over-expressing models are evaded. Introducing an FTDP-17 *tau* mutation into the mouse *tau* locus via homologous recombination is suitable for mutations affecting 4R tau isoforms, taking into account the different splicing features of mouse *tau*. But will the expression levels be high enough to show any pathological symptoms within the live-span of the mouse and is the selective vulnerability of different neuronal sub-populations comparable in mice and men? The generation of a transgenic overexpressing model is less labor intensive, faster and the chance of phenotypic changes is much larger. Therefore, most researchers, including ourselves, focused on the generation of transgenic animal models overexpressing human tau protein. A major drawback of the transgenic models is that *tau* mutations are not expressed under the control of the endogenous tau promoter. Axonal targeting signals and mRNA stability sequences are not fully present in the transgenic constructs. In advance, the most promising model would have been a human BAC transgenic model. The entire human FTDP-17 mutant *tau* gene would be expressed in mice, preferable on a mouse tau knock-out background. Previous experiments with this system, without the use of any *tau* mutations did not show any pathology (Duff *et al.*, 2000). This result further emphasizes the above-described risks of the 'knock-in approach', with a high chance of determining no obvious phenotype because of the low mutant tau expression and the short life span of the mouse. Another interesting model for human tauopathies to generate and characterize would be an inducible transgenic mouse model. In this mouse model the reversible and irreversible stages of tau pathogenesis can be studied. In a conditional mouse model of Huntington's disease was shown that blockade of expression had led to improvement of behavioral symptoms and neuropathology (Yamamoto *et al.*, 2000).

Before mutations in the *tau* gene were identified, overexpression of wild-type human tau in mice was the first attempt to develop tau pathology and filaments in mice. Several different mouse models were generated, described in detail in chapter 5, but none of the transgenic models developed tau filaments. The neuropathology in this series of tau overexpressing mice was limited and partly mimicked AD. The data did show that overexpression of wild-type human tau isoforms is sufficient to cause nerve cell dysfunction (Götz, 2001a).

The identification of *tau* mutations in patients with FTDP-17 led to the generation of several transgenic mouse lines expressing human mutated tau isoforms, resulting in model systems with large differences in pathology and clinical symptoms. The first and most severe model system was the JNPL3 mouse, carrying a human 4R0N tau transgene with the P301L mutation under the control of the PrP promoter, showing severe motor and behavioral problems, gliosis, neuronal loss and the formation of NFT's (Lewis *et al.*, 2000). All mouse models described in chapter 5 differ in the use of tau construct (isoform, mutation), the choice of promoter and genetic background, the presence or absence of intron 13 and the resulting expression levels (due to copy number) of the transgene. In my opinion, many of the observed differences can

be explained by the above-mentioned features. The differences in tau construct (the presence or absence of the two amino-terminal inserts) and the use of a different promoter may explain why there is no overt phenotype in the mice of Götz *et al* (P301L mutated human tau, 4R2N isoform and mThy-1 promoter), compared to the mice of Lewis *et al* (P301L mutated human tau, 4R0N isoform and PrP promoter) (Götz *et al.*, 2001a; Lewis *et al.*, 2000). Although the expression levels are comparable, the expression patterns in particular cell types can vary. The two amino-terminal inserts have specific calpain recognition motifs, which may subject tau to a more rapid proteolysis. The presence or absence of the intron sequence between exons 13 and 14 in the construct is probably partly responsible for the observed differences. This intron sequence is normally present in the human *tau* mRNA and known to be involved in the stabilization of the mRNA and the targeting of tau into the axonal terminals. Interestingly, recent studies in the JNPL3 mice showed that backcrossing into another genetic background (C57Bl6) resulted in a severe delayed phenotype (J. Lewis, personal communication). However, experiments with the mouse models generated in our own laboratory (described in chapter 6) showed no obvious differences when the genetic background of the animals was changed. In conclusion, several different features together account for the observed phenotype in the different mouse models and it is hard to make a good comparison. Partly, this can be solved by re-examining all mouse models in one laboratory and by one neuropathologist, thereby ruling out the technical differences between individual laboratories and persons. Probably, each individual mouse model has specific features that can be used to study different aspects of FTDP-17 or tauopathies, for instance drug testing, studies of the onset of the disease and the search for modifying factors or genes. Interestingly, the mouse model described in chapter 6 (expressing *tau* G272V and ΔK280 mutations under the NSE and mThy-1 promoter) shows limited tau pathology, but is the only model described until now that partly mimics the affected areas of human patients with FTDP-17. However, one of the key characteristics of human tauopathies, the selective and massive neuronal loss, is so far not reproduced in one of the transgenic model systems. Murine neurons in general may be less susceptible to degeneration compared to human cells.

The last few years several simple animal models for mutant tau proteins were developed, including fruit fly, zebra fish and nematode (chapter 5). A great advantage of these simple models is the fast and efficient production of transgenic animals. A good example is the targeted expression of human tau protein in neurons in the fruit fly *Drosophila melanogaster* that produces characteristic FTDP-17 defects in expressing neurons (Jackson *et al.*, 2002; Wittmann *et al.*, 2001). Notably, the amount of neuropathology in these flies is severe, compared to many mouse models described above. The levels of mutant tau expression are probably more important in determining the amount of tau pathology then the life-span and short development-time of model animals compared to humans. Simple model systems allow a fast correlation between changes in the nervous system and behavioural defects, although they can be different then in human patients. Possible interacting proteins, or proteins involved in the disease mechanism can be screened and tested quickly in these model systems.

8.5 Searching for tau interacting proteins

To obtain more insight in the molecular pathways disturbed in FTDP-17 and other tauopathies, the yeast two-hybrid system can be used to identify proteins that interact with the tau protein. In this procedure the properties of the yeast transcription activator GAL4 are used, which harbors separable domains for DNA-binding (DBD) and transcriptional activation (AD) (Fields & Song, 1989). Two plasmids are constructed expressing different hybrid proteins, a 'bait' plasmid encoding the GAL4-DBD fused to the protein (or part of the protein) of interest, and a 'prey' plasmid encoding GAL4-AD fused to protein sequences of a constructed cDNA library. When the plasmids are co-transformed in specific yeast strain cells, reporter genes will be transcribed when the bait-proteins and prey-proteins interact. In an attempt to find new interacting proteins for the wild-type tau protein, in our laboratory we have screened a human brain library and a total *C. elegans* cDNA library. The *C. elegans* library was chosen to reduce the amount of false positive clones. From both libraries, in total more than 400.000 (human brain) and 200.000 (*C. elegans*) individual clones were tested, making use of different domains of wild-type tau protein. Unfortunately, until now we failed to identify any specific interacting protein. It should be noted however, that the protein-protein interactions in this conventional two-hybrid system take place in the nucleus of the yeast, without the possibility of posttranslational modifications while tau is normally expressed in the cytoplasm of the cell. Possibly, a newly developed two-hybrid system (Cytotrap), where protein-protein interactions take place in the cytoplasm of the yeast, is more suitable for experiments with the tau protein (Aronheim *et al.*, 1997).

In addition to our own results, in the literature only one study has been described where a putative tau interacting protein has been identified using the two-hybrid approach (Hoenicka *et al.*, 2002). Taking into account the time passed since the identification of the tau protein and the relatively common use of the two-hybrid system, more laboratories could have obtained negative, unpublished results. To my opinion, the Alu-derived amino acid sequence found in the single study described in literature (Hoenicka *et al.*, 2002) is far away from a novel tau-interacting protein. These sequences are very common, and the interaction has been obtained to the Alu sequence alone, without the specificity of a particular protein containing this Alu repeat sequence. The significance of proteins identified with the yeast two-hybrid system should be confirmed with other *in vitro* and *in vivo* systems, which has not been properly performed in this study. No conclusions regarding the interaction of this protein with tau can be drawn, before these control experiments are performed.

Although the system we used may not be suitable for screening with the tau protein, the yeast two-hybrid system has proved its usefulness in the understanding of other neurodegenerative disorders. For example, successful studies were performed with the DJ-1 protein, recently shown to be mutated in autosomal recessive forms of early-onset Parkinson's disease (Bonifati *et al.*, 2003; Takahashi *et al.*, 2001). In principle, interacting proteins identified with the yeast two-hybrid system, including them from which the significance is yet unknown, can still in the future be helpful in elucidating molecular and cellular pathways.

8.6 Tau degradation

A well-established observation in aging is the tendency of cells to accumulate abnormal or modified proteins. Frequently it was suggested that the functional decline and death of cells in aging is, in part, a consequence of an impaired proteolytic efficiency. Indeed, the efficiencies of the two principal pathways for protein turnover, the lysosome and proteasome, decline with age. Lysosomes and other organelles in non-dividing cells, like neurons, can saturate during aging and therefore their proper function can be reduced. Abnormal levels of protease activity can lead to the unwanted cleavage of key structural proteins unbalance of regulatory enzymes and signalling molecules, or the generation of cytotoxic proteolytic fragments. Calpain-dependent cleavage of certain substrates may also be altered by changes in the post-translational modifications of the substrate that arise as cells age.

Degradation of mutated tau protein was shown to be impaired, both in human patients (Rizzu *et al.*, 2000) and also in a transgenic mouse model (chapter 7). It can be postulated that these proteolytic tau fragments are constantly generated and efficiently removed. At a certain age, the intracellular degradation machinery slows down and the fragments may stay longer in the cytoplasm of the cell and reach sufficient levels to have toxic effects. These toxic effects can either be to seed the formation of aggregates, or interfere with other processes in the cell, such as axonal transport. It was shown that tau fragments, including the proline-rich middle region and the microtubule binding domains, could induce apoptosis when expressed in COS cells (Fasulo *et al.*, 1998). Tau is known to be susceptible to both calpain and caspase degradation (Canu *et al.*, 1998). Mutations in the *tau* gene can make the protein more resistant to calpain or caspase degradation in respect to the wild-type protein, and can cause enhanced accumulation of intermediate proteolytic fragments. In an *in vitro* assay was indeed reported that mutant tau proteins, including the P301L mutated tau, are more resistant to calpain degeneration compared to normal 4R tau (Yen *et al.*, 1999). Recently it was found that different amino terminally cleaved tau fragments were present in the sarkosyl-insoluble fraction in PSP and CBD (Arai *et al.*, 2004). However, the fragments described in human FTDP-17 patients with the tau P301L mutation and the proteolytic fragments described in chapter 7 were mainly observed in the sarkosyl-soluble, cytoplasmic fraction and not detectable in the sarkosyl-insoluble fraction (Rizzu *et al.*, 2000).

8.7 The role of aggregates

The formation of large intra-neuronal and -glial tau aggregates can act as a physical barrier to axonal transport and other essential neuronal functions or can have a direct toxic effect on cells. It is a matter of debate whether tau aggregates are the cause or the result of neurodegenerative processes, or even only a harmless epiphenomenon. In my opinion, in correlation with experiments described in this thesis and recent literature, the explanation that tau aggregates are the result of neurodegeneration is the most likely. Large immunohistochemical studies in brains of AD patients showed that tau neurofibrillary deposition closely mirrors the extent and spread of neurodegeneration, in that way ruling out the explanation of tau aggregates being a harmless epiphenomenon (Lee *et al.*, 2001).

Experiments with tau transgenic fruit flies showed that although the transgenic flies show all the signs of neuronal degeneration, including the adult onset, progressive pathology and enhanced toxicity of mutated protein, no tangles could be observed (Jackson *et al.*, 2002; Wittmann *et al.*, 2001). A tauopathy model of the nematode *C. elegans* showed that impaired locomotion occurs before the detection of insoluble tau or aggregates. Of course, it could be argued that flies or nematodes are not good models to study human tauopathies, but it is more likely that the neurodegenerative mechanism relies more on the defective, soluble tau. Aggregated tau is not essential for initial tau toxicity, and cells can protect themselves from this mutant, soluble tau by segregating them into aggregates, thereby increasing their own survival time.

Abnormal protein aggregation is observed in a large number of neurodegenerative disorders, and intracellular aggregates of tau protein are only one example (Lee *et al.*, 2001). Other examples are the extracellular accumulation of β-amyloid in the form of plaques in Alzheimer's disease, intracytoplasmic filamentous aggregates of α-synuclein in Lewy body disorders, ubiquitinated huntingtin aggregates in Huntington's diseases and prion proteins aggregates in spongiform encephalopathies (Goedert *et al.*, 1998; Prusiner, 1998; Trojanowski *et al.*, 1998). In all known neurodegenerative diseases in which inclusion bodies are involved, they are formed from proteins that are normally present and functioning in the brain. Whether the aggregation of these proteins is toxic or the loss of normal function is not clear. A possible common aetiological mechanism can be a decrease in protein degradation. Protein aggregation seems to inhibit the ubiquitin-proteosome system leading to a positive feed-back loop resulting in cell death (Bence *et al.*, 2001). Recently was found that DJ-1, a protein mutated in autosomal recessive early-onset parkinson's disease colocalized with tau inclusions in a diverse group of tauopathies, further extending the view that different neurodegenerative disorders may have a similar pathological mechanisms (Rizzu *et al.*, 2004). Because aggregation of proteins in the brain is such a common theme in neurodegenerative disorders, insight into the pathogenesis of any one of these disorders may have implications for our understanding of the mechanisms that underlie all these diseases.

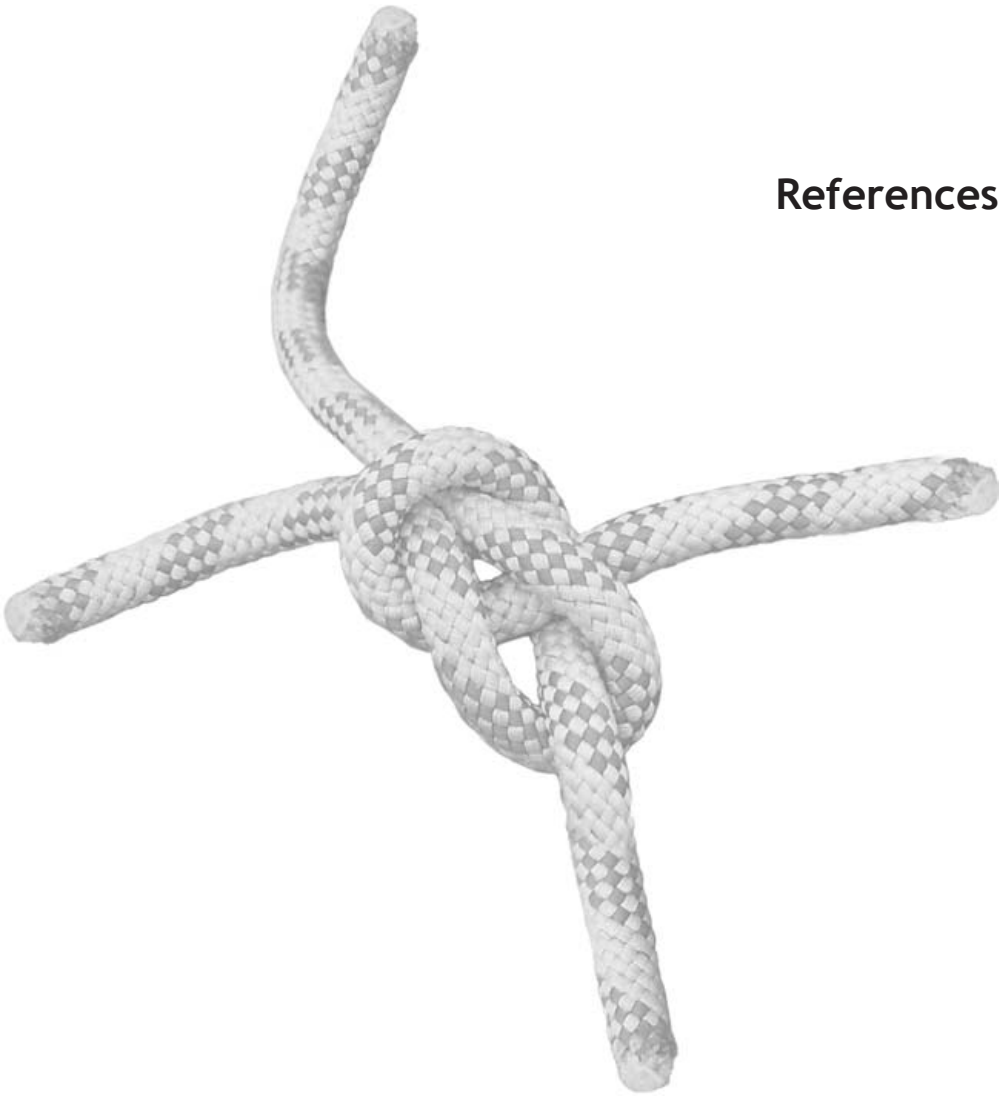
8.8 Future prospects

One of the most striking and still unanswered questions is why mutations in the *tau* gene affect only specific populations of neurons, while other seem perfectly healthy. The fact that different diseases present with typical clinical symptoms is largely related to the subgroup of neurons, which are affected by the disease. Why are certain cell types more vulnerable than others at certain stages of neuronal development and migration? Hence, developmental factors that contribute to the fate of a neuron could be additional risks or modifying factors of FTDP-17 and other neurodegenerative disorders. *Tau* mutations lead to specific cellular alterations, including altered expression, function and biochemistry of tau protein. The large variation in age at onset and duration of the disease between and within families with FTDP-17 suggests the presence of an effect due to genetic or environmental factors. Identification of protective or detrimental modifying factors will lead to a better understanding of the underlying pathways, and ultimately to the development of therapeutic interventions.

However, this kind of experiments requires large sample sizes and therefore human families are less suitable. The family with the L315R tau mutation described in chapter 4, although currently far too small, would be an excellent family to use for screening. More powerful and productive screens could be performed in transgenic animal models. Mouse models described in chapters 5 and 6 could be suitable for such a genetic screen. Moreover, in *Drosophila* unbiased genetic screens for enhancers and suppressors of neurodegeneration are already performed for other neurodegenerative disorders, such as Huntington's disease and Parkinson's disease. Genes predominantly involved in misfolding, impaired degradation and abnormal aggregation of proteins are often found in these screens (Shulman & Feany, 2003).

The underlying goal of many researchers in the field of the tauopathies and other neurodegenerative disorders is to help patients now and in the future. Elucidation of the pathophysiological pathways leading to neurodegeneration is an important step and could ultimately lead to therapeutic and preventive action. Many approaches are available and examined over the recent years (Gold, 2002). The approach to influence the phosphorylation state of tau is complicated by the fact that multiple kinases and phosphatases are involved in modulating tau. Other approaches could involve increased clearance and degradation, prevention of tau aggregation and inhibition of downstream effects of tau. An important concern is the degree to which the pathological processes need to be altered: will it require 10 or 90% inhibition.

Further investigation into the mechanisms of tau dysfunction, as well as the identification of potential disease-modifying factors, will provide additional insight into novel strategies for disease treatment and prevention.



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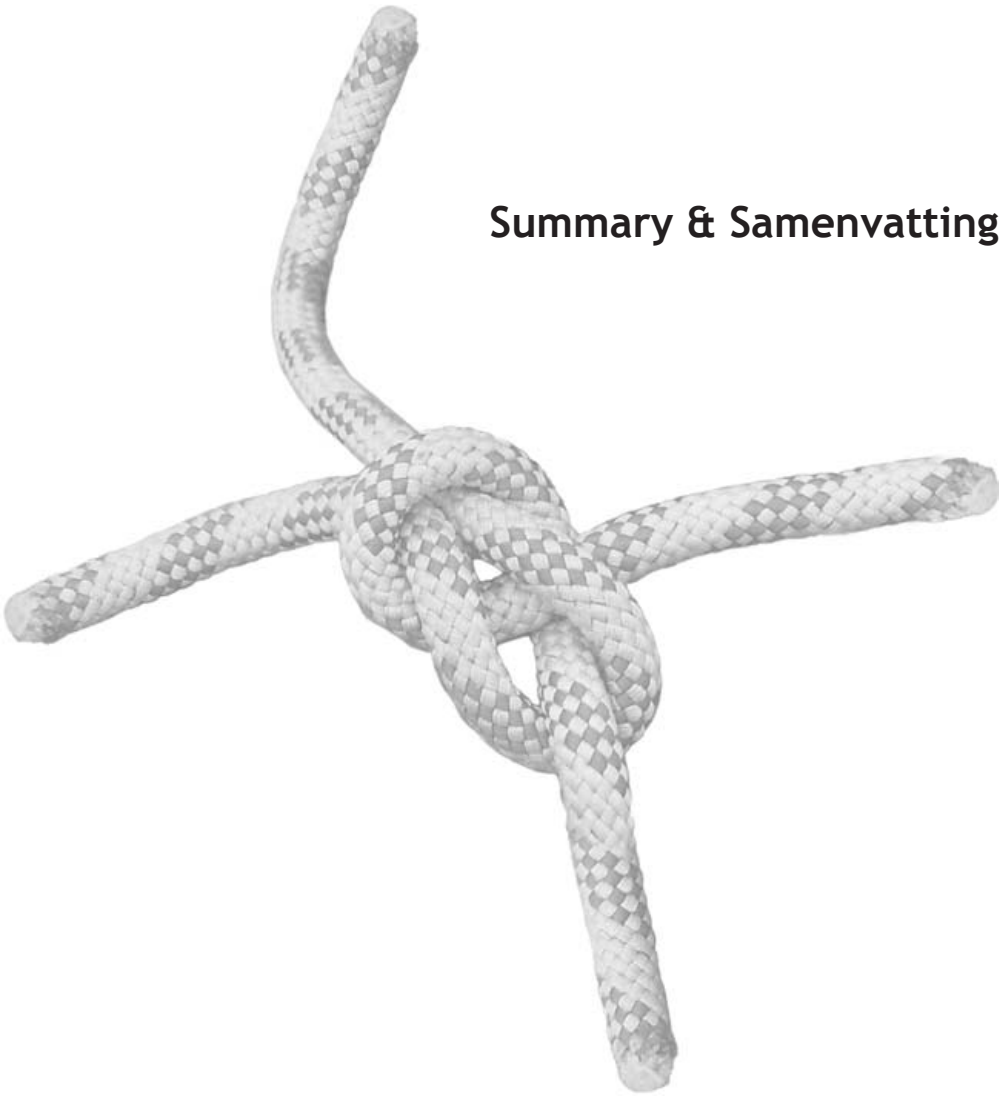

Summary & Samenvatting

Summary

Frontotemporal dementia (FTD) is a mainly presenile neurodegenerative disorder, with a prevalence of 10-20% of all dementias. Patients are characterized by progressive behavioral disturbances, aphasia and cognitive decline. Approximately 35% of the patients with FTD have intraneuronal inclusions that stain positive for the microtubule associated protein tau (MAPT). A proportion of the patients with FTD (20%) show an autosomal dominant pattern of inheritance and genetic studies with FTD families showed linkage to chromosome 17q21-22. The *tau* gene, located in this region, was an obvious candidate gene. In 1998 genetic and molecular biological analysis from several groups (including ours) showed that *tau* is indeed the responsible gene. Several exonic and intronic mutations were found in most of the FTDP-17 families. Tau pathology is also a characteristic observation in several other neurodegenerative disorders, including Alzheimer's disease and Pick's disease. This group is covered by the name 'tauopathies'. The tau protein plays an important role in promoting the assembly of tubulin into microtubules and in maintaining their structural integrity.

Many novel tau mutations have been identified over recent years, widening the clinical spectrum of the disease and providing valuable information for both diagnostic and research purposes. In this thesis the identification and characterization of two novel mutations in the *tau* gene, S320F and L351R respectively, in patients with FTDP-17 is described. These are the first mutations identified in exon 11 of the *tau* gene. Clinical, pathological and biochemical data are presented, together with functional studies measuring the *in vitro* effect of the mutated tau protein. The first mutation, S320F, was found in a small family with presenile dementia. The second mutation, L315R, was identified in two larger families with FTD. The age at onset in these families showed a large variation, ranging from 25 to 64 years. Interestingly, for the first time incomplete penetrance was established in an 82-year old mutation carrier who had no signs of dementia. This finding has a large impact on the clinical prediction of the disease based on presymptomatic DNA testing.

To study the function of the tau protein and its role in neurodegenerative processes different model systems were described by several groups. Studies were carried out with mutated *tau* gene constructs, using both mouse models and cellular over expressing systems. The individual experiments vary greatly in the choice of tau isoform, exact construct and specific mutation. Aggregates, one of the key features of the neuropathology in human patients, were only detected in a few model systems. However, most mouse models that were described show tau pathology but are not a proper model for FTDP-17; particularly, the areas involved in pathology and the behavioural changes differ. In this thesis we described the generation and characterization of mouse models with two specific *tau* mutations, G272V and ΔK280, each with a brain specific expression pattern. The neuropathological changes in these mice are limited, but mimic several features of the human disease. Interestingly, the pathology of these mice is located in the hippocampus and mediotemporal cortex, regions that are also affected in human patients. This mouse model can be used in the future for studying the onset of the disease and the search for modifying factors.

The link between neurodegeneration and disturbed degradation of a specific (group of) protein is described for several neurodegenerative disorders. In brain material of FTDP-17 patients increased degradation products for the mutated protein are present. These results suggest an alteration of the proteolytic processing of the mutated *tau* protein. Follow-up experiments are described in detail in this thesis. In transgenic tau mice, the same proteolytic fragments have been observed. These fragments were shown to be present in mice of all ages, assuming them to be unrelated to the pathological changes in the mice. The fragments were shown to be induced by the calpain degradation pathway in *in vitro* experiments. In future studies the possible toxic effects of the products can be studied. It is hypothesized that an alteration of the proteolytic processing plays a role in the pathogenesis of the disease. The importance to understand the mechanisms leading to neurodegeneration in FTDP-17 goes beyond this disease itself, considering the role of tau in several other neurodegenerative disorders, like Alzheimer's disease.

Samenvatting

Frontotemporale dementie (FTD) is een voornamelijk preseniele neurodegeneratieve aandoening, met een prevalentie van 10-20% van alle dementia. Patiënten worden gekarakteriseerd door progressieve gedragsstoornissen, afasie en cognitieve achteruitgang. Bij ongeveer 35% van de patiënten met FTD worden intraneuronale inclusies gevonden, die positief aankleuren voor het microtubuli-geassocieerde eiwit tau (MAPT). Een gedeelte van de patiënten met FTD (20%) laat een autosomaal dominant overervingspatroon zien en genetische studies met FTD families hebben een koppeling aan chromosoom 17q21-22 aangetoond. Het *tau* gen, gelegen in deze regio, was een duidelijk kandidaatgen. In 1998 heeft genetische en moleculair biologische analyse van verschillende onderzoeksgroepen (inclusief de onze) aangetoond dat *tau* inderdaad het verantwoordelijke gen was. Verscheidene mutaties in exonen en intronen werden gevonden in de meeste FTDP-17 families. Tau pathologie is ook een karakteristieke observatie in verschillende andere neurodegeneratieve aandoeningen, onder andere de ziekte van Alzheimer en de ziekte van Pick. Deze groep is bekend onder de noemer 'tauopathies'. Het tau eiwit speelt een belangrijke rol in het bevorderen van het samenvoegen van tubuline residuen tot microtubuli en daarmee het behouden van hun structurele integriteit.

De afgelopen jaren zijn vele nieuwe *tau* mutaties geïdentificeerd, die het klinische spectrum van de ziekte hebben verbreed en waardevolle informatie hebben gegeven voor zowel diagnostische als wetenschappelijke doeleinden. In dit proefschrift wordt de identificatie en karakterisatie beschreven van twee nieuwe mutaties in het *tau* gen, S320F en L315R, beschreven in patiënten met FTDP-17. Dit zijn de eerste mutaties die geïdentificeerd zijn in exon 11 van het *tau* gen. Klinische, pathologische en biochemische bevindingen worden gepresenteerd, samen met functionele studies die het '*in vitro*' effect meten van het gemuteerde tau eiwit. De eerste mutatie, S320F, werd gevonden in een kleine familie met preseniele dementie. De tweede mutatie, L315R, werd geïdentificeerd in twee grotere families met FTD. De beginleeftijd in deze families vertoonde een grote spreiding, variërend van 25 tot 64 jaar. Een interessante waarneming is dat voor de eerste keer incomplete penetrantie is aangetoond in een 82-jarige drager van de mutatie zonder tekenen van dementie. Deze bevinding heeft grote consequenties voor de klinische voorspelling van de ziekte gebaseerd op presymptomatische DNA testen.

Om de functie van het tau eiwit en de rol in neurodegeneratieve processen te onderzoeken zijn er door een aantal onderzoeksgroepen verschillende modelsystemen beschreven. Gebruik makend van zowel muismodellen als cellulaire overexpressie systemen zijn gen constructen met gemuteerd *tau* bestudeerd. De individuele experimenten vertonen grote variatie in de keus van de *tau* isovorm, het exacte construct en de specifieke mutatie. Aggregaten, een van de karakteristieke kenmerken van de neuropathologische veranderingen in patiënten, werden maar in enkele modelsystemen gedetecteerd. De meeste muismodellen die zijn beschreven in de literatuur vertonen echter wel tau pathologie maar zijn geen model voor FTDP-17; vooral de gebieden betrokken bij de pathologie en de gedragsveranderingen zijn verschillend. In dit proefschrift wordt de generatie en de karakterisatie van muismodellen met twee specifieke tau mutaties, G272V en ΔK280 beschreven, die een hersenspecifiek

patroon vertoonden. De pathologie van deze muizen werd voornamelijk gevonden in de hippocampus en de medio-temporale cortex, gebieden die ook bij de aangedane patiënten pathologie vertonen. Dit muismodel kan in de toekomst worden gebruikt voor bestudering van de aanvang van de ziekte en het zoeken naar modificerende factoren.

Voor een aantal neurodegeneratieve aandoeningen is een verband beschreven tussen neurodegeneratie en verstoorde afbraak van een specifiek eiwit of groep van eiwitten. Experimenten met hersenmateriaal van FTDP-17 patiënten toont de verhoogde aanwezigheid van afbraakproducten voor het mutante eiwit. Dit resultaat suggereert een verandering van de proteolyse van het gemuteerde tau eiwit. Vervolgexperimenten zijn in detail beschreven in dit proefschrift. In transgene tau muizen zijn dezelfde proteolytische fragmenten gevonden. Deze fragmenten komen echter voor in muizen van alle leeftijden, en zijn dus klaarblijkelijk niet gerelateerd aan de pathologische veranderingen van deze muizen. De fragmenten kunnen worden geïnduceerd door de calpain degradatie route in '*in vitro*' experimenten. In toekomstige experimenten kunnen de mogelijk toxische effecten van deze producten worden bestudeerd. Er kan worden gesteld dat een verandering van de proteolyse een rol speelt in de pathogenese van de ziekte. Het belang van het ontrafelen van het mechanisme dat leidt tot neurodegeneratie in FTDP-17 gaat verder dan deze ziekte alleen. Hierbij moet worden gedacht aan de rol van tau in een aantal andere neurodegeneratieve ziekten zoals de ziekte van Alzheimer.

Abbrevations

ABC	anterior bulbar cells
AD	Alzheimer's disease
APP	amyloid precursor protein
BAC	bacterial artificial chromosome
CaMPKII	Ca^{2+}/calmodulin-dependent protein kinase II
CBD	corticobasal degeneration
Cdk	cyclin dependent kinase
CNS	central nervous system
DDPAC	disinhibition-dementia-parkinson-amyotrophic complex
DLDH	dementia lacking distinctive histology
DNA	deoxyribonucleic acid
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
FTD	frontotemporal dementia
FTLD	frontotemporal lobar degeneration
GSK-3β	glycogen synthase kinase 3β
HDDD2	hereditary dysphasic disinhibition dementia
ISM	intron silencer modulator
Kb	kilobase
kDa	kilodalton
KO	knockout
MAPK	mitogen-activated protein kinase
MAPT	microtubule associated protein tau
MARK	microtubule-affinity regulating kinase
mRNA	messenger ribonucleic acid
NFT	neurofibrillary tangles
NMR	nuclear magnetic resonance
NT	neuropil threads
PHF	paired helical filament
PiD	Pick's disease
PIN-1	prolyl isomerase-1
PNS	peripheral nervous system
PrP	*prion* promoter
PSP	progressive supranuclear palsy
SAP kinase	stress-activated protein kinases
SDS PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SF	straight filament
SnRNP	small nuclear RNA-protein
SP	senile plaques
WT	wild type

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Curriculum Vitae

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