Mutations in frontotemporal dementia linking *tau* to neurodegeneration

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Mutaties in frontemporale dementie en de rol van *tau* in neurodegeneratie

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Chapter 1

General Introduction

Neurodegeneration

Neurodegenerative disorders are characterized by the degeneration of specific populations of nerve cells. They comprise a diverse set of diseases that contribute significantly to morbidity and mortality especially in our aging population.
Dementia in particular poses a growing social and economic problem. Prevalence values for dementia rise from 5% to more than 30% between the ages of 65 and 85 years, affecting more than 20 million people world-wide, a number expected to double within the next 30 years (1).
Progress in therapeutic approaches has been limited but genetic studies have resulted in the identification of genes involved in the pathogenesis of dementia. Although mutations in these genes explain only a small proportion of cases, the findings have been of great importance in our understanding of the disease process and opened novel areas of investigation.

In this thesis I describe how our group contributed to the continuously developing field of dementia, and frontotemporal dementia in particular.
In 1997 we reported linkage to chromosome 17 for three large Dutch kindreds with frontotemporal dementia (FTDP-17) (2) (and chapter **4.4.1** in this thesis). We then formed an international consortium with the aim to identify the gene responsible for FTDP-17 using a positional cloning approach. The finding of mutations in the gene for the microtubule associated protein tau in FTDP-17 families reported by us and other groups was a major breakthrough in the field (3-6) (and chapter **5.5.1** in this thesis). For the first time it was demonstrated that tau dysfunction can be a cause of neurodegeneration.

1.1 Frontotemporal dementia.

Historically frontotemporal dementia has been often classified as Pick's disease, even when Pick's cells or Pick's bodies were not found (7). In the mid 1980s groups from Lund, Sweden, and Manchester, UK, wrote several articles about a syndrome called either frontal lobe degeneration or frontotemporal dementia. In 1994 they published a consensus statement describing that syndrome and adopted the term frontotemporal dementia (FTD) (8).
Although the symptomatology can be remarkably broad, FTD patients typically have severe, sometimes unilateral, neuronal degeneration of the frontal and temporal cortices, and often additional degenerative changes are observed in subcortical nuclei and substantia nigra. The disease usually begins insidiously in the fifth decade of life with behavioral changes and cognitive impairment including aphasia, progressing to severe dementia and extrapyramidal symptoms,

hallucinations or psychosis (2, 9) (and chapter **4.4.1** in this thesis). In contrast to Alzheimer's disease (AD), there is a relative preservation of memory until later stages of the disease (8, 10). Microscopic neuropathology reveals neuronal loss, gliosis, spongiosis and sometimes Pick bodies and ballooned neurons. Approximately 35% of the cases have cellular inclusions that stain positively for the microtubule associated protein tau, although the nature of tau pathology varies considerably in both quantity and characteristics (11, 12).
The prevalence of FTD is unclear. Clinicopathological studies suggested that FTD represents 10 to 20% of patients with dementia, being after AD and Lewy body disease the third most common cause of dementia. In a genetic epidemiological study in the Netherlands, the prevalence of FTD has been estimated 3 per 100000 in the age group between 60 and 70 years of age (13). However, this prevalence has proved to be too low and the number increased to 6 per 100000 after using broader criteria for FTD diagnosis and additional pathological reports. FTD is mostly sporadic, although in some cases (20%) there is a strong indication of an hereditary manner. It has been shown that 40% of FTD patients have a familiar history of dementia (13-15).

1.2 Tauopathies

The new term, tauopathies (11), was recently coined to refer to a seemingly heterogeneous group of neurodegenerative disorders with filamentous tau deposits as their predominant histopathological feature. Abundant filamentous tau inclusions are the defining neuropathological characteristics of several neurodegenerative disorders including Alzheimer disease (16), Pick's disease (17), progressive supranuclear palsy (18-20) and corticobasal degeneration (21) (Table 1).

1.3 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-parkinsonism-amyotrophic complex (DDPAC) (22). Other families with a remarkable variety of clinical and pathological phenotypes were subsequently reported to be linked to 17q21-22 (2, 23-25). They were variously described as having pallido-ponto-nigral degeneration, familial parkinsonism with dementia, familial progressive subcortical gliosis, familial multisystem tauopathy and others. Clinically and

pathologically, it was not immediately obvious that these conditions might have a common pathogenesis.

A consensus conference was held in Ann Arbor in 1996 to look for similarities among these families (26). The clinical features were primarily abnormal behavior with disturbed executive function combined with relative preserved memory, orientation and visual-spatial function. These changes are followed by cognitive decline leading to a profound dementia. Parkinsonian features of bradykinesia, rigidity and postural instability, without resting tremor were seen early in the course of the disease in some patients. A description essentially identical to the picture of FTD described by the Lund and Manchester groups (8). The collective disorder was called frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17).

Neuropathologically, brains of FTDP-17 patients exhibit frontotemporal and often basal ganglia atrophy combined with depigmentation of substantia nigra. Microscopically there is a severe neuronal loss, and a gray and white matter gliosis. Abundant filamentous inclusions, which may affect both neuronal and glial cells, are the histological hallmark of FTDP-17, and the principal component of these inclusions is the microtubule associated protein tau (Figure 1). This is referred to as pathologic tau protein due to its insolubility and abnormal post-translational modification, which includes hyperphosphorylation. Therefore FTDP-17 is further classified as a tauopathy to clarify its relationship with other hereditary and sporadic disorders in which tau does not accumulate. Amyloid pathology and Lewy bodies are absent in most cases (11, 26).

The gene for the tau protein, previously cloned and localized to chromosome 17q21 (27) within or near the FTDP-17 disease locus, was considered from the beginning an obvious candidate. Genetic analysis has indeed shown that *tau* is the gene responsible. Several intronic and exonic mutations were in fact found in most of the FTDP-17 families (see chapter **5.5.1** in this thesis).

For the first time convincing evidence was provided that tau protein plays a central role in the etiology of neurodegeneration. This seminal finding has opened novel areas of investigation into the pathophysiologic mechanisms of tau dysfunction and the relationship of tau abnormalities to brain degeneration.

Table 1

Disease	Selected references
Alzheimer's disease	(16)
Corticobasal degeneration	(21, 28)
Progressive supranuclear palsy	(18-20, 29)
Pick's disease	(30)
Frontotemporal dementia and parkinsonism linked to chromosome 17	(11, 26)
Prion protein amyloid angiopathy	(31)
Postencephalitic parkinsonism	(32, 33)
Argyrophilic's grain disease	(34, 35)
Amyotrophic lateral sclerosis/parkinsonism-dementia complex of Guam	(36)
Gertmann-Straüssler-Scheinker disease with tangles	(37)
Dementia pugilistica	(30)
Myotonic dystrophy	(38)

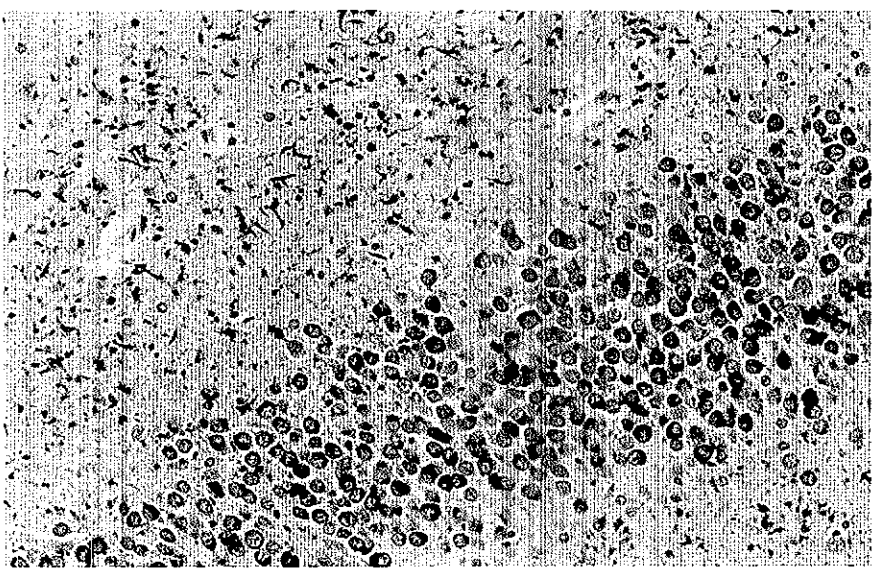

Figure 1. Tau immunostaining in hippocampal formation from a FTDP-17 patient with the P301L mutation using an anti-tau antibody.

Chapter 2

Tau Protein

Tau is part of the microtubule-associated protein family (MAP). Members of this family (i.e. tau, MAP2 and MAP4) are found in many animal species, from *Caenorhabditis elegans* to bovine and human (39-43). They are involved in promoting microtubule assembly and bundling and play a major role in stabilizing the microtubule dynamic behavior. MAP proteins are related by a common carboxy-terminal region that is ~200 amino acids long, 60-70% identical in sequence, and possess microtubule binding and assembly activities (44, 45).
Tau is an abundant protein in both central and peripheral nervous systems. In brain it is predominantly found in nerve cells and at lower level in astrocytes and oligodendrocytes (46). Within the neurons it is found mainly in the axons (47).

2.1 Gene structure.
The human *tau* gene encompasses more than 100 kb on the long arm of chromosome 17 (17q21) (27). The promoter region is characterized by the absence of a TATA sequence and by the presence of three SP1-binding sites (important in directing transcription initiation in many TATA-less promoters), in proximity of the first transcription initiation site of the *tau* gene. Two CpG islands are present, one in the promoter region and one in exon 9 (48, 49).
The *tau* primary transcript contains 16 exons (Figure 2A), however three of them (exons 4A, 6 and 8) are found only in the peripheral nervous system (PNS). Exon 4A is found in human, bovine and rodent peripheral nervous system, with a high degree of homology. Exon –1 and exon 14 are transcribed but not translated. Exons 2, 3, 10 are alternatively spliced, allowing for six combinations ($2^-3^-10^-$; $2^+ 3^- 10^-$; $2^+ 3^+ 10^-$; $2^- 3^- 10^+$; $2^+ 3^- 10^+$; $2^+ 3^+ 10^+$).

2.2 Six tau isoforms.
In the adult human brain six tau isoforms ranging from 352 to 441 amino acids are produced through alternative mRNA splicing of exons 2, 3 and 10 (Figure 2B). The most striking feature of the *tau* sequence is the presence of three (3R-tau) or four (4R-tau) imperfect tandem repeats encoded by exons 9-12. These repeats represent the microtubule binding domains. They are made of 31 or 32 amino acids, located in the carboxy-terminal half, each containing a characteristic Pro-Gly-Gly-Gly motif that is highly conserved (43, 44, 50, 51). The additional repeat of 31 amino acids (encoded by exon 10) in the isoforms with 4 repeats (4R) is inserted within the first repeat of the isoforms with 3 repeats (3R) in a way that preserves the periodic pattern (43).

Alternative splicing of exons 2 and 3 results in the absence (0N) or presence of inserted sequences of 29 (1N) or 58 (2N) amino acids located in the amino-terminus (42, 48).

Tau expression is developmentally regulated. In immature human brain, only the shortest isoform is expressed (43). The developmental shift of human tau from a simple fetal to a more complex adult pattern thus involves the transition from the expression of the isoform with three repeats containing no amino-terminal inserts, to the expression of all the six isoforms. The overall 3R/4R ratio is close to one while 2N tau isoforms are underrepresented relative to 0N and 1N (52). In cerebral cortex, a slight preponderance of 3R over 4R isoforms is observed. In the PNS, alternative splicing and inclusion of the large exon 4a in the amino terminal half of the molecule results in the expression of higher molecular weight proteins termed "big tau" (53-55).

There exist species differences in the expression of tau isoforms. Immature rodent brain expresses the shortest three repeat isoforms, like the immature human brain. However, adult rodent brain expresses only three tau isoforms, each with four-microtubule binding repeats (56, 57).

Early biophysical and microscopic data suggested that tau has very little secondary or tertiary structure in solution (58, 59), and it may be best described as a highly elongated molecule (60, 61). Minimal secondary structure was predicted from the derived amino acids sequence of the cloned *tau* cDNA (44). This conclusion was consistent with earlier observations demonstrating that tau retains its solubility, microtubules assembly and stabilizing activities even after exposure to harsh conditions such as boiling or treatment with perchloric acid (62, 63).

2.3 Structure and function of the microtubule binding domains.

By regulating microtubule properties, tau protein has a role in modulating the functional organization of the neuron, and particularly in axonal morphology, growth and polarity. Experiments with synthetic peptides and with tau protein produced by expression in *Escherichia coli*, have shown that the carboxy-terminal repeats and some adjoining sequences constitute the microtubule binding domains (64-69). In particular the first two repeats and their inter-repeat region function as a core microtubule binding domain in both 3R and 4R tau (R1, R3 and inter-repeat R1-R3 for three repeats tau; R1, R2 and inter-repeat R1-R2 for four repeats tau). They have been shown to increase the rate of microtubule polymerization and to inhibit the rate of depolymerization. The repeats bind to the microtubules

through a flexible array of distributed weak sites (51, 66). The saturated molar ratio of tau binding to polymerized tubulin dimers is ~1:2.5 (58, 60). It has been demonstrated that adult tau isoforms with 4R are more efficient at promoting microtubule assembly than the 3R isoforms (52, 65-67, 70). Interestingly the most potent part to induce microtubule polymerization is the inter-region between repeat 1 and repeat 2. This region is unique to 4R tau and thus is thought to be responsible for the higher microtubule binding affinity of these tau isoforms.
The microtubule-binding region may be also involved in other functions than microtubule assembly. Tau may also interact with microfilaments and intermediate filaments to maintain the three-dimensional cytoskeletal network.

2.4 Structure and function of the amino-terminal domains.

The two cassettes of 29 amino acids encoded by exon 2 and 3 are highly acidic and followed by a proline-rich region. The N-terminal part is referred to as the projection domain, since it projects from the microtubule surface where it may interact with other cytoskeletal elements and components of the plasma membrane (71). Inactivation of the *tau* gene by homologous recombination in mice leads to no overt phenotype, indicating that tau is not an essential protein (72, 73). However, it was observed that in the mice lacking the *tau* gene the axonal diameter in some neurons is particularly affected. This may be related to the particular length of the N-terminal domain of tau isoforms in specific axons. In fact projection domains of tau determine spacing of microtubule in axons and might well increase axonal diameter (74), being therefore crucial in the stabilization and organization of certain types of axons.
There is evidence that tau protein allows interactions with the neural plasma membrane (71). Recently this interaction is being defined as involving a binding between the proline-rich sequence in the N-terminal part of the tau protein and the SH3 domains of the src-family non-receptor tyrosine kinases, such as fyn (75). Lee *et al.* describe an association between the tau-fyn complexes and the actin cytoskeleton. These data are in favor of a role for tau protein in src-family tyrosine signaling pathway that may modify the cell shape by acting on the submembranous actin cytoskeleton.

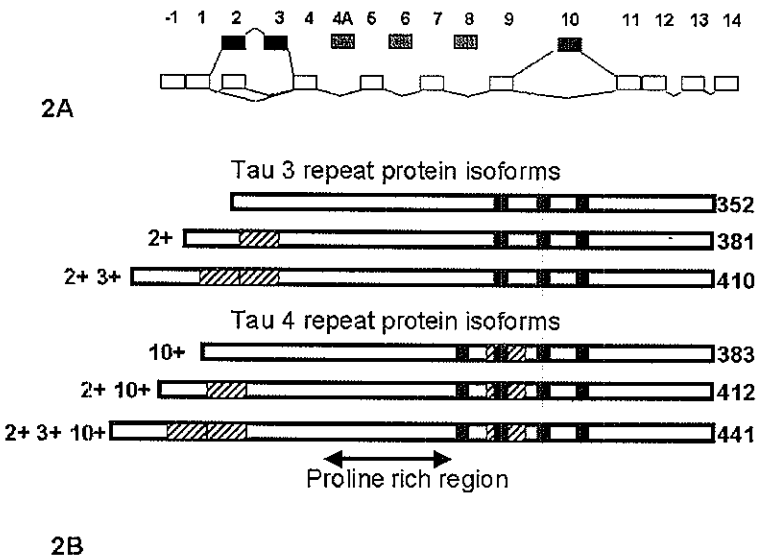

Figure 2. Schematic representation of the human *tau* gene (**2A**) and the six human CNS tau isoforms generated by alternative splicing (**2B**). Alternative splicing of exons 2, 3 and 10 (black boxes) produces the six alternative tau isoforms. Exons 4A, 6 and 8 (in gray boxes) are not transcribed in the human CNS. Exon 4A, however, is transcribed in PNS leading to the larger tau isoforms, termed "big tau"(see text). The striped boxes in **2B** represent the amino terminal inserts. The black boxes represent the microtubule binding domains. The black box with strips represents the repeat encoded by exon 10.

2.5 Post-translational modifications.

Tau is a phosphoprotein with 79 putative serine or threonine phosphorylation sites present on the longest brain tau isoform (76, 77).

Using phosphorylation dependent antibodies against tau, mass spectrometry and sequencing, at least thirty phosphorylation sites have been described (reviewed in (76, 77). Most of these sites are localized outside the microtubule-binding domains and they are serine or threonine residues followed by a proline.

Phosphorylation of tau is developmentally regulated (78, 79). Tau from developing brain is phosphorylated more than tau from adult brain suggesting selective dephosphorylation of the shortest tau isoform during brain maturation.
The different states of tau phosphorylation result from the activity of specific kinases and phosphatases toward these sites. Most of the kinases involved in tau phosphorylation are part of the proline-directed protein kinases (PDPK), which include mitogen-activated protein kinase (MAPK) (80, 81), glycogen synthase kinase 3β (GSK-3β) (82), cyclin-dependent kinases including cdc2 and cdk5 (83, 84) and stress activated protein kinases (SAP kinases) (85, 86). Non Ser/Thr-Pro sites can be phosphorylated by many other protein kinases, including Ca^{2+}/ calmodulin-dependent kinase II (CaMPK II) (87, 88) and microtubule-affinity regulating kinase (MARK) (89). However, most of the evidence to implicate the specific kinases in the phosphorylation of tau comes from *in vitro* studies and it remains unclear what role they play *in vivo*. Even if further work is necessary to determine the relative contributions of individual kinases to tau phosphorylation *in vivo* two kinases in particular, GSK-3β and cdk5 are emerging as promising candidates to regulate *in vivo* tau phosphorylation.
GSK-3β is a Ser/Thr kinase that is abundant in brain and associates with microtubules (90-92). In non-neuronal cells co-transfection of human *tau* and *GSK-3β* induces hyperphosphorylation of tau associated with a loss of microtubules binding (93). Direct inhibition of GSK-3β by lithium salts reduces tau phosphorylation and affects microtubules stability (94).
Cdk5 is a Ser/Thr protein kinase highly enriched in neurons. It colocalizes to the cytoskeleton and contributes to the phosphorylation of tau (83, 95). Recently it was shown that cdk5 complexes with tau in a tau-phosphorylation dependent manner and that tau anchors cdk5 to microtubules (96). Moreover cdk5–mediated phosphorylation of tau stimulates further phosphorylation of tau by GSK-3β (97, 98).

Tau protein either from brain tissue or neuroblastoma cells is rapidly dephosphorylated by endogenous phosphatases such as PP-1, PP-2A, PP-2B (calcineurin) and PP-2C (99-101). Like kinases, phosphatases have many direct and indirect physiological effects and counterbalance the action of kinases. Of the major phosphatases activities in brain tau is predominantly dephosphorylated by the trimeric form of phosphatase 2A (102, 103). Both PP-1 and PP-2A bind to tau and this interaction mediates an association with microtubules (104, 105). PP-2A has been demonstrated to bind directly to microtubules, an interaction that

regulates the activity of this phosphatase *in vitro* (106). Further, inhibition of either PP-2A and PP-1 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 (107).
The binding of tau to microtubules is partially modulated by its phosphorylation state. Phosphorylated tau protein is less effective than non-phosphorylated tau protein on microtubule polymerization. Thus the more phosphorylated tau is, the less able it is to bind to microtubules and to promote microtubule assembly (108, 109). Some sites, in particular Ser 262 and Ser 396 might play a dominant role in reducing the binding of tau to microtubules (108, 110). However the phosphorylation of neither site is sufficient to completely eliminate tau binding (111). Interestingly, both sites are phosphorylated in fetal tau and they are hyperphosphorylated in all six tau isoforms that form abnormal filaments in neurofibrillary tangles. Hyperphosphorylation is an invariant feature of the filamentous tau deposits that characterize many neurodegenerative disorders (112).

Another post-translational modification that takes place in tau is O-glycosilation, a modification characterized by the addition of an O-linked N-acetylglucosamine residue of Ser or Thr in the proximity of Pro residues. The functional significance of O-GlcNac modification is not fully understood, but it is implicated in transcriptional regulation, cell activation, cell cycle regulation, and the proper assembly of protein multimeric complexes (113).
Tau is also subject to other post-translational modifications such as ubiquitination, racemization and glycation (114-117). However, these events are likely to occur after filament formation and may serve to stabilize the filaments (118).

Chapter 3

Tau Related Dementia and Pathological Aggregation of Tau Protein

The aggregation of tau isoforms into intraneuronal filamentous inclusions is the most obvious pathological event in several neurodegenerative disorders collectively referred to as tauopathies. They include Alzheimer's disease (AD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease and several others disorders. Until recently, it was thought that an abnormal phosphorylation of tau protein was responsible for its aggregation. However, normal tau protein is phosphorylated in fetal and adult brain and does not aggregate to form filamentous inclusions. Moreover, non-phosphorylated recombinant tau protein forms filamentous structures under physiological conditions *in vitro*, when sulfated glycosaminoglycan or other polyanions are present. These data suggest that, in addition to phosphorylation, other mechanisms are involved in the formation of pathological tau filaments.

3.1 Tau in Alzheimer's disease.

Alzheimer's disease is the most frequent form of dementia that affects approximately 10% of the population older than 65 years of age (119). Memory loss or aphasia, apraxia and behavioral disturbance are the presenting symptoms followed in time by defects in other cognitive domains, dementia and finally death. In brains of deceased patients two main types of lesions are observed, senile plaques (SPs) and neurofibrillary tangles (NFTs) (Figure 3A). Although these deposits have been known ever since Alois Alzheimer described them in 1911 (120), it is only over the past 15 years that their chemical composition has been determined. Abnormal aggregates of amyloid-β peptide (Aβ), a proteolytic product of the amyloid precursor protein (APP), are the main component of senil plaques (121, 122). Cases of familial AD mutations have been found in the *APP* gene, suggesting that it plays a central role in the etiopathogenesis (123). SPs are diffusely and variably distributed throughout the cerebral cortex.

Neurofibrillary lesions form within nerve cells of the cerebral cortex, the hippocampal formation and some subcortical nuclei leading to degeneration and death of the cells. These lesions are found in nerve cell bodies and apical dendrites as neurofibrillary tangles, in distal dendrites as neuropil threads (NTs) and abnormal neurites which are often, but not always, associated with amyloid plaques.

Senile plaques density often does not correlates well with the clinical presence and severing of dementia, particularly when so-called diffuse, or primitive plaques are considered (124-126). In cognitively normal individuals amyloid deposits can be present and sometimes their numbers may be higher than in the average AD patient.

On the other hand the stereotypical distribution and amount of NFTs and NTs do correlate well with the observed neurodegeration, beginning in the medial temporal structures and gradually involving both neocortex and allocortex (16).
Ultrastructurally, the dominant components of neurofibrillary lesions in AD are paired helical filaments (PHFs) as well as straight filaments (SF) (Figure 3B and 3C). Electron micrographs of negatively stained isolated PHFs show two ribbons twisted around one another in a helix with a periodicity of 80 nm and a width varying from 8 to 20 nm (127). Straight filaments have a diameter of 15-18 nm and lack this helical periodicity. Both PHF and SF tau filaments contain complete tau molecules as proved by their immunoreactivity to antibodies against the N- and C-terminal regions of tau.
Despite the fact that many phosphorylation sites are common to PHF-tau protein and normal tau, there are biochemical characteristics that differentiate them and support the concept of pathological tau protein. PHF-tau protein is hyperphophorylated at more sites which can be visualized by a few phosphorylation-dependent antibodies such as AT100 (100), AP422 (128), PHF-27 (129) or the TG/MC antibodies (130). With the exception of Ser422, these sites found in PHF-tau are conformation-dependent epitopes.
Interestingly, tau's electrophoretic profile is often disease-specific. Tau protein is very hydrophilic and it can be easily extracted from brain or cultured cells. Normal adult human tau is resolved by dodecyl sulphate (SDS) gel electrophoresis into 6 bands of 45-60 kD in size. In comparison, filamentous tau extracted from diseased brains is relatively insoluble. Tau protein derived from AD inclusions is detected mainly as three bands of 60, 64 and 68 kD (56, 131-133). A 72 kD band is also present in low amount (Figure 4A). After dephosphorylation six tau bands are seen that align with the six recombinant human brain tau isoforms (134). Several approaches have helped delineating which tau isoforms make up each PHF-tau band (135-137). The shortest and the longest tau isoforms make up the 60 kD and 72 kD bands, respectively. The 64 kD band contains one or more of isoforms with three repeats and 1N and 2N or four repeats with 1N and 2N. The 68 kD band consists of tau isoforms with three repeats and 2N or four repeats and 2N, or both. PHF-tau from AD brains thus contains all six tau isoforms, each in a hyperphosphorylated state.
Treatment of filaments with pronase removes a fuzzy coat consisting of the N- and C- terminal regions of tau and leaves a pronase-resistant core, which retains the characteristic appearance of the starting filament (134, 138). Chemical analysis has shown that the proteolitically protected core of the PHF tau contains

various fragments of three- and four-repeats tau, consisting of slightly more than three repeats from the microtubule binding region, typically about 95 amino acids long from the region 297-391 in the numbering of the longest isoforms (139).

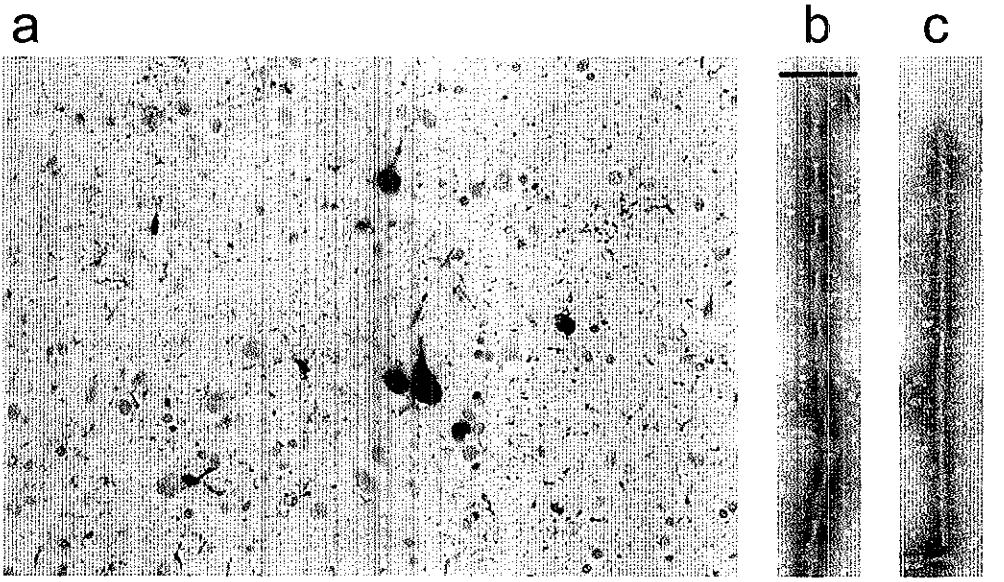

Figure 3. A: NFT in AD patient (immunostaining with anti-tau antibody). **B** and **C**: electron micrographs of sarkosyl insoluble PHF (3B) and SF (3C) in AD patient.

3.2 Tau in progressive supranuclear palsy.

Progressive supranuclear palsy is an extrapyramidal disorder characterized by akinetic-rigid syndrome with supranuclear gaze palsy and sometimes pyramidal tract dysfunction, pseudobulbar signs and cognitive decline of the frontal lobe type (29). Neuropathologically PSP is characterized by neurodegeneration and gliosis in striatum, pallidum, and substantia nigra.. Within these brain regions there is a high density of fibrillary tau pathology including NFTs of round or globose shape, and NTs (140, 141).

The insoluble hyperphosphorylated tau from the affected brain areas migrate as two major bands of 64 and 68 kD and a variably detected band of 72 kD (142) (Figure 4B). These bands represent tau isoforms containing 4R (143). Two-dimensional analysis revealed that the isoelectric point of the pathological tau doublet in PSP is less acidic than the isoelectric point in AD. However, most of the phosphorylation sites found in PHF-tau are also encountered in pathological tau protein from PSP patients (17). The predominant form of filament seen in sectioned material is straight with a width of about 15 nm, but PHF-like filaments (144) and wider twisted ribbons have also been reported (145).

PSP usually occurs as a sporadic disorder but familial forms have been described. Linkage for chromosome 17 for familiar forms of PSP has been excluded (146). In PSP the selective aggregation of 4R tau has been reported to be associated with increased expression of exon 10 in vulnerable regions (147), as demonstrated for several FTDP-17 kindreds (3, 148, 149).
Genetic changes in the *tau* gene may contribute to the risk of developing PSP since Conrad *et al.* identified a polymorphic dinucleotide repeat region after exon 9 of the *tau* gene associated to sporadic PSP cases (150). Subjects with the homozygous *tau* allele A0, characterized by 11 TG repeats were over-represented in PSP patients (95,5%) versus normal controls (57,4%) and AD patients (49,7%0). Subsequent studies have confirmed this correlation in the Caucasian population (146, 151-154), as well as its association with an earlier age of disease onset (155). More detailed studies using multiple tau polymorphisms spanning the whole *tau* gene including the promoter region (156-158) showed association with an extended haplotype (H1/H1) in complete linkage disequilibrium with the *tau* gene, making difficult to pinpoint the exact location of the biologically relevant defect. The more common haplotype, H1, is significantly over-represented in unrelated Caucasian individuals with PSP.
One explanation for these findings could be that the H1/H1 haplotype harbors a mild risk factor since it is also present in >50% of the health control population. Alternatively, the result could be explained by the possibility that a subtype of the H2 allele, which is virtually absent in PSP cases, harbors a protective effect. The biological relevance of this is unknown, and it is unclear whether the *tau* gene is simply a marker for a mutation in a nearby gene or whether pathogenic sequences in the *tau* gene remain to be identified. Mutation analyses have not yet revealed pathogenic mutations (156, 157, 159).

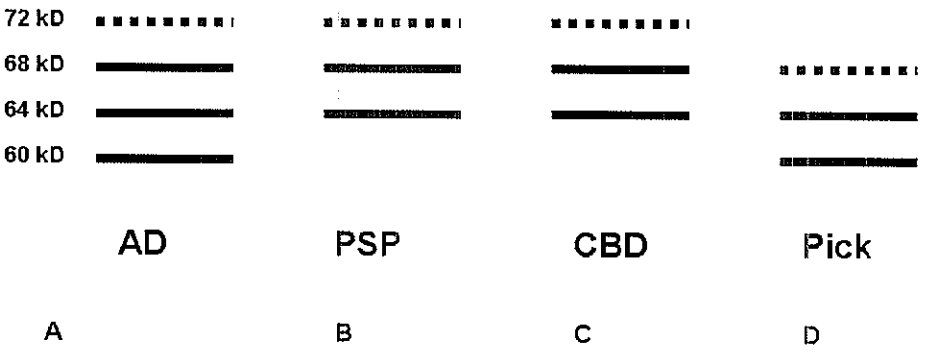

Figure 4. Schematic representation of Western blot banding patterns of insoluble tau from different tauopathies. Non-dephosphorylated insoluble tau from brain of AD patients run as three mayor band of 60, 64, 68 kD, and a minor and variable band of 72 kD (**4A**). Non-dephosphorylated insoluble tau from brain of PSP and CBD patients run as two mayor bands of 64 and 68 kD. The 72 kD band is variable detected. (**4B** and **4C**). In brain from Pick's patients two mayor band of 60 and 64 kD are observed and a minor variable band of 68 kD (**4D**). A dashed bar is used to depict the minor and variable 68 and 72 kD tau bands.

3.3 Tau in corticobasal degeneration.

Corticobasal degeneration is a rare, sporadic and slowly progressive neurodegenerative disorder and it is clinically characterized by unilateral extrapyramidal motor dysfunction, limb dystonia, alien hand syndrom and parietal dysfunction like apraxia (160). Dementia might be present at the onset of symptoms (161, 162). Neuropathological examination reveals an asimmetric parietal atrophy of the brain with prominent neuronal and glial intracytoplasmatic filamentous tau pathology (163). One of the most striking features of CBD is the extensive accumulation of tau immunoreactive neuropil threads through both the gray and white matter (28).

There is a clear clinical and pathological overlap between PSP and CBD, overlap that extends at the biochemical level. The tau electrophoretic profile of CBD is in fact very similar to that of PSP, with a main doublet of 64 and 68 kD and a minor band of 72 kD (Figure 4C). Tau isoforms are exclusively with four repeats and more acidic than those in PSP (143). Isolated filaments have a greater width and a longer, more irregular twisted than PHFs (164).

A further similarity between PSP and CBD was recently described (165, 166). It was shown that CBD is associated with the A0 allele of the *tau* gene as well as the H1 haplotype just like PSP, providing therefore evidence that the same tau haplotype is a risk factor for developing CBD.

Considering the overlap between the two disorders it is tempting to speculate that PSP and CBD rather than representing two distinct neurological entities may be different phenotypic expressions of the same neurodegenerative disease process that reflect the effects of additional modifying factors. The finding that the tau pathology of PSP and CBD bears striking resemblance to that of cases of FTDP-17 with mutations that destabilize the exon 10 splicing regulatory element RNA might support this hypothesis.

3.4 Tau in Pick disease.

Pick's disease is a rare neurodegenerative disorder first described by Arnold Pick in 1892 (167). It is also regarded as a subtype of FTD, defined neuropathologically by the presence of tau immunoreactive Pick bodies (7). A primary progressive aphasia followed by personality changes is described in the original description by Pick and in several clinicopathological reports over the last decades. Neuropathologically classic Pick's disease is characterized by marked temporal atrophy with or without frontal atrophy. In affected regions there is a striking neuronal loss with characteristic round intracytoplasmatic neuronal inclusions, Pick bodies, in both cortical and subcortical areas (8, 168). The filaments seen in sectioned Pick bodies appear to be mostly 15 nm straight filaments, but twisted filaments have also been described (168). The biochemical composition of the insoluble tau is distinct from that detected in AD, CBD and PSP. Tau protein isolated from brains with Pick's disease reveal two major bands of 60 and 64 kD and a minor band of 68 kD (169) (Figure 4D). These two bands specifically lack the microtubule binding repeat encoded for by exon 10 and thus are composed predominantly by 3R-tau (170). Isoelectric point of pathological tau protein in Pick's disease is less acidic than the isolelectric point of PHF-tau in AD, indicating that the protein is less phosphorylated. This result is strengthened

by the absence of tau phosphorylation on Ser262 in Pick bodies as reported by some authors (171). However, others have not confirmed this finding (172).

Extended association studies performed on a collection of pathologically confirmed Pick cases revealed no apparent association with *tau* H1 and H2 haplotypes (156, 173). These results suggest that polymorphisms in the *tau* gene do not alter the risk for Pick's disease.

Chapter 4

Linkage Analysis and Positional Cloning Related to FTDP-17

4.1 Genetic mapping in 17q21-22

In the last decade many disease genes have been identified using a positional cloning approach, which is based on the knowledge of gene physical location in the genome. Some genetic disorders present cytogenetically visible chromosomal abnormalities, such as large deletions or re-arrangement, giving a direct indication of the chromosomal region involved in a disease. However, in many cases, as in FTDP-17, a disease-causing gene has to be mapped to a chromosomal location by screening the genome with polymorphic markers in multiplex families. Polymorphic markers have more than one allelic variant and they can be therefore used to monitor familial segregation and determine which marker alleles present in the parents are transmitted to the offspring.
Statistical analysis (linkage analysis) is then performed to establish whether or not a certain allele of a polymorphic marker is segregating with the disease trait in a significant way.

In a genetic-epidemiological study of FTD in the Netherlands, aimed to obtain a full ascertainment of FTD patients, two large families (Families I and III) with dementia were identified, whereas a third one (Family II) was reexamined. The latest family has been described before as hereditary Pick's disease despite the absence of Pick's bodies (174). The clinical picture in affected individuals met the criteria for FTD.
Previous genetic studies in other FTD families with similar clinical profiles (see chapter **1.3** in this thesis) prompted us to investigate whether our families were linked to the same region on chromosome 17q21-22. We selected 12 polymorphic markers from the CEPH/Genethon linkage map. Positive lod scores (LOD) were obtained for all three families for a number of markers in the region. None of the individual family was powerful enough to provide significant evidence for linkage itself, but with the combined data of the three families, significant lod scores were obtained for the marker D17S932 ($Z = 4.0$ at $\theta = 0.0$) and D17S934 ($Z = 4.8$ at $\theta = 0.0$). A substantial contribution to these high lod scores came by using in the analysis unaffected individuals that are at risk for the disease. Therefore we reanalyzed markers D17S932 and D17S934, giving all the unaffected individuals diagnosis unknown. The maximum lod score obtained then for marker D17S932 was 2.9 at $\theta = 0.0$ and 3.6 at $\theta = 0.0$ for marker D17S934.
To determine the size of the critical region for the gene responsible, we performed haplotype analysis only in affected individuals for all 12 markers positioned according to the physical map. By comparing marker data we could place the

disease locus telomeric from D17S946 and centromeric from D17S791. The distance between both markers is 5 cM (see chapter **4.4.1** in this thesis).

As described in chapter **1.3**, in October 1996 geneticists and clinicians with FTD families linked to an overlapping region of chromosome 17, organized a conference in Ann Harbor to establish whether these families would constitute a new distinct group with comparable clinical and neuropathological features (26). The linkage found in these families to chromosome 17q21-22 strongly suggested that a single locus is responsible for this new disorder named FTDP-17. By pooling recombination data from all reported families with linkage to chromosome 17q21-22 it was possible to locate the disease locus to a ~2cM region between markers D17S800 and D17S791 (Figure 5).
The gene for the microtubule associated protein tau was an obvious candidate gene for FTDP-17, considering the presence of hyperphosphorylated tau filaments in brains of affected individuals and the fact that it was previously cloned and localized to chromosome 17q21 (27) within the FTDP-17 disease critical region. However, several research groups (175-177) had failed to identify *tau* mutations that segregated with the disease in selected FTDP-17 families.

4.2 Physical mapping in 17q21-22.
We formed then an international consortium with groups from Stockholm, Jacksonville and St Louis, to characterize the FTDP-17 critical region and to find the gene responsible for the disease. In an initial study, in order to narrow down the critical region, we performed a refined genetic mapping in the families with critical recombinants, the Dutch family III and I (our unpublished results). The critical region was then reduced to a 0.5 cM interval flanked by markers D17S1789 and D17S1804, defining respectively the centromeric and telomeric boundaries. We were not able to map the gene for the microtubule associated protein tau within this minimal critical region.

In the classical positional cloning approach after defining the critical region the next step involves physical mapping, during which genomic clones are isolated and constructed in an overlapping contig covering the whole region. Some of the most commonly screened artificial libraries consist of yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), phage artificial chromosomes (PACs) and cosmid clones. Contigs are generated by DNA fingerprinting of shared restriction fragments, or by the use of amplification of sequence tagged sites (STSs). The last technique is especially useful with YACs

clones that in general have inserts too large with high incidence of instability or chimerism to construct detailed restriction map. Finer mapping, at the level of an extended single DNA fiber, can be achieved by using Fiber-FISH technique (178 341, 179 340, 180 339). This method is particular useful in ordering genomic clones and estimating the size of gaps between contigs, obtaining a very high resolution.

Initially we built a physical YAC contig covering the estimated critical region. Inter-Alu PCR probes generated from the YACs were then used to isolate PACs from genomic libraries. Positive PACs were isolated and analyzed by STS, microsatellite content and Fiber-FISH to determine the level of coverage of the region and the relative order of the clones. In addition P1 clones earlier mapped to the region (181, 182) were included in the map. The assembled contig was used to generate a transcript map of the FTDP-17 candidate region (see chapter **4.4.2** in this thesis).

4.3 A transcript map in 17q21-22.

During and after mapping studies, transcripts can be isolated from the critical region using different methods. In this study a combination of techniques was used such as exon trapping and hybridization-based mapping of ESTs (expressed tagged sequences) identified in databases. Exon trapping is based on the detection of coding sequences within genomic DNA, which are selected for by the functional 5' and 3' splices sites present in the DNA by the splicing machinery of COS-7 cells.

By using these approaches we localized 19 known genes, and a large numbers of ESTs to the transcript map. Furthermore seven novel genes were identified by exon trapping and isolated in their full-length sequences. These novel genes were named frontotemporal dementia candidate genes (*FTDCG 1-7*) (see chapter **4.4.2** in this thesis) (183).

A number of genes mapped to the contig during our positional cloning efforts have been considered as candidate genes for FTDP-17 due to their expression pattern and predicted role in neurodegenerative disease process. Several of these genes, such as *GFAP* (glial fibrillary acidic protein), *VHR* (dual-specific phosphatase), *RAPIP8* and *FTDCG2*, have been sequenced in several FTDP-17 families of the consortium. However, we were not able to identify any mutations segregating with the disease in the genes investigated.

As mentioned before the *tau* gene was an obvious candidate. Nevertheless we initially excluded it because previous sequencing efforts yielded negative results and it mapped outside the minimal critical region. However, the diagnosis of the affected individual which showed recombination with marker D17S1804, changed from probable FTD to possible Alzheimer's disease. Consequently we lost the telomeric boundary of the minimal critical region and the *tau* gene was still a full candidate.

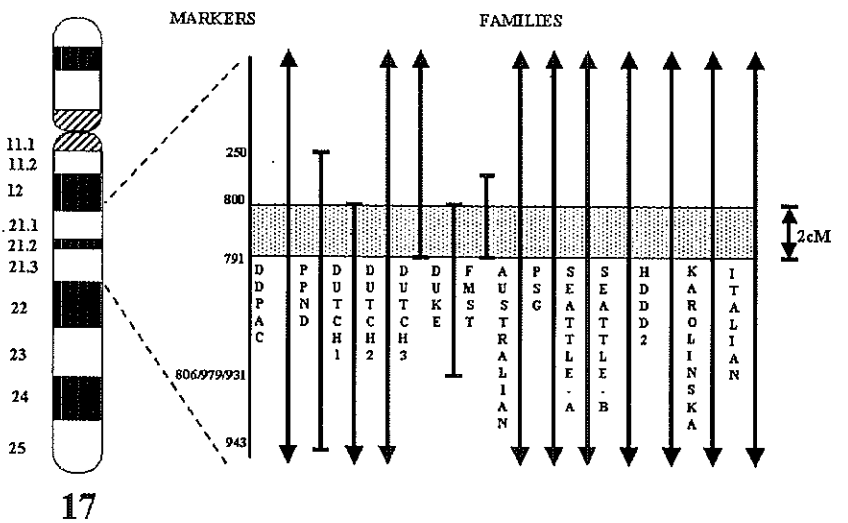

Figure 5. The location of the FTDP-17 locus is shown on an ideogram of a metaphase chromosome. The locations of the genetic markers shown are based on the 1996 Genethon map and they are not drawn in scale. Next to each family identification is a line that indicates the probable location of the disease gene for that family. Arrows indicate that the disease gene could be in a region that extends beyond the map shown. The minimal sharing region between families (~2 cM) is indicated with a black bar.

Chapter 4.4

Publications

Chapter 4.4.1

Hereditary frontotemporal dementia is linked to chromosome 17q21-22: a genetic and clinicopathological study of three Dutch families.

P. Heutink, M.Stevens, P. Rizzu, E. Bakker, J.M. Kros, A. Tibben, M.F. Niermeijer, C.M. van Duijn, B.A.Oostra, J.C. van Swieten

Ann Neurol 1997;41:150-159

Hereditary Frontotemporal Dementia Is Linked to Chromosome 17q21–q22: A Genetic and Clinicopathological Study of Three Dutch Families

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Hereditary frontotemporal dementia (HFTD) is a rare autosomal dominant form of presenile dementia characterized by behavioral changes and reduced speech. Three multigeneration kindreds with this condition, in the Netherlands, were investigated for clinicopathological comparison and linkage analysis. Frontotemporal atrophy on computed tomographic scanning and/or magnetic resonance imaging was usually present. Single-photon emission computed tomography (SPECT) showed frontal hypoperfusion in the early phase of the disease. Brain tissue showed moderate to severe atrophy of frontal and temporal cortex with neuronal loss, gliosis, and spongiosis. Pick bodies were lacking in all cases of the 3 families. The mean age of onset varied significantly between families. We report here evidence for linkage to chromosome 17q21–q22 with a maximum lod score of 4.70 at $\Theta = 0.05$ with the marker D17S932. Recombination analysis positions the gene for HFTD in a region of approximately 5 cM between markers D17S946 and D17S791. Three other neurodegenerative disorders with a strong clinical and pathological resemblance have recently been mapped to the same chromosomal region, suggesting that a group of clinically related neurodegenerative disorders may originate from mutations in the same gene.

Heutink P, Stevens M, Rizzu P, Bakker E, Kros JM, Tibben A, Niermeijer MF, van Duijn CM, Oostra BA, van Swieten JC. Hereditary frontotemporal dementia is linked to chromosome 17q21–q22: a genetic and clinicopathological study of three Dutch families. Ann Neurol 1997;41:150–159

Hereditary frontotemporal dementia (HFTD) is the familial form of frontotemporal dementia (FTD), a rare, mostly sporadically occurring and predominantly presenile dementia [1–4]. The characteristic frontal and temporal lobar atrophy was originally described by Arnold Pick in 1892 [5]. Gans [6] in 1923, and later others, reported families with an autosomal dominant inheritance pattern [7–10]. A diagnosis of Pick's disease, the best known type of FTD, is now set aside for cases with so-called Pick bodies [11, 12], and several authors introduced diagnostic descriptions for new entities of frontal atrophy without Pick bodies [13–17]. The main clinical features of FTD are personality changes, a disinhibited and inappropriate behavior, hyperorality, stereotyped and perseverative behavior, emotional and social indifference, aspontaneity, loss of judgment and insight, and speech reduction [1, 13]. Molecular genetic studies of HFTD failed to find mutations in the genes implicated in Alzheimer's disease or the mutation in the prion protein gene involved in Creutzfeldt-Jacob disease [18–20]. More recently, linkage to chromosome 17 has been reported in a family with disinhibition–dementia–parkinsonism–amyotrophy complex (DDPAC), 2 families with progressive subcortical gliosis (PSG), and a family with pallido–ponto–nigral degeneration (PPND) [21–23]. The disease in these families shows a strong clinical and pathological resemblance to HFTD, as reported in this study [24–26].

To investigate whether HFTD could also be linked to the same region on chromosome 17q21–q22, we performed a linkage study with 3 large families with

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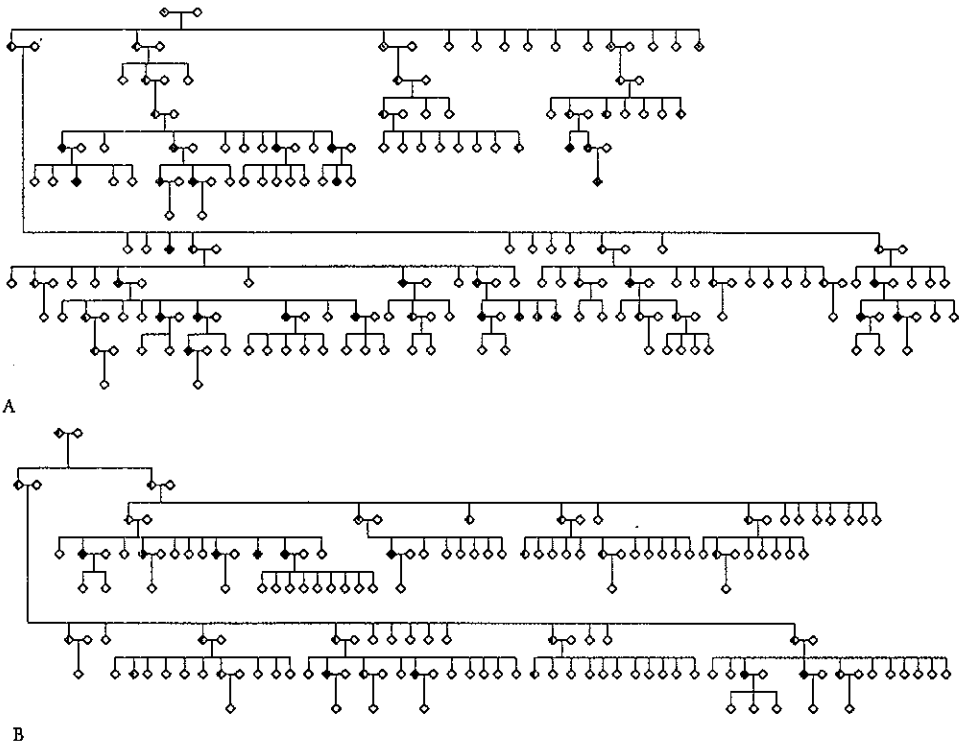


Fig 1. (A) Pedigree of Family I. (B) Pedigree of Family III. As the disorder shows clear autosomal dominant inheritance, sex designation of family members has been omitted for privacy reasons. ◇ = unaffected; ◇ = two different spouses; ◈ = possibly affected; ◐ = affected (dementia); clinical information insufficient to establish diagnosis FTD; ◆ = affected (fronto-temporal dementia); sufficient clinical information and/or pathologically confirmed.*

HFTD that were ascertained in the Netherlands. Here, we report evidence for linkage of HFTD to chromosome 17q21–q22, the same chromosomal region where DDPAC, PPND, and PSG have previously been localized.

Materials and Methods

Family Studies

In a genetic–epidemiological study of FTD in the Netherlands, we aimed to obtain a full ascertainment of FTD patients by addressing all neurologic, psychiatric, geriatric, and nursing homes to report their patients twice a year. In this study, 2 large families (Families I and III) with dementia were identified, whereas a third one (Family II) was reexamined. These families were selected for linkage analysis because of their strong clinical and pathological similarities. The second family has been described before as hereditary Pick's disease, despite the absence of Pick bodies [2–4]. Dementia is transmitted as an autosomal dominant disorder in all 3 families [4] (Fig 1B). The clinical picture in affected individuals meets the criteria for FTD [1]. The age at which behavioral changes were reported by more than 1 relative was considered as age of onset. Diagnosis of living patients was established using extensive neuropsychological testing and brain computed tomographic (CT) scanning and/or magnetic resonance imaging (MRI); diagnosis of deceased patients was established either on pathology findings or on available medical records and family interviews.

Family I

The first family consists of 2 sisters with dementia in the first generation and 49 of 160 offspring (28 men and 21 women) of these 2 sisters in the subsequent five generations (see Fig 1A). The diagnosis of FTD was established in 8 living patients. Sufficient clinical information and family history were available on 18 affected relatives to allow establishing age of onset and diagnosis of HFTD, whereas the type of dementia could not be specified by lack of detailed information in the remaining affected family members. A neuropsychological assessment was performed in 15 of 18 patients.

Ten patients had CT scanning; 3 cases had a single-photon emission computed tomographic (SPECT) scan and 3 an MRI scan. Neuropathological examination in all 14 autopsied cases confirmed HFTD.

Family II

This family with HFTD was previously documented by Schenk and others [2–4]. There are seven generations with 34 affected relatives (14 men and 20 women). CT scans were performed in 6 patients and SPECT scanning in 1 patient. Neuropathological examination in 15 patients confirmed the clinical diagnosis of HFTD. After the last report another 2 affected cases were identified (V14 and V21) [4].

Family III

Dementia was first recognized in 1 male, and subsequently another 29 affected relatives were identifed (see Fig 1B). Relevant clinical data are available on 10 patients, CT scanning and neuropsychological assessment in 7 patients, and SPECT scanning in 2 patients. Neuropathological studies in 1 patient confirmed the diagnosis of HFTD.

After written consent, DNA was isolated from peripheral blood leukocytes as described by Miller and colleagues [27]. Blood was taken from 5 patients from Family I, 2 patients from Family II, and 5 patients from Family III. DNA samples were also obtained from 62 healthy relatives with 50% risk for developing dementia (31, 15, and 16 individuals, respectively).

This study was approved by the Medical Ethics Committee of the University Hospital Rotterdam.

Pathology

Brain specimens of different cortical regions, basal ganglia, and pons from 11 cases (5 from Families I and II each, and 1 from Family III), were reexamined for Pick bodies, ballooned cells, and immunohistochemical features. Immunohistochemistry included the following antibodies: ubiquitin (1:125; Novocastra), τ (1:400; Dako, no. A 024), an antibody against paired helical filaments (PFHs) (1:100; ICN), an antibody against somatodendritic microtubule-associated protein (MAP-2) (1:170; Zymed), and β-amyloid antibody (1:600; Novocastra). Staining with a conventional peroxidase anti-peroxidase method was done. Age- and sex-matched controls (normal and with Alzheimer's disease) were used for comparison.

DNA Studies

Simple sequence length polymorphisms (SSLPs) from chromosome 17q21–q22 were amplified from 50 ng of genomic DNA. One primer from each pair was end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The amplification was performed essentially as described [28] except for markers D17S946 and D17S932 for which 7.5% dimethyl sulfoxide was added to the reaction mix. Analysis of SSLPs was performed on a denaturing 6% acrylamide gel.

Chromosome 17 markers were obtained from the CEPH/Genethon linkage map [29]. Marker order was obtained by combining data from the CEPH/Genethon linkage map and the Whitehead physical map [30] and is shown in Figure 2.

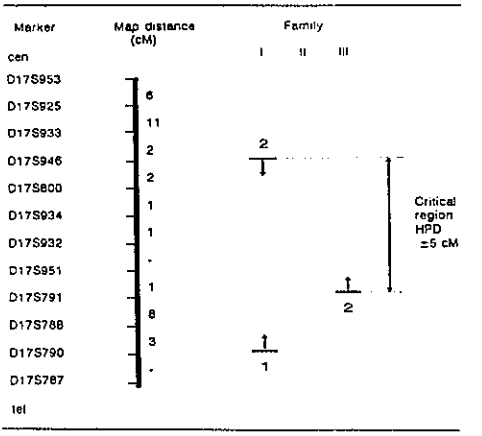


Fig 2. Schematic map of the hereditary frontotemporal dementia (HFTD)-linked region on chromosome 17q21–q22 with sex-averaged distances based on the CEPH/Genethon linkage map [29]. For each family, the number of recombinational events in affected individuals is indicated with an arrow *accompanied by the number of recombinations observed. The* double-sided arrow *indicates the minimum critical region for HFTD.*

Table 1. Liability Classes Used in the Linkage Analysis

Liability Class	Penetrance
Class 1	1.00 < married in or affected
Class 2	0.95 < unaffected, at risk >50 yr
Class 3	0.75 < unaffected, at risk, between 40 and 50 yr
Class 4	0.10 < unaffected, at risk <40 yr

Linkage Analysis

Pairwise lod scores were calculated for each family by using the MLINK program of the LINKAGE programs package (version 5.1) [31], assuming HFTD to be an autosomal dominant disease with a gene frequency of 0.0001. The mean age of onset in the available families was 51 years (range, 43–75 years). The late age of onset was accounted for by defining 4 liability classes with different penetrance values (Table 1). Phenocopy rate was estimated to be 0.1% for individuals older than 50 years of age and 0.01% for individuals younger than 50 years of age. Mutation rate was set at zero and equal recombination rates for males and females were assumed because of the variable recombination ratio between males and females in this region [29, 30]. Marker allele frequencies were kept equal because allele frequencies from the Dutch population were not available. Calculation of pairwise lod scores with allele frequencies calculated from individuals marrying into the HFTD kindreds did not substantially alter results (<10%).

Results

Demographic Data and Clinical Features

The age of onset was comparable in Families I and II (mean, 50.4 and 46.5 years, respectively); all but 1 patient developed symptoms before the age of 57. In Family III, the mean age of onset was 63.4 years (range, 57–75 years), which is significantly different ($p < 0.001$). The mean duration of illness was similar for the 3 families (8.2–8.7 years; range, 4–16 years). The mean age of death in Family III (71.9 years; range, 63–81 years) again differed significantly ($p < 0.001$) from those in Families I and II (58.6 and 54.7 years, respectively). The mean ages of onset and of death were constant over consecutive generations. The male-to-female ratio of patients in Family II was 2 to 5, but equal to 1 in Families I and III. There was a remarkable uniformity in clinical symptoms and progression of the disease within each family.

Disinhibition, including aggressive behavior, stealing, jocularity, and/or obsessional behavior, was the presenting symptom in all patients of Family I and Family II. Loss of initiative was the prominent presenting feature in Family III and developed later in Families I and II. Hyperorality, roaming behavior, restlessness, and stereotyped behavior developed often during the course of the illness. Spontaneous speech became gradually reduced in all, resulting in a state of mutism. Mild memory problems were common.

Neurological examination was always normal in the early phase of the disease, except sometimes for frontal release reflexes. The progression of the disease was quite similar in these families. Pyramidal and extrapyramidal signs occurred in several patients in the late phase of the disease (Table 2). Neuropsychological assessment showed frontal dysfunction in all patients of the 3 families. Perseveration, impaired attention, decreased mental shifting, impaired executive skills, and speech reduction reflecting frontal lobe dysfunction were found in combination with intact orientation and memory functions at neuropsychological assessment.

Electroencephalography, serum levels of vitamins, syphilis reactions, and/or thyroid function tests, performed in most patients, were always normal. CT scanning after a mean duration of illness of 2.5 years (range, 1–5 years) showed frontal atrophy (mild in 6, moderate to severe in 9 patients) in 15 cases from the 3 families, whereas CT scan was normal in 4 patients. Increased signal intensities in the subcortical white matter of the frontal lobe on T2-weighted MRI images were found in 2 patients of Family I. Frontotemporal hypoperfusion on SPECT was found in all 6 investigated patients.

The brain weights at autopsy from Families I and II were strongly reduced (mean, 1,035 and 920 gm, respectively); 1 case of Family III was 1,170 gm. Moderate to severe atrophy of the frontal lobe was present

Table 2. Comparison of Clinical and Pathological Features Between Three Chromosome 17–linked Neurological Disorders and Three Families with Hereditary Frontotemporal Dementia

	PPND	DDPAC	PSG	HFTD
Number of patients	26	12	8	49
Mean age of onset (yr)	43	45	46	51
Presenting symptoms				
Personality and behavioral changes	10	12	6	49
Dementia	5	–	–	–
Parkinsonism	14	1	–	–
Subsequent manifestations				
Dementia	26	12	8	49
Supranuclear palsy	15	–	–	–
Extrapyramidal signs	25	11	7	10
Pyramidal signs	16	3	1	7
Amyotrophy	–	1	–	–
Neuropathology	n = 4	n = 6	n = 7	n = 30
Macroscopy				
Frontal atrophy	4*	6	7	30
Microscopical involvement				
Frontal cortex	∓	++	++	++
Temporal cortex	∓	++	++	++
Caudate nucleus	+	nm	∓	+
Substantia nigra	4	6	4	9
Hippocampus	∓	∓	+	–
Amygdala	∓	++	nm	∓
Thalamus	∓	nm	∓	∓
Spinal cord	nm	∓	∓	–
Ballooned cells	nm	+	–	+
Pick bodies	nm	nm	–	–

Numbers indicate the number of patients for whom feature was observed.

nm = not mentioned; – = not affected; ∓ = not generally affected; + = generally affected; ++ = prominently affected; * = mild generalized atrophy described. PPND = pallido–ponto–nigral degeneration; DDPAC = disinhibition–dementia–parkinsonism–amyotrophy complex; PSG = progressive subcortical gliosis; HFTD = hereditary frontotemporal dementia.

in all cases of Families I to III, as well as atrophy of the anterior part of the temporal lobe. The caudate nucleus was atrophied in 9 cases of Families I and II each, and in 1 patient of Family III. Neuronal loss, gliosis, and spongiosis were found in the frontal and temporal cortex, in the absence of senile plaques and neurofibrillary tangles. The substantia nigra was degenerated in 4 brains of Families I and II each, and in 1 patient of Family III. Neuronal loss in the olivary nuclei was found in 3 brains. Some cases of Families I and II showed white matter changes (demyelination and/or gliosis). Ballooned cells in the cortex and/or basal ganglia were found in a number of cases of Families I and II, whereas these cells were absent in 1 case of Family III. Pick bodies were lacking in brains of the 3 families, except sporadically in 1 brain from Family II. At reexamination of 10 available brain specimens, no Pick bodies were found at all. Immunohistochemical studies were negative for τ or ubiquitin. In 2 pa-

Table 3. Two-Point Lod Scores for Chromosome 17 Markers and HFTD

Marker	Family	Recombination Fraction (Θ)						
		0.000	0.010	0.050	0.100	0.200	0.300	0.400
D17S953	I	−3.96	−1.31	−0.59	−0.29	−0.04	0.02	0.08
	II	−1.29	−1.14	−0.77	−0.52	−0.25	−0.10	−0.02
	III	−2.18	−1.96	−1.35	−0.87	−0.36	−0.12	−0.02
	Total	−7.43	−4.41	−2.71	−1.68	−0.65	−0.20	−0.04
D17S925	I	−0.43	−0.37	−0.19	−0.05	0.07	0.08	0.05
	II	−8.16	−1.21	−0.54	−0.29	−0.10	−0.03	−0.01
	III	−1.89	−1.70	−1.17	−0.76	−0.30	−0.08	0.01
	Total	−10.48	−3.28	−1.90	−1.10	−0.33	−0.03	0.05
D17S933	I	−3.55	−1.33	−0.63	−0.34	−0.14	−0.06	−0.01
	II	−8.14	−0.63	−0.02	0.16	0.20	0.11	0.03
	III	−0.96	−0.83	−0.46	−0.21	0.00	0.04	0.01
	Total	−12.65	−2.79	−1.11	−0.41	0.06	0.09	0.03
D17S946	I	1.88	1.84	1.67	1.43	0.95	0.48	0.12
	II	0.34	0.33	0.29	0.24	0.17	0.10	0.04
	III	−2.30	−1.96	−1.20	−0.71	−0.24	−0.06	−0.01
	Total	−0.08	0.21	0.76	0.96	0.88	0.52	0.15
D17S800	I	−0.35	−0.32	−0.23	0.14	−0.04	0.00	0.01
	II	−0.18	−0.18	−0.15	−0.12	−0.07	−0.03	−0.01
	III	0.92	0.94	0.98	0.96	0.77	0.47	0.15
	Total	0.39	0.52	0.60	0.70	0.66	0.44	0.15
D17S934	I	2.76	2.71	2.50	2.22	1.58	0.91	0.32
	II	1.35	1.33	1.23	1.10	0.82	0.49	0.16
	III	0.17	0.20	0.27	0.26	0.16	0.05	−0.02
	Total	4.28	4.24	4.00	3.58	2.56	1.45	0.46
D17S932	I	2.64	2.59	2.38	2.10	1.49	0.86	0.29
	II	1.61	1.57	1.44	1.27	0.90	0.50	0.14
	III	−3.98	0.36	0.89	0.96	0.79	0.47	0.14
	Total	0.27	4.52	4.71	4.33	3.18	1.83	0.57
D17S951	I	2.07	2.05	1.93	1.74	1.26	0.71	0.21
	II	0.68	0.67	0.61	0.53	0.37	0.21	0.07
	III	−0.45	−0.29	0.08	0.27	0.34	0.22	0.07
	Total	2.30	2.43	2.62	2.54	1.97	1.14	0.35
D17S791	I	1.55	1.54	1.42	1.25	0.85	0.46	0.16
	II	0.80	0.77	0.68	0.57	0.38	0.21	0.07
	III	−5.12	−0.70	0.05	0.31	0.38	0.25	0.08
	Total	−2.77	1.61	2.15	2.13	1.61	0.91	0.31
D17S788	I	−2.81	−0.79	−0.17	0.03	0.12	0.08	0.02
	II	−∞	−0.42	0.17	0.34	0.36	0.23	0.07
	III	−13.03	−2.86	−1.35	−0.72	−0.19	−0.02	0.01
	Total	−∞	−3.27	−1.35	−0.35	0.29	0.29	0.10
D17S790	I	−1.82	−0.02	0.60	0.77	0.73	0.48	0.18
	II	−∞	−0.20	0.38	0.53	0.50	0.33	0.12
	III	−5.62	−1.43	−0.74	−0.45	−0.19	−0.09	−0.03
	Total	−∞	−1.65	0.24	0.85	1.04	0.72	0.27
D17S787	I	2.60	2.57	2.43	2.20	1.65	1.02	0.39
	II	−∞	−0.41	0.18	0.35	0.37	0.24	0.08
	III	−8.40	−2.46	−1.47	−0.92	−0.35	−0.11	−0.03
	Total	−∞	−0.30	1.14	1.63	1.67	1.15	0.44

HFTD = hereditary frontotemporal dementia.

tients scattered mild diffuse and granular staining for MAP-2 was visible in both neurons and glia.

Linkage

Three linkage reports [21–23] of variable forms of frontal lobe dementia prompted us to investigate whether the families with HFTD described in this study were linked to the same region on chromosome 17q21–q22. We selected 12 SSLPs from the CEPH/Genethon linkage map. There was a discrepancy in marker order between the CEPH/Genethon linkage map and the Whitehead physical map for markers D17S932 and D17S934 [29, 30]. In our study we used the marker order obtained by physical mapping data from the Whitehead map. Positive lod scores were obtained for a number of markers in the region for all 3 families. Table 3 summarizes the pairwise lod scores between HFTD and the 12 chromosome 17q markers. None of the individual families was powerful enough to provide significant evidence for linkage by itself, but with the combined data of the 3 families significant lod scores were obtained for the marker D17S932 ($Z = 4.70$ at $\Theta = 0.05$) and D17S934 ($Z = 4.28$ at $\Theta = 0.00$).

A substantial part of the information that contributed to these lod scores in these families comes from unaffected individuals that are at risk for the disease. We therefore reanalyzed D17S932 and D17S934, giving all the unaffected individuals a diagnosis unknown. The maximum lod score obtained with this analysis for marker D17S932 was 2.99 at $\Theta = 0.00$. For marker D17S934, the maximum lod score obtained was 3.46 at $\Theta = 0.00$.

The marker order and intermarker distances of the available genetic and physical maps do not always concur. Therefore multipoint linkage analysis can generate a confidence interval that later would prove to be inaccurate. To strengthen our findings, we performed haplotype analysis for all 12 markers positioned according to the physical map. In a number of cases the haplotype of the markers for the affected individuals could not be determined because of the unavailability of first-degree relatives (data not shown). As a result it could not be determined whether marker alleles were identical by descent (IBD) or just identical by state (IBS). To determine the size of the critical region for the gene responsible for HFTD, we therefore compared marker data for affected individuals only (Fig 3A–C).

The 5 patients from Family I share a common allele for 6 of the markers tested (see Fig 3A). On the centromeric side, no sharing was found for D17S953, D17S925, and D17S933, and D17S946. On the telomeric side, no sharing was obtained for D17S790 and D17S787. The 2 patients in Family II (see Fig 3B) share a common allele for all 12 markers that were tested, and no recombination event could be detected by comparing inherited alleles. The linkage analysis, however, revealed recombination events in this family for markers D17S788, D17S790, and D17S787 (see Table 3). In Family III (see Fig 3C), the patients share at least one allele for almost all markers except for markers D17S791, D17S788, and D17S787. At least two recombination events must have taken place in this family, since only 3 of 5 patients share a common allele for D17S791.

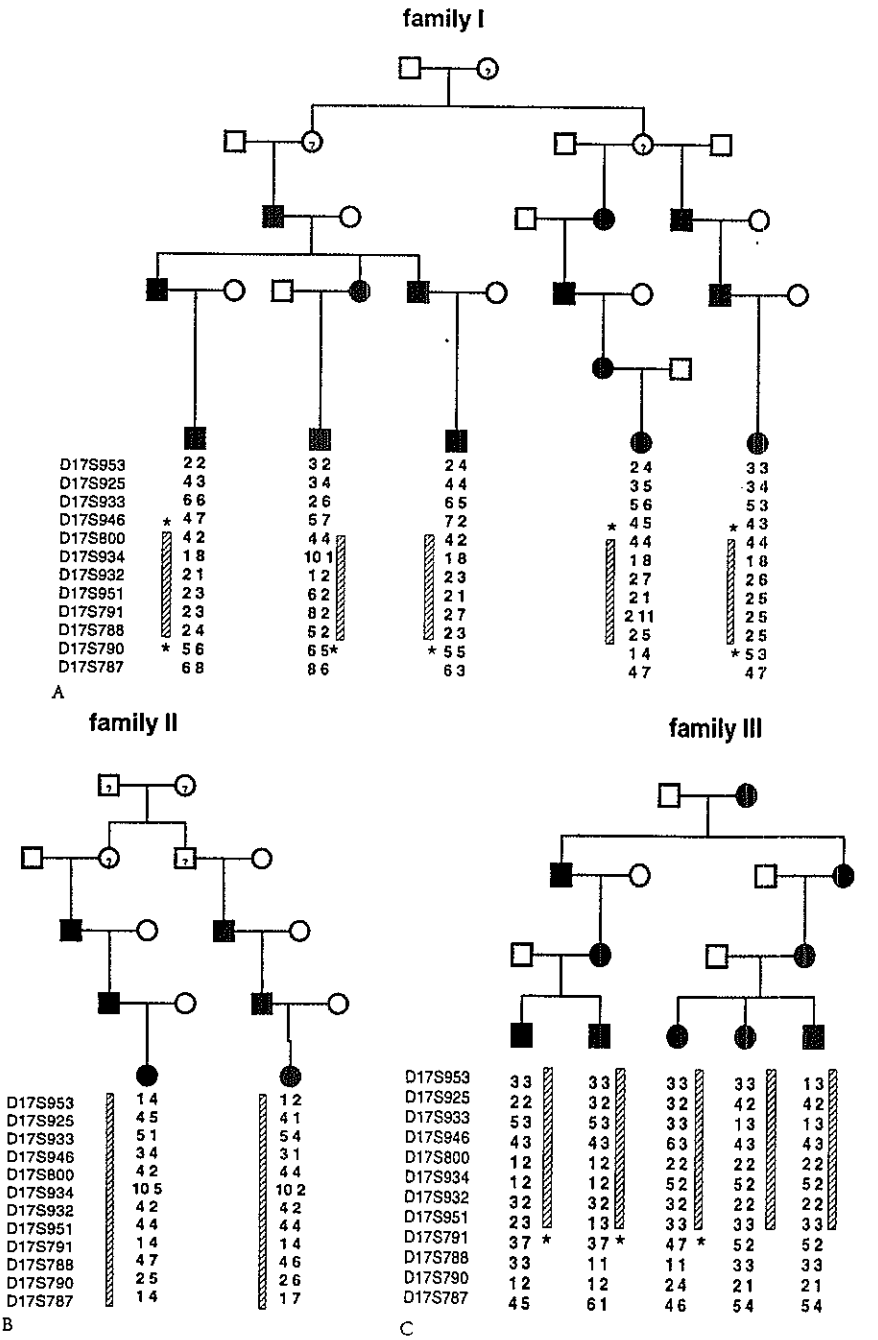
These data place the HFTD locus telomeric from D17S946 and centromeric from D17S791. The distance between both markers is 5 cM on the sex average linkage map (see Fig 2) [29]. A common ancestor for the 3 families could not be traced genealogically. The patients from the 3 families do not share a common disease haplotype. This indicates that HFTD in the 3 families is caused by independent mutational events.

Discussion

The 3 families with HFTD described in this study show strong clinical and pathological similarities. All patients presented with behavioral changes followed by mutism, and sometimes by pyramidal and/or extrapyramidal signs in the final phase. Selective atrophy of the frontal and temporal lobe is the characteristic feature on CT/MRI and at neuropathological examination in all 3 families. Anterior hypoperfusion on SPECT was a common finding. The unifying pathological feature in all 3 families was the selective frontotemporal atrophy with aspecific features. Although ballooned cells were present in some cases, Pick bodies were lacking in all.

There are also some clinical differences between the 3 families. The mean age of onset in Family III was significantly higher than in the other 2 families. Disinhibition was the presenting clinical symptom in Families I and II, but in patients of Family III, loss of initiative was the first symptom. Caudate atrophy, degeneration of the substantia nigra, and white matter involvement found in some brains of the 3 families might reflect intrafamilial phenotypical variation. Similar observations are reported in hereditary dysphasic dementia and other conditions with a descriptive diagnosis [16, 24, 26]. Ballooned cells were present in some brains of Families I and II. That they were lacking in the only autopsied case of Family III does not indicate a pathological distinction from Families I and II, since ballooned cells were not found in several brains from these families either.

Family II has been cited as hereditary Pick's disease (HPD) in McKusick Mendelian Inheritance of Man [32]. However, if Pick bodies are essential for diagnosis of Pick's disease, according to the criteria, this family should not be considered to have Pick's disease, since Pick bodies were lacking in all cases. It is even doubtful if other earlier reported families did have HPD, since


family I
D17S953 22 32 24 24 33
D17S925 43 34 44 35 34
D17S933 66 26 65 56 53
D17S946 * 47 57 72 * 45 * 43
D17S800 42 44 42 44 44
D17S934 18 101 18 18 18
D17S932 21 12 23 27 26
D17S951 23 62 21 21 25
D17S791 23 82 27 211 25
D17S788 24 52 23 25 25
D17S790 * 56 65* * 55 14 * 53
D17S787 68 86 63 47 47
A
family II
D17S953 14 12
D17S925 45 41
D17S933 51 54
D17S946 34 31
D17S800 42 44
D17S934 105 102
D17S932 42 42
D17S951 44 44
D17S791 14 14
D17S788 47 46
D17S790 25 26
D17S787 14 17
B
family III
D17S953 33 33 33 33 13
D17S925 22 32 32 42 42
D17S933 53 53 33 13 13
D17S946 43 43 63 43 43
D17S800 12 12 22 22 22
D17S934 12 12 52 52 52
D17S932 32 32 32 22 22
D17S951 23 13 33 33 33
D17S791 37 * 37 * 47 * 52 52
D17S788 33 11 11 33 33
D17S790 12 12 24 21 21
D17S787 45 61 46 54 54
C

most of these families did not show Pick bodies [7–10]. Taking into account the contribution of Arnold Pick to the recognition of this type of dementia [5], one might also redefine and reintroduce the diagnosis of Pick's disease for all cases with frontotemporal atrophy. In that case, frontal atrophy with Pick bodies should be considered a subtype.

We report linkage of HFTD in 3 Dutch families to chromosome 17q21–q22. All families generated positive lod scores with several markers from this chromsomal region. None of the families is informative enough to generate a significant lod score by itself, but combining the data from all families gives significant evidence for linkage. The clinical heterogeneity of Family III compared with Families I and II is not reflected in the linkage results. Family III also generated positive lod scores for a number of markers (see Table 3). Two unaffected individuals in this family share part of the haplotype that is found in patients (data not shown). These individuals are 70 and 75 years of age respectively, and in the linkage analysis we assumed 95% penetrance of the disease phenotype at that age. For this reason these individuals are regarded by the linkage program as likely recombinant cases, resulting in low lod score curves that had their peak at a considerable recombination value from markers. Considering the later onset of the disease in this family, the linkage parameters that were used are probably too conservative for this specific family. It is still unclear whether these individuals will still develop the disease phenotype. Further support that Family III is indeed linked to chromosome 17q21 comes from the fact that all 5 patients share a common allele for more than 20 cM on chromosome 17q21–q22 (see Figs 2 and 3C).

Comparison of marker alleles revealed recombination events in affected individuals with a number of markers. Recombination events with the markers D17S946 and D17S791 define the boundaries of the critical region. According to the CEPH/Genethon linkage map, these markers are separated by a genetic distance of approximately 5 cM. The 3 families do not share a common "disease" haplotype, suggesting that independent mutations are responsible for the onset of the disease in these families.

For a number of markers, it could not be determined whether the shared alleles were IBD or IBS. We are currently constructing hybrid cell lines of all available patients in order to separate the disease chromosome and the healthy chromosome. This will enable us to determine whether shared alleles are IBD or IBS. These data could reduce the critical region further.

A large number of genes have been localized on chromosome 17q. Several of them are involved in neurological functions or diseases and could be regarded as candidate genes for HFTD. Glial fibrillary acidic protein is an intermediate filament protein that is highly specific for cells of astroglial lineage (glial fibrillary tangles). The level of protein expression is elevated in patients with Alzheimer's disease, Down's syndrome, and scrapie infection [33–35]. The exact localization of this gene on chromosome 17q is unclear [36]. The nerve growth factor receptor (NGFR) is able to bind nerve growth factors and is an essential component in the survival and maintenance of sympathetic and sensory neurons [37, 38]. NGFR was located on a single restriction fragment of 500 kb with the HOX2B gene [39]. According to the mapping data in the Human Genome Database [40], the HOX2B gene is localized within the critical region for HFTD.

The microtubule-associated protein τ that was localized on the long arm of chromosome 17 appears to be involved in the maintenance of axonal cytoskeletal structure. The gene is expressed in neurons and its transcript is subject to alternative splicing and posttranscriptional modifications. These modifications can lead to the formation of the PHF, which is a major component of neurofibrillary tangles. Neurofibrillary tangles in Alzheimer's disease [41, 42] and neurofilaments in Pick bodies, as observed in sporadic patients with Pick's disease, stain intensively with antibodies against phosphorylated τ [43]. However, in brain tissue of cases of HFTD from this study, no Pick bodies were found; ballooned cells were observed, but they did not stain with antibodies against τ. Genetic mapping on radiation hybrids places τ between markers D17S190 and D17S409 [44]. These markers are not part of the CEPH/Genethon map that was used for this study, and it is difficult to determine whether τ or one of the other genes mentioned above is localized within the critical region. We are currently mapping these genes into the CEPH/Genethon linkage map by using the GeneBridge 4 radiation hybrid mapping panel [45].

This study demonstrates linkage in 3 families with HFTD to chromosome 17q21–q22. Recently, linkage

Fig 3. Marker data for chromosome 17q21–q22 markers for all available patients in the 3 families with hereditary frontotemporal dementia. Markers are oriented from the centromeric side to the telomeric side. The hatched boxes *indicate the maximum region of allele sharing between patients. *Alleles that are shared only by some patients in the family. Only relatives leading to a common ancestor are indicated. (A) Family I. (B) Family II. (C) Family III.* Filled symbols *represent affected individuals,* question marks *in symbols indicate that insufficient data were available to determine diagnosis,* open symbols *indicate unaffected individuals.*

was reported to the same region of the three hereditary neurological disorders with a very strong clinical and pathological resemblance (see Table 2): DDPAC [21], PSG [22], and autosomal dominant parkinsonism and dementia with PPND [23]. Now an important question is whether these chromosome 17q21–q22 disorders are caused by mutations in the same gene or whether there are different genes in this region that are responsible for these disorders.

The three conditions show considerable clinical and pathological overlap with HFTD in age of onset, presenting and subsequent symptoms, and most affected brain regions [24–26] (see Table 2). All four conditions share a presenile age at onset, and most patients show personality and behavioral changes as an initial manifestation. The mean age of onset between families varies, but this difference is smaller than the intrafamiliar differences in age of onset. Brain tissue reveals only general features of degeneration like neuronal cell loss and gliosis. Pick inclusion bodies are absent in all cases. Varying degrees of frontal lobe atrophy were present in all cases of DDPAC, PSG, and HFTD. In the PPND family, mild generalized atrophy with mild neuronal loss and gliosis was found. Degeneration of subcortical structures (caudate nucleus, hippocampus, and substantia nigra) showed a rather similar pattern in all families. There are, however, also some differences within the "chromosome 17 families." First, parkinsonism was the only presenting symptom in 14 of 26 affected family members of the PPND family but only in 1 of 69 patients of the other families. During the course of the disease, parkinsonism is also observed in the majority of the patients with DDPAC and PSG and in 10 of 49 patients with HFTD. The absence of parkinsonism in the majority of HFTD patients must be interpreted with caution because most patients had their neurological examination in an early phase of the illness. The actual frequency of parkinsonism might have been higher in later phase of the illness.

Second, 5 patients from the PPND family had dementia as the presenting symptom, whereas in the other families these were personality and behavioral changes. To determine if these manifestations show overlap requires a detailed comparison of clinical data. Finally, there was a significant difference in age of onset between the first 2 families and the third family of the present study. Even if there are some clinical and pathological differences between the phenotypes of HFTD, DDPAC, and PSG, these are no basis for a sharp differentiation into separate entities. Also the presentation of parkinsonism in PPND seems different from the other families, but it needs additional study to establish if this is phenotypic variation or locus heterogeneity.

At this moment it is unknown if these four conditions are genetically related. The published data do not allow definition of an overlapping critical region for the four disorders. The critical region for the responsible gene(s) for PSG and DDPAC has not yet been determined; multipoint linkage analysis for DDPAC suggested a localization between D17S800 and D17S787, but these borders are based on healthy "at risk" individuals and must be interpreted cautiously. The critical region for PPND was determined based on recombination events in affected individuals. This region, between markers D17S250 and D17S943, partly overlaps with the critical region for HFTD reported in this study.

The currently available data suggest that all four disorders might very well be caused by different mutations in the same gene or even by variant expressions of a single mutation. Another explanation might be different genes, localized in close proximity of each other, being responsible for the four linked neurological diseases. The answer to this question requires further dissection of this region and the identification of its genes.

In conclusion, HFTD is part of a group of neurodegenerative diseases with striking clinical and pathological similarities. The critical regions for all four disorders show considerable overlap on chromosome 17q21. The available clinical and genetic data suggest that this group of disorders might be considered phenotypic variants of the same disorder. The discussion of whether the group of "chromosome 17–linked neurological diseases" should be considered as separate entities, as subtypes of HFTD, or even hereditary Pick's disease, will only be resolved after the identification of the responsible gene defect for these disorders.

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Chapter 4.4.2

Construction of a detailed physical map of the FTDP-17 candidate region on chromosome 17q21

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Construction of a Detailed Physical and Transcript Map of the FTDP-17 Candidate Region on Chromosome 17q21

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Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) is an autosomal dominant condition clinically characterized by behavioral, cognitive, and motor disturbances. Until now, at least 13 different FTDP-17 families that show linkage to chromosome 17q21 have been described. To characterize the FTDP-17 candidate region, flanked by the markers D17S1789 and D17S1804, we constructed a physical map in P1 and PAC clones. A detailed transcript map was generated by positioning known genes and EST clusters to the physical map. In total, we investigated 150 STSs mapped to this region. In addition, novel transcripts were isolated by exon-trapping. We were able to localize 19 known genes and a number of ESTs to this chromosomal region. Furthermore, seven novel genes were identified for which we isolated the full-length sequence. © 1999 Academic Press

INTRODUCTION

Frontotemporal dementia (FTD) accounts for 3–10% of all dementias and can be described as nonspecified degenerative dementia with frontal and temporal lobar atrophy (Brun, 1987; Knopman, 1993). Thirty to 50% of the FTD cases seem to have a positive family history of dementia (Gustafson, 1987; Neary *et al.*, 1990; Stevens *et al.*, 1998), and two disease loci have so far been identified for autosomal dominant forms of the disease. At least 13 families have been described showing linkage to chromosome 17q21, which now is referred to as "frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17)" (for review see Foster *et al.*, 1997). In addition, a large Danish kindred with unspecified dementia has been mapped to chromosome 3 (Brown *et al.*, 1995).

Examination of the haplotype analysis for all 13 FTDP-17 kindreds that display significant evidence of linkage to chromosome 17q21 revealed a 2-cM region that cosegregates with disease in all individual families. Assuming that FTDP-17 is a single gene disorder, a candidate region for the FTDP-17 gene defect could thus be defined between D17S800 and D17S791. Extensive haplotype analysis in two Dutch FTDP-17 families enabled us to refine the candidate region to a 1.5-cM interval flanked by the markers D17S1789 and D17S1804 (unpublished results).

Generation of a high-resolution map of a genomic region is often essential for the positional cloning of disease genes. It provides a good tool for generating novel markers, which is important for minimizing a candidate region by haplotype analysis. Moreover, it is essential for the identification of novel transcripts by techniques such as exon-trapping and cDNA selection. In addition, it can serve as a template for large-scale genomic sequencing. We here present a physical map of the 1.5-cM FTDP-17 candidate region, flanked by the markers D17S1789 and D17S1804, in P1 clones and P1-derived phage artificial chromosomes (PACs). In addition, we generated a detailed transcript map by mapping known genes and expressed sequence tags (ESTs) onto the physical map of the candidate region. This detailed map is currently being used as a template for the genomic sequencing of the complete region and is of interest for identifying the responsible gene defects for other disease genes that have been mapped to chromosome 17q21.

MATERIALS AND METHODS

Isolation of PAC clones. YAC clones from the Whitehead Institute and CEPH chromosome 17 physical map were used as template for inter-*Alu* PCR according to Nelson *et al.* (1989). A gridded PAC

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TABLE 1

Novel Polymorphic Markers Isolated from the FTDP-17 Candidate Region

Locus	Primer sequences	Maximum heterozygosity	Size (bp)
CEN-5	F: 5′ TTTGCTGGCTCCAAGTCCTC 3′ R: 5′ CTGGTCATGCGTGCTTGTCC 3′	0.621	152–156
CEN-58	F: 5′ CTGTTGCTTTCCCTCCTCC 3′ R: 5′ GCACAGAGGACTCTACAGAAGC 3′	0.756	153–167
CG-15	F: 5′ CCCAACTTCCTGTACCTC 3′ R: 5′ GTGAGCTGAGAATCGTGC 3′	0.132	190–200
TEL-2	F: 5′ CACCAGGGTCAACATCTC 3′ R: 5′ CCGTCTCTGTTGTCATTTC 3′	0.466	252–262
TEL-3	F: 5′ GGCTGGTCTCAAACTCCTG 3′ R: 5′ CTGCAGTCCAACCTGGTAG 3′	0.764	167–183
TEL-4	F: 5′ CGTGTCATTGCGAGTGTAC 3′ R: 5′ TTCCCATTTCTCCCTTCTAG 3′	0.890	136–168

library provided by Professor P. de Jong (Ioannou *et al.*, 1994) was then screened by hybridization with purified inter-*Alu* PCR products according to standard techniques.

End-clone sequencing. Isolation of P1/PAC DNA was carried out according to the "PAC Manual" (Genome Systems). Prior to sequencing, an extra precipitation step with 0.8 M NaCl/6.5% PEG was included. Direct sequencing was then performed using the dye Terminator cycle sequencing kit (Amersham). Sequence analysis was performed on ABI Prism 377 (Perkin–Elmer).

Fiber-FISH. The Fiber-FISH slides were prepared according to Datson *et al.* (1996). The probes derived from P1/PAC clones were labeled with either biotin-11–dUTP or digoxigenin-11–dUTP in accordance with the standard nick-translation protocol (Boehringer Mannheim). Hybridization was carried out according to standard techniques. Bicolor immunofluorescence detection of biotin and digoxigenin in FITC and Texas red was performed according to Florijn *et al.* (1995). Finally the slides were mounted in Vectashield containing DAPI (0.2 mg/ml). Digital images were taken with a Photometrics CCD camera placed on a DMRXA Leica fluorescence microscope using a PL Fluotar 100× NA 1.3–0.60, equipped with multibandpass filters for simultaneous observation of FITC, Texas red, and DAPI.

Isolation of novel polymorphic markers. DNA from P1/PAC clones was partially digested with *Sau*3AI. Fragments of 0.5–1.5 kb were isolated and cloned into pUC18 vector to generate a genomic library from the candidate region. Approximately 20,000 clones from this library were screened with a ^{32}P-labeled $(CA)_{12}$ oligonucleotide probe using standard techniques. Positive clones were isolated and sequenced to identify tandem repeats and flanking genomic sequences. To analyze potential polymorphic sites, primers were designed to amplify across the tandem repeat, and PCR was performed on genomic DNA from ~50 control individuals. Estimates of allele frequencies (data not shown) and maximum heterozygosity were made for each polymorphism using data from this control population (Table 1). The location of each polymorphism within the candidate region (Fig. 1) was determined by PCR-based mapping to genomic clones from the physical contig.

Mapping of STSs and transcripts. PCR conditions were optimized for each sequence tagged site (STS) or EST to be mapped. PCR products were then amplified on 50 ng of DNA isolated from each P1/PAC clone, using human genomic DNA as positive control. To confirm mapping data further, amplified fragments of STSs and ESTs were purified and hybridized to Southern blots of the digested P1/PACs from the FTDP-17 contig.

P1/PAC Southern blot hybridization. Genomic clones were digested with *Eco*RI and separated on 0.7% agarose gel electrophoresis in 0.5× TBE at 20 V for 16 h. The DNA was then transferred to nylon membrane (Amersham) by capillary blotting. Probes for each STS/EST to be tested were amplified by PCR. PCR fragments was purified with a Qiaquick PCR purification kit (Qiagen) prior to labeling. Labeling and hybridization were performed as described earlier.

Exon trapping. PAC DNA from the FTDP-17 candidate region physical map was digested with *Bam*HI/*Bgl*II and shotgun cloned into the exon-trapping vector pSPL-3 (Life Technologies). Plasmid DNA was prepared from the PAC/pSPL-3 sublibrary, which then was transfected into COS-7 cells. Total RNA was prepared from the COS-7 cells 24 h after transfection and then used in the RT-PCR amplification of trapped exons with vector-specific primers. The trapped exons were cloned using uracil DNA glycosylase with the pAMP10 cloning system (Life Technologies). The putative exons were then sequenced, and artifacts due to the isolation of vector sequences or repetitive elements were discarded.

Northern blot hybridization. Northern blots of total RNA from multiple tissues (MTN, Clontech) were screened with ^{32}P-labeled PCR-generated probes derived from each of the transcripts/exons being studied. Probes were labeled as described earlier. Hybridization was performed in ExpressHyb solution according to the manufacturer's protocol (Clontech).

cDNA library screening. Three different cDNA libraries were used to increase the probability of isolating positive clones: normalized human infant brain library (Bento Soares), human fetal brain phage library (Clontech), and human adult brain library (Gibco, Life Technologies). The libraries were plated and blotted according to the manufacturer's protocol. The positive clones were sequenced as described earlier and the maximum open reading frame (ORF) was identified by the DNA STAR program package.

RACE-PCR. If further 5′ (or 3′) cDNA sequence was required to isolate the full-length cDNA from a certain transcript, we used the technique of 5′ (or 3′) RACE (rapid amplification of cDNA ends). A human fetal cDNA library was amplified using the Clontech Marathon Ready cDNA kit according to the manufacturer's instructions. The amplified products were directly cloned into a pCR 2.1 vector using the TA cloning kit (Invitrogen). Direct sequencing was then performed as described earlier.

RESULTS AND DISCUSSION

Contig Assembly

YAC clones that span the D17S800–D17S791 region were initially used to develop a physical coverage of the region. Inter-*Alu* PCR probes (Nelson *et al.*, 1989) derived from the YACs were then used to isolate PACs from genomic libraries. Positive PACs were isolated and analyzed by STS and microsatellite content and color Fiber-FISH to determine the level of coverage of

the candidate region. In addition, P1 clones earlier mapped to the region (Neuhausen *et al.*, 1994; Miki *et al.*, 1995) were included in the map. Gaps remaining in the contig were filled in by isolating additional clones from libraries using STSs that were generated from end sequences of P1/PAC clones that flank each gap. In conclusion, a complete physical map was generated in 13 PAC clones and 13 P1 clones (Fig. 1), which was an excellent tool for generating novel polymorphic markers, for use as a template for the mapping of ESTs and known genes, and for isolation of novel transcripts.

Mapping of Genes and Transcripts

A large number of known genes and ESTs had already been mapped to chromosome 17q21 using the radiation hybrid panels, as part of the effort to generate a gene map of the human genome (Schuler *et al.*, 1996). An examination of the human map for the region D17S800–D17S791 (on the NCBI Web site: www.ncbi.nlm.nih.gov/SCIENCE96/) revealed that approximately 150 STSs for different EST clusters had been mapped to genomic regions overlapping the FTDP-17 candidate region. Furthermore, a number of candidate genes, such as VHR (dual-specific phosphatase), MOX-1 (human Mox-1 protein), myosin light chain 1, DLG3 (human homolog 3 of *Drosophila* large discs protein), PPY (pancreatic peptide Y), EPB3 (anion band 3), and GP2B (glyco protein 2B), had been identified and mapped on contigs that overlap our candidate region, as part of the previous efforts to clone the BRCA1 (breast cancer 1) gene (Albersten *et al.*, 1994; Miki *et al.*, 1994, 1995; Frideman *et al.*, 1995; Harshman *et al.*, 1995; Osborne-Lawrence *et al.*, 1995).

To localize the EST clusters and known candidate genes to our contig, we performed PCR for all STSs on each of the YAC, P1, and PAC clones in the contig. In addition, hybridization of STS probes to filters blotted with digested clones was performed. A positive map location was thus indicated by positive PCR amplification of a certain STS from the relevant YAC, P1, or PAC clone or a positive signal on the P1/PAC blots. We investigated all STSs mapped between D17S800 and D17S791 and known genes localized close to our candidate region (Albersten *et al.*, 1994; Miki *et al.*, 1994; Frideman *et al.*, 1995; Harshman *et al.*, 1995; Osborne-Lawrence *et al.*, 1995; Schuler *et al.*, 1996).

We were able to confirm the reported mapping data of VHR, MOX-1, myosin light chain 1, DLG-3, PPY, EPB3, and GPIIB. In addition, 12 other known genes were mapped to genomic clones in our contig (Table 2; Fig. 1). Furthermore, we localized 9 ESTs to our contig as described in Table 2. Some of these transcripts were later characterized, and their full-length sequence could be identified (Table 3).

Exon Trapping

To isolate additional expressed sequences encoded within the FTDP-17 candidate region, exon-trapping experiments were performed. Initially, more than 4000 primary clones were isolated from the P1/PAC clones in the FTDP-17 candidate region. After artifacts due to the isolation of vector sequences or repetitive elements were discarded, the remaining putative "exons" were compared to databases of known genes and ESTs, using the BLAST suite of programs on the NCBI Web site. This analysis allowed us to confirm our previous mapping data by identifying trapped exons from all known genes and ESTs that we previously had localized to the region. In addition, we could map Connexin 45 to the telomeric part of the contig (Fig. 1) and we isolated 25 exons from putative novel transcripts.

Identification of Novel Genes

Once a novel transcript in the form of an EST or trapped exon product was mapped to the FTDP-17 candidate region, homology searches in dbEST database using the BLASTN program on the NCBI Web site was performed to identify additional ESTs that were derived from the same gene. If further sequence information was required to isolate the full-length sequence, cDNA library screening and RACE-PCR were performed as described under Materials and Methods.

A number of novel transcripts were identified in this way. These novel genes are entitled *frontotemporal dementia candidate genes* (*FTDCG 1–7*) (Fig. 1, Table 3). Furthermore, two additional brain cDNA clones (*FTDCG8* and *FTDCG9*) (Fig. 1, Table 3) were isolated due to hybridization of trapped exons. These transcripts lack homology to any known gene or EST, and full characterization of these genes has not yet been performed.

FTDCG1

FTDCG1 was initially mapped to the contig as an EST (WI-17645, GenBank Accession No. H01677). The full-length sequence for this gene was generated by assembling EST sequences from UNIgene (NCBI Web site) and homologous mouse ESTs identified by BLAST to two contigs. The gap between the contigs and weak sequences were filled in by PCR amplification and sequencing on human brain cDNA. The full-length sequence reveals an ORF of 1.1 kb that shows weak similarities to rat glucose-6-phosphatase (G-6-P) (GenBank Accession No. L37333). However, the expression patterns of these two genes seem to differ. While G-6-P is expressed mainly in liver, this novel gene seems to be ubiquitously expressed (including expression in brain) since ESTs listed in UNIgene come from a variety of tissues.

FTDCG2

FTDCG2 was identified independently both by exon-trapping and by RACE-PCR from the EST WI-16312 (GenBank Accession No. T17249) that was mapped to the FTDP-17 region as an end-clone sequence from the

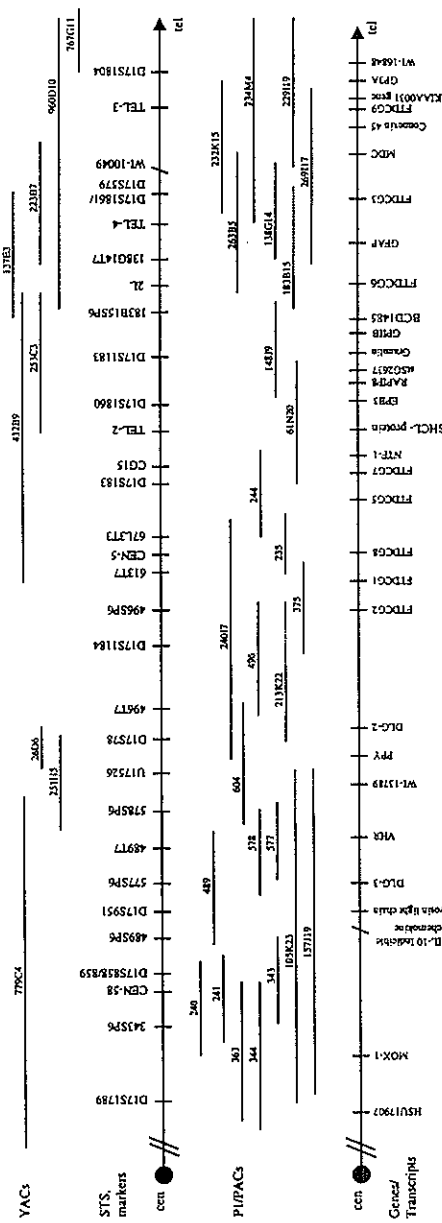

FIG. 1. Schematic diagram of the detailed physical map and transcript map of the FTDP-17 candidate region flanked by markers D17S1789 and D17S1804 on chromosome 17q21. Contigs of overlapping genomic clones; YACs, PACs, P1s, and Whitehead clones are represented by horizontal lines. Locations of STSs and polymorphic markers (upper horizontal arrow) and location of genes and transcripts (lower horizontal arrow) are indicated by vertical lines. The final order of STSs, polymorphic markers, and transcript was generated by combining data from mapping experiments and BLAST results from homology searches to clones sequenced by the Whitehead Institute. Inconsequent mapping data are shown by dashed lines.

TABLE 2

Genes and ESTs Mapped to the FTDP-17 Candidate Region

Gene/EST	GenBank Accession No.	Location in physical map		
		PAC clone	P1 clone	Whitehead clone (GenBank Accession No.)
MOX-1	U10492	122K13, 157J19	363, 344	AC004149
IL-10 inducible chemokine	U91746		343	AC004675
Myosin light chain	X05450	105K23	489	
DLG-3	U37707		578	AC003098
VHR	L05147	105K23	489, 577, 578, 604	AC003098
PPY	M11726	240I7	604	AC002094
DLG-2	X82895		244	
SHCL protein	X68148			AC004596
NTF1	X53390	61N20	244	AC003102, AC004596
EPB3	X12609			AC003102, AC003043
RPIP8	U93871	61N20	375	AC003043
Granulin	L32588			AC003043
GPIIB	J02764			AC003043
GFAP	J04569	269I17, 234M4, 232K15, 229I19, 183B15		
MDC	D31872	183B15, 232K15, 234M4, 269I17, 229I19		AC005180
Connexin 45	U03493			AC005180
KIAA0031 gene	D21163	22L20, 232K15, 234M4, 269I17, 229I19		AC005180
GP3A	M32673	234M4, 229I19, 269I17		
FHF-3	U66199			AC002348
WI-13789	AA047813		577	
WI-16848	G22522	229I19		
WI-17645	H01677		375	AC004150
WI-16312	T17249	213K22	375, 496	AC004150
86JS	AF039240			AC004596
510/77	AF039237			AC004596
stSG2637	AA064737	61N20, 148J9		AC003043
BCD 1485	M86036	148J9		

PAC 213K22. The full-length sequence was generated from a 1.7-kb cDNA clone, RACE PCR fragments, and ESTs identified by BLAST analysis (GenBank Accession Nos. AF039691 and AF039241) of sequences from trapped exons. Northern blot hybridization using the 1.7-kb cDNA clone containing the 3′ end of the transcript showed a transcript of 5.5 kb, expressed mainly in brain and skeletal muscle. This novel gene shows 80% homology to the 8-kb KIAA0288 gene (GenBank Accession No. AB006626), which is described as a putative member of a family of histone deacetylases.

FTDCG3

The transcript *FTDCG3* was identified through the isolation of several trapped exons. Contigs from the 5′ and 3′ ends of the gene were generated by aligning ESTs identified by BLAST analysis (GenBank Accession Nos. AA622743, AI144462, and AA205292) together with the exon sequences. Full-length sequence was then generated by internal PCR amplification between the two contigs. Northern blot analysis shows a major transcript of 7.5 kb and minor transcripts of 5 and 6 kb, and the full-length sequence reveals an ORF of 1.2 kb. No significant homologies were found to indicate a possible function for this gene.

FTDCG5

The transcript *FTDCG5* was initially identified as a trapped exon. BLAST analysis showed 100% homology to several ESTs, one of which was previously mapped to the BRCA1 locus (GenBank Accession No. AF039237). Full-length sequence was generated by sequencing different cDNA clones isolated from libraries. No significant homologies were found to indicate a possible function for this gene.

FTDCG7

The 86JS (GenBank Accession No. AF039240) sequence was identified by BLAST analysis from sequence analysis of trapped exons. A 3-kb transcript

TABLE 3

Novel Genes Identified in the FTDP-17 Candidate Region

Gene	Origin	Size by Northern blot	ORF	Location in physical map: PAC	Location in physical map: P1	Whitehead clone (GenBank Accession No.)
FTDCG1	WI-17645		1.1 kb		375	AC004150
FTDCG2	WI-16312, trapped exon	5.5kb	4 kb	213K22	375, 496	AC004150
FTDCG3	Trapped exon	7.5kb	1.2 kb	234M4, 229I19, 269I17		AC005180
FTDCG4	U17907	4.0kb	1 kb	105K23, 157J19	344, 363	
FTDCG5	Trapped exon, AF039237	2.4kb	0.49 kb			AC004596
FTDCG6	Trapped exon	2.4kb	Pseudogene	183B15		
FTDCG7	Trapped exon, AF039240	4.4kb	>3.2 kb			AC004596
FTDCG8	Trapped exon	8.0kb	—		235	AC004150
FTDCG9	Trapped exon	6.0kb	—	229I19, 269I17, 232K15		AC005180

including the 3′ end sequence was identified by BLAST analysis, which was then used to design primers for 5′ RACE-PCR, to generate the full-length sequence. This gene shows weak homology to a tobacco cell wall hydroxyproline-rich glycoprotein (GenBank Accession No. 119714).

Candidate Gene Analysis

A number of genes that have been mapped to the contig during our positional cloning efforts have been considered as candidate genes for FTDP-17 due to their expression pattern and predicted role in a neurodegenerative disease process. Several of these genes, such as GFAP, VHR, DLG-2, RAPIP8, and *FTDCG2,* have been sequenced in several FTDP-17 families in the search for disease-causing mutations (data not shown). Extensive sequencing of another strong candidate, the *tau* gene, which we were unable to localize to the contig, showed that both missense mutations and 5′-splice mutations segregated with the disease in several FTDP-17 families (Hutton *et al.,* 1998). However, in some of the FTDP-17 families, including the Dutch family defining the telomeric recombinant (D17S1804), no such mutations could be identified. We therefore need to investigate other parts of the *tau* gene as promoter regions, intron sequences, or large deletions before excluding it as *the* candidate gene in these families. The other possibility would be that a second disease-causing gene could be present in the FTDP-17 candidate region on chromosome 17q21.

General Discussion

In the initial process of positional cloning, a high-resolution contig is important for identification and localization of candidate genes and ESTs in a genomic region. The contig presented in this study is currently being used as a template for large-scale sequencing of the FTDP-17 candidate region, which will generate the sequence information needed for identification of promoter regions and intron/exon boundaries for the candidate genes.

We have performed extensive exon-trapping experiments to isolate expressed sequences from the physical contig. Moreover, frequent EST database searches were performed. Comparing these methods, we find that for this specific region on chromosome 17q21, the major part of the data could be found in the publicly available databases. The new genome maps (Schuler *et al.,* 1996; Deloukas *et al.,* 1998) thus provide an excellent tool for identification of ESTs within a specific genetic region. Furthermore, the previous efforts in characterizing the partly overlapping BRCA1 region generated a large number of EST sequences to be further characterized.

During the later stage of this project, the Whitehead Institute released sequence information from BAC/PAC clones that overlaps with the genomic region described in this study. This information was used to refine and confirm our mapping data, and the combination of all data provides us now with a more exact order of the genes and transcripts localized in the region.

The genes identified in this genomic region seem to be of different origins. No cluster of gene families was observed. Telomeric of this region, a cluster of homeobox genes was observed, and centromeric of the region, a keratin gene cluster was observed. Homology searches with other species have generated very little useful information. At the different mouse genome databases, The Jackson Laboratory (http://www.informatics.jax.org/) and the Whitehead Institute (http://www.genome.wi.mit.edu/), information about the mouse genomic region

homologous to human 17q21 was available. Several genes homologous to candidate genes within our candidate region were mapped to mouse chromosome 11 (60–68 cM). The order did, however, differ somewhat, probably due to rough mapping data.

The described region on chromosome 17q21 is likely to remain in focus as it is a highly gene-dense region that harvests several genes with implications of biological significance for other disorders. Some genetic disorders, such as Van Buchem disease, have already been mapped close to the FTDP-17 candidate region (Balemans *et al.*, 1997). It is possible that candidate genes for this type of osteosclerosis could be identified among the genes located in the FTDP-17 candidate region.

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Chapter 5

Identification of Missense and 5' Splice Mutations in the *Tau* Gene

5.1 Mutations in the *tau* gene.

We sequenced the 11 coding exons and flanking intronic regions of the *tau* gene in 40 individuals from all the families in the consortium. Eight of these families have previously showed linkage to chromosome 17q21-22 (26). We detected three missense mutations: G272V, P301L and R406W, in exon 9, 10 and 13 respectively. In addition we identified three intronic mutations in a cluster of four nucleotides 13-16 bp 3' of the exon 10 5' splice site (see chapter **5.5.1** in this thesis) (3). We failed to identify mutations in some FTDP-17 families of the consortium, including HFTD family III.

Our findings were independently confirmed by other groups (4-6) The mutations found by others and us in the majority of FTDP-17 families provided direct evidence that tau dysfunction can lead to neurodegeneration.

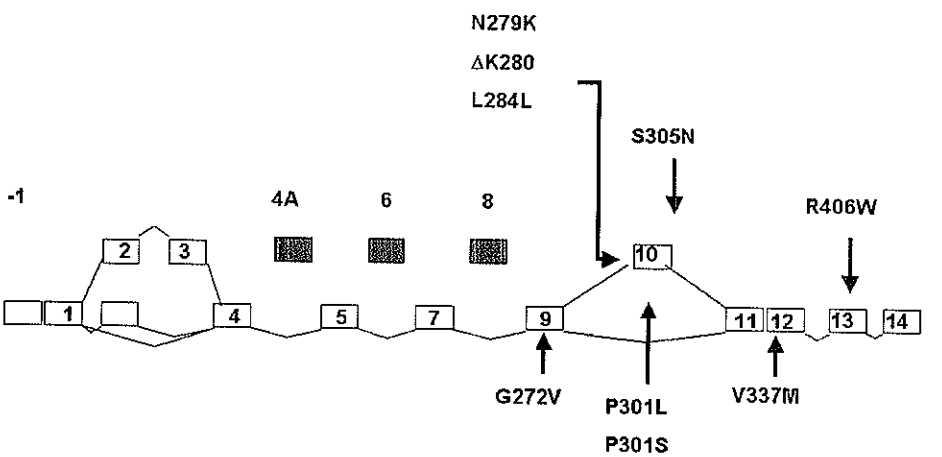

Figure 6: schematic diagram of the genomic organization of the *tau* gene. Only some of the known missense mutations are indicated. All the missense mutations found to date are located in exons 9 to 13.

The *tau* mutations found to date in FTDP-17 are either missense or deletion mutations in the coding region, or intronic mutations located close to the splice-donor site of the intron following exon 10. Missense mutations are located in or near the microtubule-binding domain of tau that consists of the repeats and

flanking regions (Figure 6). Mutations in exons 9, 12 and 13 affect all six tau isoforms. By contrast, mutations in exon 10 affect tau isoforms with four microtubule-binding repeats or their expression.

Intronic mutations were found at positions +3, +12 +13, +14, +16 of the intron following exon 10, with the first nucleotide of the splice donor site taken as +1 (Figure 7A), and at position +33 in the intron after exon 9.
It need to be added that so far *tau* mutations have been shown to give rise to a clinical picture that resembles frontotemporal dementia, Alzheimer's disease, progressive supranuclear palsy, Pick disease, corticobasal degeneration or progressive subcortical gliosis. The factors determining these different clinical phenotypes remain to be discovered.

5.2 Neuropathology of FTDP-17.
All cases of FTDP-17 examined to date have shown the presence of an abundant filamentous pathology made of hyperphosphorylated tau proteins (4, 112, 184). The morphologies of tau filaments and their isoforms compositions appear to be determined by whether *tau* mutations affect mRNA splicing of exon 10, or whether they are missense location located outside or inside exon 10 (see table2).

Table 2. *Tau* mutations

Mutation	Tau pathology	Western blot	'Filament	Possible path. mechanisms	References
K257T (Ex 9)	Pick type inclusions	3R>4R	N.T.	↓ MT-bind.	(185, 186)
I260V (Ex 9)	N/A	N/A	N/A	N/A	(Hutton, pers. comm.)
G272V (Ex 9)	Pick type inclusions	N/A	N/A		(3, 187)
			S. T.	↑ 4R	(148, 149, 188)
N279K (Ex 10)	Neuronal and glial deposits	64, 68 kD ↑ 4R			
ΔK280 (Ex 10)	N/A	N/A	N/A	↓ 4R and ↓ MT-bind.	(188, 189)

L284L (Ex 10)	Neuronal and glial deposits +Aβ deposits	N/A	N/A	↑ 4R	(188)
N296N (Ex 10)	CBD type inclusions	N/A	N/A	↑ 4R	(190)
ΔN296 (Ex 10)	N/A	N/A	N/A	N/A	(191)
P301L (Ex 10)	Neuronal and glial deposits (mostly PT)	64, 68 and 72 kD; ↑ 4R	N.T.	↓ MT-bind and MT-as.	(3, 6, 187, 192-195)
P301S (Ex 10)	Neuronal and glial deposits	N/A	S.F.	↓ MT-bind and MT-as.	(196, 197)
S305N (Ex 10)	Ringed NFTs, glial coiled bodies	N/A	S.F.	↑ 4R	(149, 198)
S305S (Ex 10)	Subcortical globose NFTs (neurons, glia)	N/A	S.F.	↑ 4R	(199)
S320F (Ex 11)	Pick type inclusions	↓ 3R0N + 4R2N	S.T.	↓ MT-binding.	(Rosso, unpublished results)
V337M (Ex 12)	Neuronal NFTs	AD like (60, 64, 68, 72kD)	PHF	↓ MT-binding and assembly	(5, 192, 200)
E342V (Ex 12)	Pick bodies and some NFTs	↑ 4R0N	PHF	↑ 4R no N-terminal inserts	(201)
G389R (Ex 12)	Pick type inclusions	N/A	S.T.	↓ MT-bind ↑calpain digestion	(186, 202)
R406W (Ex 12)	Neuronal NFTs	AD like (60, 64, 68, 72kD)	PHF	↓ MT-binding and MT-assembly	(3, 189, 200, 203)
+3 (5'	Neuronal and	↑4R	T.R.	↑ 4R	

splice ex 10)	glial deposits				(4, 204)
+12 (5' splice ex 10)	Neuronal and glial deposits	↑4R	T.R.	↑ 4R	(205)
+13 (5' splice ex 10)	N/A	N/A	N/A	↑ 4R	(3)
+14 (5' splice ex 10)	Neuronal and glial deposits	↑4R	S.T.R	↑ 4R	(3)
+16 (5' splice ex 10)	Neuronal and glial deposits	64, 68 and 72 kD; ↑4R	T.R.	↑ 4R	(3, 206-208)
+33 (5' splice ex 9)	N/A	N/A	N/A	N/A	(189)

N/A: Non available **N.T.**: Narrowed Twisted. **S.T.**: Straight Filaments. **S.T.R.** : Straight Twisted Ribbons

5.3 Effects of *tau* mutations

5.3.1 Missense mutations: their effect on tau-microtubule interactions and tau polymerization into filaments.

Based on their location it can be predicted that most coding mutations might have an effect on binding of tau to tubulin and promotion of microtubule assembly and stability. The G272V and V337M mutations for example, are in or near highly conserved motifs in the hinge region (70) of microtubule binding repeats in exon 9 and 12 respectively. The hinge regions are terminal motifs of the microtubule repeats and border the inter-repeat sequences that link adjacent microtubule binding domains. They may provide critical secondary structure for interaction of tau with binding areas on the microtubules. Some of the missense mutations in exon 10 substitute amino acids with significant changes in charge or bulk. This would predict to affect tau's interactions with microtubules.

Several studies using *in vitro* microtubule assembly and /or binding assays (186, 189, 192, 200, 209, 210) (and chapter **5.3.1** in this thesis) were performed to assess the functional consequences of the mutations. They all support the hypothesis that certain *tau* mutations tend to weaken tau capacity to bind to microtubule and lower its support of assembly microtubules.

The microtubule-binding domain of tau is not only important for microtubule assembly, it is also essential for tau-self interaction to form tau polymers (61). Filaments can be generated *in vitro* by incubation of tau fragments containing the microtubule-binding domain. *In vitro* studies demonstrated that in the presence of the reducing agent dithiothreitol (DTT), unsaturated free fatty acids, glycosaminoglycans and RNA, normal tau could be transformed into filaments (211-214).

The possibility that *tau* mutations may alter tau-tau-interactions was investigated by comparing the amount of tau filaments formed in the presence of full length wild type recombinant tau, with those formed in the presence of mutant tau and synthetic peptides, derived from each of the four microtubule-binding repeats of tau. The results obtained by three independent studies all demonstrated that in presence of heparin 4R tau with P301L mutation generates more filaments than other mutants (215-217). Similar to normal tau, assembly of filaments from V337M or R406W mutant tau has a marked lag-phase. Such a lag-phase was not apparent in polymerization of P301L mutant tau. The results indicate that this mutation affects the nucleation phase polymerization. The P301L mutation has the most prominent stimulatory effect on tau nucleation, while R406W mutation has an inhibitory effect. Besides the nucleation phase, *tau* mutations affect the elongation phase of tau filament assembly. This is most noticeable with the R406 mutation. Therefore, despite a delay in assembly of filaments with R406W mutant tau, the amount of filaments detected after four days incubation was comparable to that produced by P301L mutant.

5.3.2 Mutations that affect 3R vs 4R ratio.

Mutations in the intron following exon 10 influence its alternative splicing, leading to a change in the ratio of protein isoforms with 3R or 4R (chapter **5.5.1** in this thesis) (3, 4, 148).

The mechanism by which most of these mutations act is probably by destabilizing a short stem-loop structure that spans the splice site (Figure 7A). The stem loop is proposed to compete with the U1 snRNP for binding to the 5'splice site of exon 10 (3). Exon trap assays with constructs containing *tau* mutations, gel migration assays and RNAse mapping are all in agreement with this model (3, 149, 218). Interestingly, the relative proportions of 3R-tau and 4R-tau from other species correlates with the predicted stability of this stem-loop structure (218).

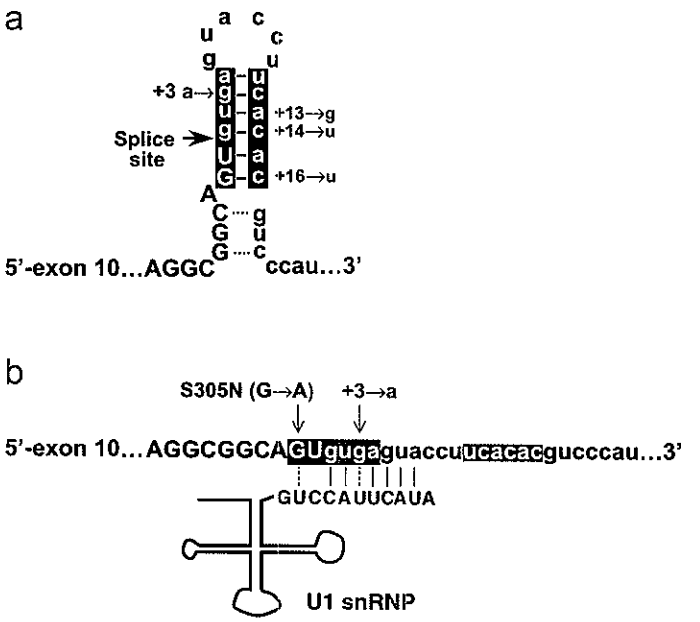

Figure 7. A and **B**: exonic RNA sequence is indicated with capital letters. Intronic RNA sequence with lowercase letters. **7A**: representation of the predicted stem loop and some of the intronic mutations (+3, +13, +14 +16) around the exon 10 3' splice site. **7B**: The S305N mutation and the exon 10 +3 mutation are an example of mutations that affect the binding of the U1 snRNP complex to the splice site. The 5'terminus of the U1 snRNA of this complex has a sequence that base pairs with the splice donor consensus sequence: AGgu(a/g)agu. Both mutations increase the base pairing of U1 snRNA.

Increased production of transcripts encoding exon 10 has also been demonstrated in brain tissue from patients with *tau* intronic mutations (3, 219). This is in turn reflected by a change in the ratio of 3R to 4R tau isoforms, resulting in a net overproduction of 4R tau isoforms (3, 4, 200, 207)

The exact structure and length of the stem-loop, however, has been difficult to determine and different RNA conformations and stem lengths have been proposed. A study using UV melting assays and NMR spectroscopy of a 25-nucleotide-long RNA (extending from position 5 to +19) strongly supports the hypothesis that a stem-loop is indeed formed. The determination of the three dimensional structure of this *tau* exon 10 splicing regulatory element RNA has shown that it consists of an upper and a lower stem that are separated by a bulging A, with an apical stem of six nucleotides (219). Long stem structures, however, cannot be excluded since the length of the oligonucleotides used in these experiments was limited.

Known intronic mutations are located in the upper part of the stem of the *tau* exon 10 regulatory element. All intronic mutations reduce the thermodynamic stability of the stem-loop structure, but to various extents. The largest drop in melting temperature was observed for the +3 mutation. The +14 mutation also produced a large reduction in melting temperature, whereas the effects of the +13 and +16 mutations were smaller.

Some of the mutations occurring in exon 10 also affect the binding of the U1 snRNA complex to the splice site. The 5'terminus of the U1 snRNA complex has a sequence that base pairs with the splice donor consensus sequence: AGgu (a/g) agu. The S305N (AGT to AAT) changes the last amino acid in exon 10. This sequence forms part of the stem-loop structure where the mutation produces a destabilizing G to A transition, at position –1. This mutation increases the base pairing of U1 snRNP since GUgugagua changes to AUgugagua, which is a stronger splice site (149, 188). In a similar way, the +3 mutation increases U1 snRNP binding by a better matching sequence: GUguaagua (149, 188, 218) (Figure 7B). The S305S mutation, determines a T to C transition at position 0 disrupting the stem-loop structure, without a predicted effect on U1 snRNA binding (199).

The N279K and L284L mutations upstream of the stem-loop increase exon 10+ transcripts (149, 188). The N279K mutation (AAT to AAG) creates a purine-rich splice enhancer sequence that explains its effect on exon trapping experiments and soluble 4R tau in brain (148, 149, 200). Moreover the thymidine nucleotide present in the wild type sequence may function as an inhibitor of splicing (220).

The L284L mutation disrupts a UUAG sequence that might act as a splicing silencer (188). However, since mutation of this consensus sequence does not increase exon 10 splicing (221), a second possibility is that the mutation lengthens the AC-rich element within the ESE. Thus the L284L mutation might affect either an enhancing or inhibiting regulatory splicing element. The ΔK280 mutation seems to have an opposite effect to that of the N279K mutation by removing an AAG triplet resulting in a reduction of transcripts with exon 10+ (188) (and our unpublished data). The ΔK280 is particular since has a strong effect in microtubule interaction. It has a reduced ability in promoting microtubule assembly, stronger than the effect of the P301L mutation, but this might be compensated by the possibility that the mutation also reduces the splicing of exon 10.

The G to A transition at position +33 of the intron following exon 9 disrupts one of several (A/T) GGG repeats that may play a role in the regulation of the alternative splicing of exon 10 (222, 223).

All mutations that affect the alternative splicing of exon 10 have in common the fact that they affect the ratio between 3R and 4R tau protein isoforms. The proteins themselves are fully normal and functional, showing that a simple disturbance of this balance can result in neurodegeneration.

5.3.3 Characterization of P301L mutation.

We further characterized the composition of the tau aggregates by developing specific rabbit polyclonal antibodies against the P301L point mutation and its normal counterpart (see chapter **5.5.3** in this thesis) (Figure 8).

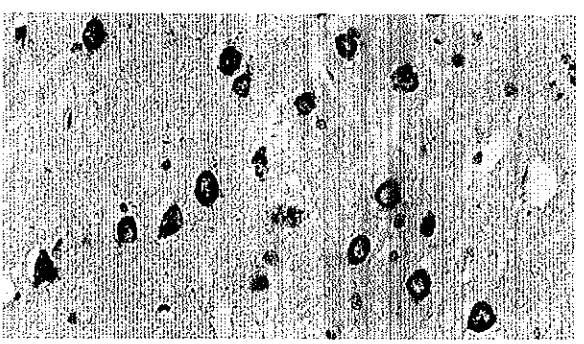

Figure 8. Sections of the temporal cortex of a FTDP-17 patient with the P301L mutation stained with tau-P301L antibody.

In immunohistochemistry experiments we clearly showed the presence of both mutated and normal 4R tau protein in the perinuclear deposits in the affected area of frontal and temporal cortex and hippocampal formations. No clear differences were observed in the cellular localization of the mutated P301L protein versus the normal 4R tau. This result was confirmed by our biochemical experiments where we determined the relative ratio of mutated versus normal tau protein in the sarkosyl-soluble and –insoluble protein fractions from several brain regions.

We showed that, although mutated and normal tau protein tau is present in the insoluble deposits of the cerebral cortex, the main component of these deposits is the mutated tau protein (Figure 9).

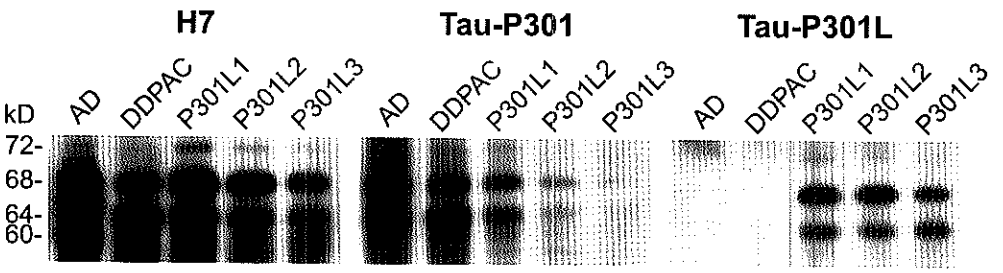

Figure 9. Immunoblot of sarkosyl-insoluble tau from the frontal cortex of one AD, one DDPAC and three FTDP-17 patients carrying the P301L mutation (P301L1, P301L2, P301L3), using the phosphorylation independent antibody H-7, the tau-P301 and the tau-P301L antibodies.

In the soluble fraction of frontal and temporal cortex the overall ratio of 3R versus 4R tau isoforms remain unchanged, but we observed a strong reduction in the level of mutated P301L protein. The most notable depletion was observed in the frontal lobe (~5-fold) and in the temporal lobe (on average ~3-fold), the most affected area of the

brain. In cerebellum, where no tau aggregates were observed, the reduction of mutant protein was the smallest (2-fold) (Figure 10). Our findings suggest that the low level of mutated protein in the soluble fraction is due to a selective depletion, resulting from the selective aggregation of mutant protein in the sarkosyl-insoluble deposits.

In addition, we observed below 45 kD, an increase of tau-imunoreactive cleavage products for the mutant protein compared with the normal one. Since we proved that the two antibodies have similar strength, the result strongly suggests an alteration of the proteolytic processing of the mutated P301L protein.

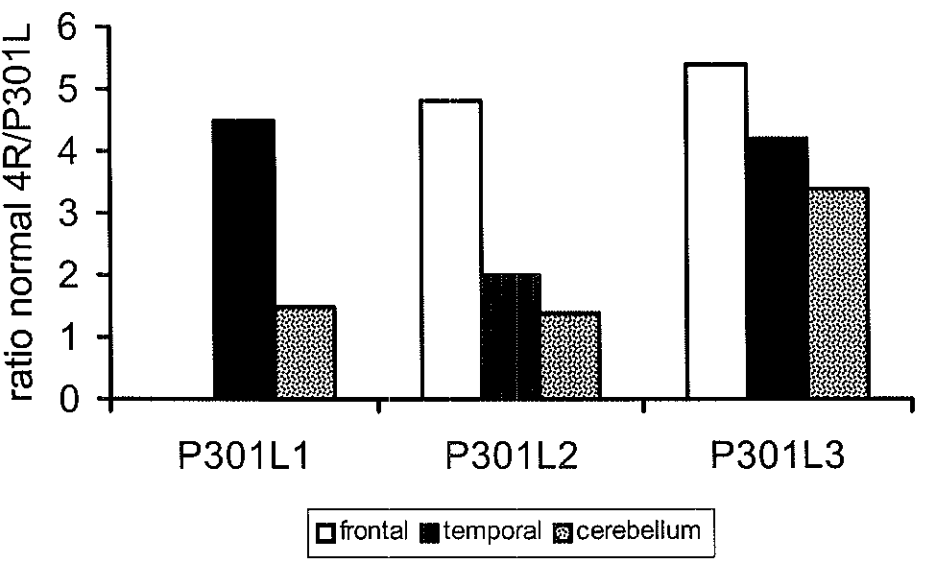

Figure 10. Graphic showing the ratio between normal 4R and P301L protein in the soluble fraction from frontal cortex, temporal cortex and cerebellum from three P301L patients (P301L1, P301L2, P301L3). The amount of P301L tau protein in the soluble fraction of frontal cortex in P301L1 patient was below detection level.

5.4 Frequency of *tau* mutations estimated in a population study of FTD in the Netherlands

In an attempt to estimate the contribution of *tau* mutations in the general FTD population, we systematically performed SSCP (single strand conformation polymorphism analysis) (224) and sequence analysis in a large group of patients (90) ascertained in a genetic epidemiological study of FTD in the Netherlands since January 1994 (see chapter **5.5.2** in this thesis) (189). A mutation in the *tau* gene was found in 17.8% of patients with FTD. Three distinct missense mutations, one 3-bp deletion, and one mutation in the intron following exon 9 were detected. No nonsense mutation were found and none of the previously described intronic mutations in the predicted stem loop after exon 10 (3, 4) were detected (see table 3)

Table 3.
Mutations identified in the *tau* gene in FTD patients from the Netherlands

	Nucleotide change	Amino acid change	No. of cases	No. with positive family history
Exon 9	G→T	G272V	2	2
3' Exon 9	+33 G→A		1	1
Exon 10	C→T	P301L	11	11
Exon 10	ΔAAG	ΔK280	1	0
Exon 13	C→T	R406W	3	3

Positions are numbered according to the longest human brain tau isoform

All patients with a mutation had a positive family history of dementia, except the patient with the ΔK280 mutation, who had a positive family history of Parkinson disease.

The G272V mutation was found in 2 members of the HTDF II family, for which segregation of the mutation with the disease has been reported (see chapter **5.5.1** in this thesis) (3). The P301L mutation was observed in 11 individuals, 5 of which are members of the HFTD family I. Nine of the 11 total cases with P301L mutation share a common ancestor, on the basis of a genealogical study and construction of haplotypes for polymorphic markers in and around the *tau* gene. The base change G→A at position +33 in the intron after exon 9 was observed only in one patient from a small family with no additional affected family members available for study. Thus, additional evidence that this mutation is

pathogenic and not merely a rare polymorphism can be obtained only by in vitro exon trap assay.

In summary while the biochemical and structural characteristics of the tau aggregates in FTDP-17 appear to be somewhat predictable based on our understanding of the functions of tau protein and *tau* gene splicing, the mechanism that lead to assembly of tau in brain remains to be discovered. However experiments using cultured cells and animal models are providing additional insight into the pathogenesis of this disorder.

Furthermore is not clear why the clinical and neuropathological phenotype of individuals with FTDP-17 mutations ranges from FTD (including Pick's disease) to CBD, to PSP and to a multisystem neurodegeneration. A possible explanation might be that other genetic and/or epigenetic factors might influence and modify the effects of the *tau* mutations.

The specific genetic and/or environmental modifiers that might mediate the phenotype are not yet identified, but again the generation of model systems might facilitate this research.

Chapter 5.5

Publications

Chapter 5.5.1

Association of missense and 5' –splice–site mutations in *tau* with the inherited dementia FTDP-17

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Association of missense and 5′-splice-site mutations in *tau* with the inherited dementia FTDP-17

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Thirteen families have been described with an autosomal dominantly inherited dementia named frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17)[1–9], historically termed Pick's disease[10]. Most FTDP-17 cases show neuronal and/or glial inclusions that stain positively with antibodies raised against the microtubule-associated protein Tau, although the Tau pathology varies considerably in both its quantity (or severity) and characteristics[1–8,12]. Previous studies have mapped the FTDP-17 locus to a 2-centimorgan region on chromosome 17q21.11; the *tau* gene also lies within this region. We have now sequenced *tau* in FTDP-17 families and identified three missense mutations (G272V, P301L and R406W) and three mutations in the 5′ splice site of exon 10. The splice-site mutations all destabilize a potential stem–loop structure which is probably involved in regulating the alternative splicing of exon10 (ref. 13). This causes more frequent usage of the 5′ splice site and an increased proportion of *tau* transcripts that include exon 10. The increase in exon 10$^+$ messenger RNA will increase the proportion of Tau containing four microtubule-binding repeats, which is consistent with the neuropathology described in several families with FTDP-17 (refs 12, 14).

FTDP-17 is a condition characterized clinically by behavioural, cognitive and motor disturbance[1]. At autopsy all patients with FTDP-17 have pronounced frontotemporal atrophy with loss of neuronal cells, grey and white matter gliosis and superficial cortical spongiform changes. Variable Tau inclusions are observed in the brains of most FTDP-17 patients. Tau is also the major component of the paired helical filaments[15] that make up the characteristic tangles seen in the brains of patients with Alzheimer's disease and with other neurodegenerative disorders. The Tau isoforms that predominate in human brain are encoded by eleven exons[11]. A total of six different major *tau* mRNA transcripts are generated as a result of alternative splicing and encode proteins of 352–441 amino acids[16]. Alternative splicing of exon 10 generates Tau protein with 3 or 4 microtubule-binding motifs that are imperfect repeats of 31 or 32 residues[15].

Previous studies[1,3–5] found no evidence of mutations in the *tau* gene that were associated with FTDP-17, but these were done on individual families and were mainly restricted to the coding region of the gene. We have now extended the analysis of the *tau* gene to additional families with FTDP-17 and to other regions of the gene. The 11 *tau* coding exons and flanking intronic regions were initially sequenced in 40 individuals from families with frontotemporal dementia from Scandinavia (9 families)[17], the Netherlands (3 families), the USA (5 families), Australia (1 family; Fig. 1a) and from Greater Manchester in the UK (22 families)[17]. Eight of these families had previously displayed evidence for linkage to chromosome 17 (refs 1–3, 7).

We detected two missense mutations (G272V and P301L, numbered from the longest Tau isoform) that occur in two of the microtubule-binding repeat domains of Tau[15,18]. The P301L mutation (Fig. 1b) in exon 10 was found in two families (Table 1): a large Dutch kindred hereditary frontal temporal dementia I (HFTDI)[7], and a small kindred from the United States (FTD003). This substitution occurs in a highly conserved region of the Tau sequence, where a proline residue is found in all mammalian species from which *tau* has been cloned so far. The P301L mutation will only affect the 4-repeat Tau isoforms because exon 10 is spliced out of mRNA that encodes the 3-repeat isoforms[11]. Analysis of Tau aggregates in affected brains from the FTD003 family (P301L) reveals that these consist mainly of 4-repeat isoforms, consistent with a mutation affecting exon 10 (P.D., manuscript in preparation).

The G272V mutation was found in a second large Dutch kindred HFTD2 (Table 1), originally described as having hereditary Pick's disease[7]. This mutation also affects a highly conserved residue within the microtubule-binding domain, encoded by exon 9. Within the imperfect repeat sequence that makes up the four microtuble-binding domains, the G272V and P301L mutations affect positions that are separated by only one residue. Thus, for P301L the invariant PGGG motif in the binding repeat becomes LGGG, and for G272V it becomes PGVG. In contrast to the P301L mutation (exon 10), the G272V mutation (exon 9) will affect all Tau isoforms. The G272V and P301L mutations segregate with disease in each of the relevant families. Both mutations were absent from 192 Dutch controls and the P301L mutation was also absent from 150 US controls. These results indicate that the G272V and the P301L mutations are probably pathogenic.

A third *tau* missense mutation (R406W) was detected in exon 13 in a single family from the United States (FTD004)[19] which alters a highly conserved residue near the carboxy terminus. This mutation segregates with the disease in this family and was absent from 150 US controls. The distribution of Tau-positive inclusions in FTD004

Table 1 Families with segregating mutations in the *tau* gene

Family	Origin (founder)	Affecteds*	Generations	Mean onset age	Mutation
HFTD2†	Netherlands	34(15)	7	47	G272V
HFTD1†	Netherlands	49(14)	5	50	P301L
FTD003	USA	3(2)	2	45–50	P301L
Man19	UK	3(1)	2	55	Ex10 splice + 13
DDPAC†	Ireland	13(7)	3	44	Ex10 splice + 14
Aus1†	Australia (UK)	28(5)	5	53	Ex10 splice + 16
FTD002†	USA	3(1)	2	40	Ex10 splice + 16
Man6	UK	2(1)	1	48	Ex10 splice + 16
Man23†	UK	10(2)	3	51	Ex10 splice + 16
FTD004	USA	10(2)	4	55	R406W

* Confirmed post mortem in brackets.
† Families with prior evidence of genetic linkage to chromosome 17.

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meets the NINDS neuropathological criteria for progressive supranuclear palsy (PSP)[19]. Electron microscopy, however, revealed that the Tau filaments in this family are Alzheimer's-like paired helical filaments[19] and not the straight Tau filaments normally observed in PSP[12]. The neuropathological phenotype in this family is also similar to that of the *lytico* and *bodig* diseases of Guam[20]. It may be important that the R406W mutation lies near key residues that are phosphorylated in Tau from paired helical filaments (Ser 396, Ser 404)[21].

In addition to the three missense mutations (G272V, P301L and R406W), we also found three heterozygous mutations in a cluster of 4 nucleotides 13–16 base pairs (bp) 3′ of the exon-10 5′ splice site (Fig. 3). Six families (Table 1) had mutations at these 3 sites, including 4 families who had previously shown linkage to chromosome 17 (DDPAC[2], AusI (ref. 3), FTD002 and Man23). In each of the 6 families, the relevant mutation was found to segregate with the disease (Fig. 1a): none of these intronic variants was observed in 150 US and 23 British caucasian controls. These results indicated that this cluster of mutations (Fig. 2a, b) could be pathogenic, but it was not obvious how such intronic mutations could influence exon-10 splicing and thus the function of Tau.

To test whether these 5′-splice-site mutations could be affecting alternative splicing of exon 10 and thus altering the proportion of *tau* mRNA containing this exon, we used the polymerase chain reaction with reverse transcription (RT-PCR) to estimate the ratio of *tau* exon10$^+$ RNA to exon10$^-$ RNA. Seven FTDP-17 brains were analysed: four were from families with 5′-splice-site mutations (DDPAC (2 brains)[2], ManF23 and AusI (refs 3, 9)) and three were from families with the P301L mutation (FTD003 (2 brains) and HFTD1 (ref. 7)). RT-PCR was done between exons 9 and 11 and, in a separate reaction, between exons 9 and 13. Both amplifications generated two products, one corresponding to *tau* transcripts containing exon 10 and one to *tau* transcripts lacking exon 10. In both PCRs the four FTDP-17 brains carrying 5′-splice-site mutations gave a two- to sixfold higher proportion of *tau* exon10$^+$ RNA compared with 7 control brains (Fig. 2c). FTDP-17 brains without splice-site mutations (P301L mutants) gave similar ratios to control brains (Fig. 2c), indicating that the increased ratio in brains with the splice-site mutations is not secondary to the disease process. It needs to be investigated whether the variability between the splice-site mutant brains (two- to sixfold increase over controls) reflects the variable nature of the disease phenotype in these families, particularly the wide range in onset age. Our results indicate that the 5′-splice-site mutations in FTDP-17 families increase the proportion of *tau* mRNA containing exon 10 and encoding the 4-repeat isoforms.

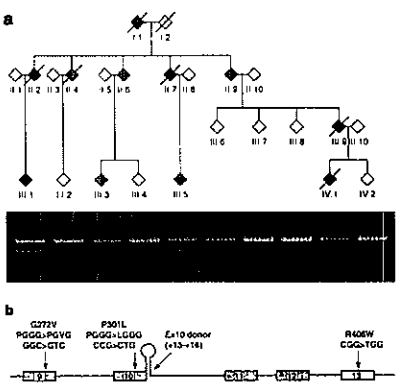

Figure 1 *tau* mutations in FTDP-17. **a**, Segregation analysis of the exon-10 5′ splice site +16 mutation in the AusI kindred. The mutation is indicated by a band at 148 bp on the agarose gel whereas the normal allele is represented by a band at 200 bp. Unaffected individual III.6 carries the mutation and disease haplotype. **b**, Diagram of the *tau* gene (exons 9–13) showing locations of missense and 5′-splice-site mutations. Shaded boxes denote exons encoding microtubule-binding domains. The effect of two missense mutations G272V and P301L on the PGGG motif in the microtubule-binding domains is shown.

We used exon-trapping assays to test the effect of the 5′-splice-site mutations on alternative splicing of *tau* exon 10 (ref. 22). Wild-type and the three mutant (+13, +14 and +16) versions of exon 10 were analysed. These sequences, including ~40 bp of intronic sequence at either end, were amplified and cloned into the splicing vector pSPL3b, which contains exons from the rabbit β-globin and HIV *tat* genes. A multiple cloning site in an intron between the two *tat* exons allows test DNA to be introduced. An SV40 promoter in pSPL3b drives the generation of artificial mRNAs when the construct is transfected into COS7 cells, trapping any functional exons in the cloned DNA between the two *tat* exons so they can be detected by RT-PCR (Fig. 3).

Exon-trapping analysis[22] of wild-type *tau* exon-10 constructs (Fig. 3) gave multiple bands, the most prominent at 177 bp corresponding to vector-only *tat* to *tat* exon splicing (exon10$^-$ mRNA). Weaker products at 270 bp corresponded to exon 10$^+$ transcripts. In contrast, each of the *tau* exon-10 5′ splice mutant constructs gave products mainly of 270 bp, corresponding to exon 10$^+$ transcripts. With the mutant constructs, *tat* to *tat* spliced vector-only transcripts (177 bp) were greatly reduced, consistent with increased usage of the exon-10 5′ splice site. *tau* exon 10–vector-only transcripts were almost eliminated with the +13 and +14 mutants. These results show that the 5′-splice-site mutants increase splicing of exon 10 *in vitro*.

The mechanism by which the intronic mutations increase exon 10$^+$ *tau* mRNA is not fully understood. However, we examined the sequence of the exon-10 5′ splice site and found a short potential stem–loop structure (12-base stem, 6-base loop) which spans the splice site (Fig. 2a, b). The three mutations occurred in the stem of this structure and should destabilize it. It has been reported that short stem–loop structures can sequester 5′ splice sites and lead to usage of alternative 5′ splice sites[23]. This suggested that the stem–loop structure could be involved in regulating exon-10 alternative splicing, presumably by competing with the U1 snRNP (or with other factors recognizing this site) for binding to the exon-10 5′ splice site[24]. Blocking U1 snRNP (or another factor) binding would result in failure to define exon 10 (through U1 and U2 snRNP binding) and lead to skipping of this exon[24]. This would allow the splicing of exon 9 to exon 11 and the generation of *tau* transcripts lacking exon 10. We propose that the ratio of transcripts with and without exon 10 reflects the stability of this stem–loop structure, which in turn determines the ratio of 4-repeat and 3-repeat Tau. Mutations in the step–loop that are associated with FTDP-17 because of their destabilizing effect on the structure will promote recognition of the 5′ splice site[24] and the inclusion of exon 10 in *tau* mRNA. This should increase the proportion of *tau* transcripts containing exon 10, just as we found in our RT-PCR analysis of FTDP-17 splice-mutant brains and in the exon-trapping analysis, and hence the ratio of 4-repeat to 3-repeat isoforms. This idea is consistent with the observation that soluble Tau in many FTDP-17 families consists of more of the 4-repeat isoforms than that from control brains[14]. The absence of this type of splice-site mutation in other FTDP-17 families (including families with missense mutations) is consistent with the finding that, in at least one FTDP-17 family, the relative abundance of Tau isoforms with 3- and 4-repeats is similar to that of soluble Tau in control brains[12].

Our results have several implications for frontotemporal dementia and neurodegeneration. First, we have identified mutations that cause FTDP-17, one of the major autosomal-dominant

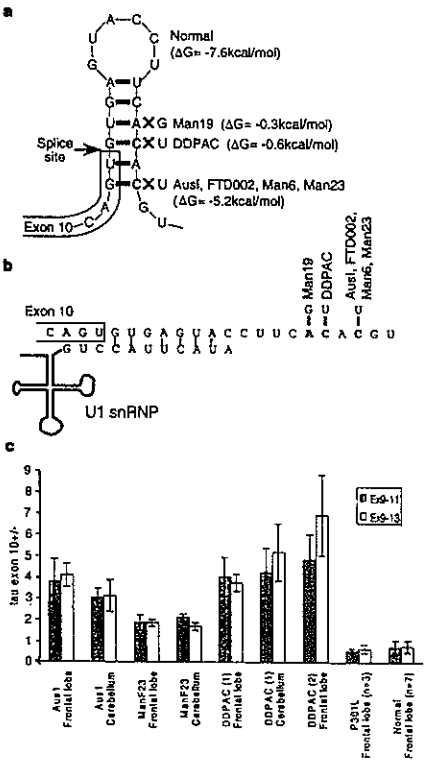

Figure 2 *tau* exon-10 5'-splice-site mutations. The splice site in the **a**, the predicted stem-loop structure, and **b**, in the linear form. Mutations are marked and the predicted free energy of the stem-loop is indicated[28]. Predicted binding of U1 snRNP to the 5' splice site is shown. **c**, RT-PCR analysis of the molar ratio between *tau* mRNA with and without exon 10. RNA from frontal lobe (4 cases) and cerebellum (3 cases) from FTDP-17 brains with splice mutations (DDPAC (2), ManF23 and Aus1) has ratios >1.6 (left, 14 bars); RNA from frontal lobe of normal brains (n = 7) and P301L mutation brains has ratios <0.8 (right, 4 bars). Analysis of total *tau* mRNA revealed no significant differences.

loci associated with neurodegeneration[1]. Frontotemporal dementia, of which FTDP-17 is a familial form, accounts for 3–10% of dementia cases[3]. Our data also indicate that the recently reported[12] Tau-sequence variant V337M in a family with FTDP-17 (Seattle A) is probably pathogenic. Because no mutations were previously found in other families, it was suggested that this might be a benign polymorphism, although a pathogenic effect in this particular family was not excluded[12]. Second, and most important, our identification of pathogenic missense (G272V, P301L and R406W) and 5'-splice-site mutations associated with FTDP-17 shows that Tau dysfunction can lead to neurodegeneration. In addition, the nature of the 5'-splice-site mutations indicates that the relative levels of 4-repeat and 3-repeat isoforms are crucial to the functioning of Tau, consistent with alternative splicing of exon 10 being developmentally regulated[13]. Other tauopathies[25], including Pick's disease, PSP, corticobasal degeneration and *lytico* and *bodig* diseases of Guam[20], should be tested for evidence of *tau* gene dysfunction—

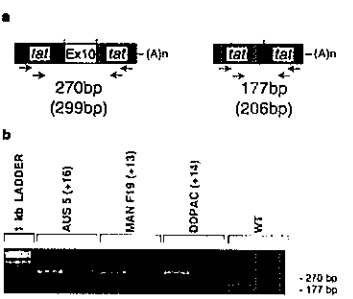

Figure 3 5'-splice-site mutations increase incorporation of *tau* exon 10 into artificial mRNAs. **a**, Diagram of major RT-PCR products generated by exon trapping analysis of *tau* exon 10. PCR products result from splicing of pSPL3b vector-derived *tat* exons with (270 bp) and without (177 bp) *tau* exon 10. In addition, there are minor products of 299 bp and 206 bp that contain an additional 29 bp of vector sequence. The position of nested RT-PCR primers is indicated by arrows. **b**, Results of exon trapping analysis of *tau* exon 10. Wild-type constructs (WT) gave strongest signals from bands (177 bp, 206 bp) corresponding to exon 10⁻ vector-only splicing. Splice-site mutants gave strongest signals corresponding to exon 10⁺ transcripts (270 bp, 299 bp).

an association between an intronic polymorphism in the *tau* gene and PSP has already been reported[26,27]. Our results also have implications for Alzheimer's disease because Tau paired-helical-filament pathology is a hallmark of this disease. Finally, the nature of the mutations identified in the FTDP-17 families indicate that abnormalities in Tau microtubule binding are probably important in the pathogenesis of this group of tauopathies. It is not clear whether abnormal microtubule binding would lead directly to cell death (through microtubule destabilization), or whether it would merely result in an increase in unbound Tau that could then form pathogenic fibrils which disrupt cellular function. □

Methods

***tau* gene sequencing.** *tau* exons (1–4,5,7,9–13) were amplified from genomic DNA from family members with primers designed to flanking intronic sequence. PCRs contained a final concentration of 0.8 μM for each primer and 1 unit of *Taq* 'Gold' polymerase (Perkin Elmer). Amplification was done using a 60–50 °C touchdown protocol over 35 cycles with a final extension of 72 °C for 10 min. PCR products were purified using the Qiagen PCR kit and their concentration estimated on an agarose gel. DNA (100 ng) for each exon was sequenced on both strands using the Rhodamine dye terminator cycle sequencing kit (Perkin Elmer) and relevant PCR primers. Sequencing was performed on an ABI377 automated sequencer. Heterozygote base cells were made using Factura software (Perkin Elmer) and sequence alignment was performed with Sequence Navigator (Perkin Elmer).

Mutation detection. Mutations were detected in families and controls using sequencing or by PCR–RFLP analysis. Missense mutation P301L was detected by *Bst*NI or *Sma*I digestion of exon-10 PCR product, the mutant allele contains a *Bst*NI site, the normal allele a *Sma*I site. The G272V mutation was detected by *Acl*I digestion of exon 9 PCR products, the mutation eliminates the *Acl*I site. The R406W missense mutation was detected by *Nco*I digestion of exon 13 PCR product, the mutant allele contains the *Nco*I site. The exon 10 splice donor +16 mutation was detected by *Nsp*I digestion, again the mutant allele is cleaved by this assay. In contrast, the exon-10 5' splice site +13 and +14 mutations eliminate an *Afl*III site from the amplification product. After digestion, genotyping was done on 3% Metaphor (FMC) agarose gels.

RT-PCR analysis of exon 10 alternative splicing. Total RNA was prepared from a section of frontal lobe from seven normal brains and from the frontal lobes of seven FTDP-17 brains using the Trizol reagent and protocol (Life Technologies). Four of the FTDP-17 brains were from families with 5'-splice-site

mutations (DDPAC (2 brains), ManF23 and Aus1) and 3 were from families with the P301L point mutant (FTD003 (2 brains) and HFTD1). Reverse transcription was performed using the Superscript preamplification kit (Life Technologies) on 1–4 μg of brain RNA with an oligo(dT) primer. PCR was performed between exon 9 (forward, 5′-ATCGCAGCGGCTACAGCAG-3′) and exon 11 (reverse, 5′-TGGTTTATGATGGATGTTGCC-3′) and between exon 9 and exon 13 (reverse 5′-TCTTGGCTTTGGCGTTCTC-3′). In each case, the 5′ end of the forward amplification primer was labelled with TET (Perkin Elmer) to allow detection by an ABI377 automated sequencer. Preliminary PCRs (not shown) were performed using a range of amplification cycles (18–37) to determine the optimum number of cycles for this analysis. Based on these results, we used 32 cycles in subsequent experiments. After amplification, PCR products were analysed on an ABI377 automated sequencer (Perkin Elmer) where they resolved into two major fragments (327 and 418 bp, exons 9–11; 487 and 578 bp, exon 9–13) corresponding to *tau* transcripts with and without exon 10. The identity of each band was confirmed by sequence analysis. The molar ratio of exon10^{+} to exon 10^{-} RNA was determined using Genescan software. Three independent PCRs (for both exons 9–11 and 9–13) were used to determine the mean and s.d. of the ratio for each brain. Results (not shown) were essentially identical when exon 10$^{+/-}$ ratios were estimated by densitometric analysis of PCR products on agarose gels using a Kodak DC120 camera kit and 1D Image Gel densitometry software.

Exon-trapping analysis of exon-10 splicing. Mutant and wild-type versions of *tau* exon 10 were amplified from the DNA of patients with each of the three different splice mutations (+13, +14 and +16) and from normal individuals. PCR products contained exon 10 and ~40 bp of flanking intron sequence at either end. PCR products were cloned into the splicing vector pSPL3b using *Xho*I and *Pst*I sites incorporated into the amplification products. Mutant and wild-type constructs were identified by sequence analysis. For exon trapping, the exon-trapping system of Life Technologies was used. Briefly, COS-7 cells were transfected in duplicate with 1 μg each construct using LipofectACE reagent (Life Technologies). Cells were collected 24 h post-transfection and RNA prepared using the Trizol reagent (Life Technologies). First-strand synthesis and nested PCR were done using reagents supplied with the system and conditions described in manufacturer's instructions, except that *Bst*X1 digestion of primary PCR products was excluded. To verify that the RT-PCR was quantitative, different amounts of primary PCR template (1–5 μl) were used and the total number of amplification cycles was varied (30–35 cycles). PCR products were analysed on 3% Metaphore (FMC) gels. RT-PCR products (Fig. 3) had their identities confirmed by sequencing.

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The antigenic structure of the HIV gp120 envelope glycoprotein

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The human immunodeficiency virus HIV-1 establishes persistent infections in humans which lead to acquired immunodeficiency syndrome (AIDS). The HIV-1 envelope glycoproteins, gp120 and gp41, are assembled into a trimeric complex that mediates virus entry into target cells[1]. HIV-1 entry depends on the sequential interaction of the gp120 exterior envelope glycoprotein with the receptors on the cell, CD4 and members of the chemokine receptor family[2–4]. The gp120 glycoprotein, which can be shed from the envelope complex, elicits both virus-neutralizing and non-neutralizing antibodies during natural infection. Antibodies that lack neutralizing activity are often directed against the gp120 regions that are occluded on the assembled trimer and which are exposed only upon shedding[5,6]. Neutralizing antibodies, by contrast, must access the functional envelope glycoprotein complex[7] and typically recognize conserved or variable epitopes near the receptor-binding regions[8–11]. Here we describe the spatial organization of conserved neutralization epitopes on gp120, using epitope

Chapter 5.5.2

High prevalence of mutations in the microtubule associated protein tau in a population study of frontotemporal dementia in the Netherlands

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High Prevalence of Mutations in the Microtubule-Associated Protein Tau in a Population Study of Frontotemporal Dementia in the Netherlands

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Summary

Mutations in microtubule-associated protein tau recently have been identified in familial cases of frontotemporal dementia (FTD). We report the frequency of *tau* mutations in a large population-based study of FTD carried out in the Netherlands from January 1994 to June 1998. Thirty-seven patients had ≥1 first-degree relative with dementia. A mutation in the *tau* gene was found in 17.8% of the group of patients with FTD and in 43% of patients with FTD who also had a positive family history of FTD. Three distinct missense mutations (G272V, P301L, R406W) accounted for 15.6% of the mutations. These three missense mutations, and a single amino acid deletion (ΔK280) that was detected in one patient, strongly reduce the ability of tau to promote microtubule assembly. We also found an intronic mutation at position +33 after exon 9, which is likely to affect the alternative splicing of *tau*. *Tau* mutations are responsible for a large proportion of familial FTD cases; however, there are also families with FTD in which no mutations in *tau* have been found, which indicates locus and/or allelic heterogeneity. The different *tau* mutations may result in disturbances in the interactions of the protein tau with microtubules, resulting in hyperphosphorylation of tau protein, assembly into filaments, and subsequent cell death.

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Introduction

Frontotemporal dementia (FTD) is a form of presenile dementia characterized by behavioral changes, cognitive decline, personality changes, speech deterioration, and, later in the disease, decline of memory. Sometimes, parkinsonian symptoms are prominent (Brun 1987; Knopman et al. 1990). Atrophy of the frontal and/or temporal cortex, as well as of the basal ganglia and substantia nigra, are the characteristic neuropathological features. Neuronal loss, gray-matter and white-matter gliosis, and superficial cortical spongiform changes generally are found in the cortex and some subcortical areas (Brun 1987; Lund and Manchester Groups 1994).

Prevalence of FTD in the Netherlands is estimated to vary between $1.2/10^6$ in the age group of 30–40 years and $28.0/10^6$ in the age group of 60–70 years (Stevens et al. 1998). FTD can occur in a sporadic form, but 30%–50% of persons with FTD have been found to have a positive family history of dementia (Gustafson 1987; Neary et al. 1988; Knopman et al. 1990; Stevens et al. 1998).

A number of families with an autosomal dominant mode of inheritance and almost complete penetrance have been described, and ≥13 have been linked genetically to chromosome 17q21-22; these are now referred to as "frontotemporal dementia and parkinsonism linked to chromosome 17" (FTDP-17 [MIM 601630]; Wilhelmsen et al. 1994; Petersen et al. 1995; Wijker et al. 1996; Yamaoka et al. 1996; Baker et al. 1997; Poorkaj et al. 1998; Foster et al. 1997; Froelich et al. 1997; Heutink et al. 1997; Murrell et al. 1997; Lendon et al. 1998).

Most, if not all, families with FTDP-17 show microtubule-associated protein tau deposits in neurons or in both neurons and glial cells. In some FTDP-17 families, the tau deposits are identical to those found in Alzheimer disease (AD; Spillantini et al. 1996): they are present in neurons and consist of paired helical and straight filaments, which contain all six brain tau isoforms. Other families show tau deposits, in neurons and in glial cells,

Table 1

PCR Primers for Genomic Amplification of Tau Exons (5′→3′)

Exon	Forward	Reverse	Exon Size (bp)	PCR Product (bp)
1	CAACACTCCTCAGAACTTATC	CAGTGATCTGGGCCTGCTGTG	150	228
2	CACAGGGAGCGATTTTCAGC	CCACGCTGTCCTGCAAAGC	87	339
3	GGGCTGCTTTCTGGCATATG	CCTCACTTCTGTCACAGGTC	87	297
4	GGATGTGAACTTTCCTGAATG	GAGCTCAGGTCCAAATGATC	66	271
5	CAGTGAAAATGGAGTGTGAC	CAGCTGCAGAGCTCCGTGGC	56	136
7	CTAGGAGGCCAAGGGTCAC	GAGAGCTTCAGCTTCCTCTAAG	127	300
9	CGAGTCCTGGCTTCACTCC	CTTCCAGGCACAGCCATACC	266	379
10	GGTGGCGTGTCACTCATCC	GGTGGCGTGTCACTCATCC	93	200
11	CTTCTCATTGAGTTACACCC	CTCACCAGGACTCCTCCAC	82	174
12	AGATGCTCTTGTGTGTGTTGTG	CAGCATCCAACCCACCCTAC	113	173
13	CTTTCTCTGGCACTTCATCTC	CCTCTCCACAATTATTGACCG	208	299

which consist of wide, twisted ribbons and contain only tau isoforms with four microtubule-binding repeats (Spillantini et al. 1997).

The microtubule-associated protein tau is believed to function in the assembly and stabilization of microtubules. The tau protein isoforms found in human brain samples are encoded by 11 exons (Andreadis et al. 1992). A total of six different major tau mRNA transcripts are generated, resulting from alternative splicing, which encode proteins of 352–441 amino acids (Goedert et al. 1989*a*). The alternative splicing of exon 10 generates tau proteins with three or four microtubule-binding motifs that are imperfect repeats of 31 or 32 residues each (Goedert et al. 1989*b*).

Recently, we and others sequenced the *tau* gene in FTDP-17 families (Hutton et al. 1998; Poorkaj et al. 1998; Spillantini et al. 1998*b*) and identified missense mutations in coding exons 9, 10, 12, and 13, which are predicted to affect the microtubule-binding properties of the tau protein (MIM 157140). Mutations also were found in a predicted stem-loop structure at the 5′ side of the intron, between exons 10 and 11, causing a shift in the normal splicing ratio of transcripts containing exon 10, and resulting in a higher proportion of four-repeat tau isoforms. In the present study, we report the contribution of *tau* mutations as a cause of FTD in a large group of patients ascertained in a genetic epidemiological study of FTD in the Netherlands.

Patients and Methods

Patients

Ninety patients with FTD were identified in a genetic-epidemiological study in the Netherlands (population ~15 million), between January 1994 and June 1998. Part of this study has been described elsewhere (Stevens et al. 1998). Neurologists, psychiatrists, and physicians at nursing homes were asked to report all patients with FTD with age at onset <65 years, irrespective of family history. We excluded secondary cases of FTD mentioned in family history, to avoid referral bias resulting from familial clustering. The diagnostic criteria were the clinical and neuroimaging findings defined by the Manchester and Lund groups (1994). Progressive behavioral changes and speech disturbances were characteristic early symptoms in all patients, whereas memory problems were initially absent. The diagnosis of FTD was supported by neuropsychological test results at ascertainment or by review of previous neuropsychological reports, when available. All patients showed frontotemporal atrophy, on computed tomography or magnetic resonance scan, or showed anterior hypoperfusion on single-photon-emission–computed tomography. Two independent neurologists, blinded for family history and neuroimaging, checked the clinical diagnosis, and a neuroradiologist with no knowledge of the patients' family history of FTD evaluated the presence of neuroimaging changes. Data on dementia and other neurodegenerative disorders in first-degree relatives were collected by use of a family questionnaire.

The study was approved by the Medical Ethics Committee of The University Hospital Dijkzigt. Informed consent for venipuncture for DNA studies was obtained from the spouse or from a first-degree relative of each patient.

DNA studies

Blood samples were collected from 90 patients with FTD. DNA was prepared according to standard procedures (Miller et al. 1988). Exons of the *tau* gene were amplified by use of specific primers derived from the 5′ and 3′ intronic sequences (table 1). The annealing temperature for all primer pairs was 58°C. Amplification conditions were as follows: reaction volume was 50 μl, with a final concentration of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM dNTPs; *Taq* polymerase at 1.5 units/50 μl; primers at 25 pmol/μl; and 50 ng template genomic DNA. The PCR reactions

were analyzed on a 2% agarose gel to verify the size and quantity of the PCR product.

For SSCP, 3 μl PCR product was applied to the Pharmacia GenePhor Electrophoresis system. Gels were run for 70 min at 18°C and for 90 min at 5°C. Running conditions for both gels were 600V, 400 mA, and 30W, respectively. Bands then were visualized by use of a DNA silver staining kit (Pharmacia) in a Hoefer automated gel stainer. All 11 coding exons of the *tau* gene, including flanking intronic sequences, were amplified from genomic DNA of each FTD patient by use of PCR primers specified in table 1. SSCP analysis was performed on exons 1, 2, 3, 4, 5, 7, 9, 11, and 13, and the exons presenting band shifts were subsequently analyzed by direct sequence analysis of the PCR products, on an automated DNA sequencer (ABI 377), by use of the BigDye terminator cycle sequencing kit. Exons 10 and 12 of all 90 cases of FTD were sequenced directly on both strands.

Oligonucleotides with a length of 15 bases for allele-specific oligo (ASO) hybridization were designed for the mutated and normal sequence. ASO hybridizations were performed at 42°C for 1 h. Filters were washed, until a final stringency of 0.3XSSC/0.1SDS was obtained, for 15 min at 42°C.

Microtubule Assembly Assays

Site-directed mutagenesis was used in the four-repeat 412–amino acid isoform of tau (expressed from cDNA clone htau46; Goedert et al. 1989*a*), to change the proline residue at position 301 to a leucine (P301L) and to delete the lysine at position 280 (ΔK280), in the numbering of the 441–amino acid isoform of human tau. Wild-type and mutated tau proteins were expressed in *Escherichia coli* BL21 (DE3), as described elsewhere (Goedert and Jakes 1990). Bacterial pellets were resuspended in 50 mM PIPES, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 0.5 μg/ml leupeptin (pH 6.8), followed by a 2 × 1 min sonication on ice, by use of a Kontes Micro Ultrasonic Cell Disrupter. The homogenates were centrifuged at 27,000 × *g* for 15 min, and the supernatants were filtered through a 0.45 μm Acrodisc. The filtrate was loaded onto a phosphocellulose column (bed volume 2 ml) that was equilibrated in extraction buffer. The column was washed in extraction buffer and then in extraction buffer + 0.1 M NaCl. Protein was eluted batchwise with 6 ml extraction buffer containing 0.3 M NaCl. This was followed by overnight dialysis against a saturated ammonium sulphate solution and then precipitation by a 10-min centrifugation at 50,000 rpm (Beckman TL100). The pellet was resuspended in extraction buffer and reprecipitated by addition of an equal volume of saturated ammonium sulphate solution. After centrifugation, the pellet was resuspended in 1 ml extraction buffer containing 0.5 M NaCl and 1% 2-mercaptoethanol and was then boiled for 3 min. After a 10-min centrifugation at 50,000 rpm, the supernatant was loaded onto a NAP10 column equilibrated in 80 mM PIPES, 1 mM EGTA, 0.2 mM $MgCl_2$, 1 mM DTT (microtubule assembly buffer minus GTP), and eluted with 1.5 ml of the same buffer. GTP was added until a final concentration of 1 mM. Tau protein concentrations were determined by densitometry (Molecular Dynamics), with bovine serum albumin used as the standard. In all experiments, wild-type and mutant proteins were expressed and purified in parallel.

Purified recombinant wild-type and mutated htau46 (0.1 mg/ml, 2.3 μM) proteins were incubated with bovine brain tubulin (1 mg/ml, 20 μM, Cytoskeleton, Inc.) in assembly buffer at 37°C, as described elsewhere (Hasegawa et al. 1997). The assembly of tubulin into microtubules was monitored over time by a change in turbidity at 350 nm.

Results

The group of patients with FTD consisted of 59 women and 31 men. The mean age at onset of FTD was 54.6 years ± 8.4 years, with a mean duration of illness of 5.4 years ± 2.7 years. Family history of dementia was positive in 37 patients (41.1%). The clinical diagnosis of FTD was confirmed by pathological examination in 13 cases. Pick disease was diagnosed in three cases. Nine patients died without pathological verification.

We systematically performed SSCP and sequence analysis in our collection of patients with FTD, to detect mutations in the *tau* gene. Thirteen sites were detected in patients who were heterozygous for the sequence alterations; no homozygous sequence alterations were detected. To determine whether these alterations were potential disease-related mutations or nonpathogenic polymorphisms, we looked for the presence of each sequence alteration in healthy family members or in 192 (96 males and 96 females) unaffected individuals from the Dutch population, using ASO hybridization.

Eight of the 13 sequence alterations also were found in healthy control individuals and were identified as non–disease-related polymorphisms (table 2). These eight base changes are all silent mutations that do not affect the amino acid sequence of tau protein. Five sequence alterations were not detected in healthy family members or in the 192 control individuals and therefore could be disease-related mutations (table 3).

Three different missense mutations were detected in a total of 14 patients, resulting in the following amino acid substitutions: G272V, P301L, and R406W, numbered according to the longest isoform of human brain tau (Goedert et al. 1989*a*; table 3). In addition, we found

a single amino acid deletion (ΔK280) in one patient and a base change (G→A) at position +33 in the intron after exon 9 in another patient.

In 17.8% of all patients with FTD, a mutation was detected (table 3). All patients with a mutation had a positive family history of dementia, except the patient with the ΔK280 mutation, who had a positive family history of Parkinson disease.

In genetic diseases, pathogenic mutations must be absent in healthy control individuals and must segregate with the disease phenotype. The G272V mutation, resulting from the nucleotide change 1051G→T in exon 9, was found in two patients (2.2%). Both patients were members of the HFTD II family, for which segregation of the mutation with the disease has been reported (Hutton et al. 1998).

The P301L mutation, resulting from the nucleotide change C→T in exon 10, was present in 11 cases (12.2%). Cosegregation of the P301L mutation for five affected family members has been described elsewhere, for the HFTD I family (Hutton et al. 1998). Six additional cases were found in the present study. Nine of the 11 total cases with a P301L mutation share a common ancestor, on the basis of a genealogical study and construction of haplotypes for polymorphic markers in and around the *tau* gene.

The R406W mutation, resulting from the nucleotide change C→T in exon 13, initially was observed in a single patient (1.1%). From the relatively small R406W family (HFTD IV), three affected individuals were tested, and the mutation segregated with the disease phenotype.

The ΔK280 mutation, resulting from a deletion of the nucleotides AAG in exon 10, was detected in a single case (1.1%). The base change G→A at position +33 in the intron after exon 9 was also observed, in one patient (1.1%) from a small family with no additional affected family members available for study. Thus, additional evidence that this mutation is pathogenic and not merely a rare polymorphism can be obtained only by in vitro studies.

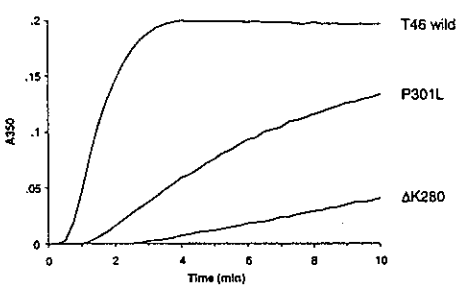

Figure 1 Effects of the ΔK280 mutation in tau on the ability of four-repeat htau46 (412-amino acid isoform of human tau) to promote microtubule assembly. Comparison with the P301L missense mutation in tau. Polymerization of tubulin induced by wild-type htau46, htau46 ΔK280, and htau46 P301L, as monitored over time by turbidimetry. A typical experiment is shown; similar results were obtained in three separate experiments.

Table 2

Polymorphic Sites Identified in the Tau Gene

Position in Tau Gene	Nucleotide Change	Frequency in FTD Cases (%)
Exon 1 position −13 from ATG	A→G	18
3′ Exon 2 + 18	C→T	18
3′ Exon 3 + 9	A→G	33
Exon 7 codon 176	G→A	18
Exon 9 codon 227	A→G	30
Exon 9 codon 255	T→C	30
Exon 9 codon 270	G→A	6
3′ Exon 11 + 34	G→A	28

NOTE.—Sequence alterations were also detected in healthy family members of patients or in a series of Dutch healthy controls.

The present study includes 30 independent families, each with at least two affected family members, that could not be linked to a common ancestor by genealogical studies or haplotype data. In seven (23.3%) of these families, a mutation in the *tau* gene was detected.

Tau protein promotes microtubule assembly, stabilizes microtubules, and affects their dynamic behavior. The G272V, P301L, and R406W mutations strongly reduce the ability of tau protein to promote microtubule assembly (Hasagawa et al. 1999). To strengthen the evidence that the ΔK280 reported here is indeed pathogenic, we performed microtubule assembly assays using recombinant wild-type four-repeat tau and tau with the Δ280K mutation. We compared their ability to promote microtubule assembly with that of the same four-repeat tau isoform with the P301L mutation. The effect of the Δ280K mutation on microtubule assembly was dramatic and larger than the effect of the P301L mutation (fig. 1). Recombinant tau protein with the Δ280K mutation showed a greatly reduced ability (<1% of activity of wild-type tau, after 2 min) to promote microtubule assembly (fig. 1). The effect was much larger than for the other known missense mutations in *tau*.

Discussion

We describe here the results of a systematic screen for mutations in the microtubule-associated protein tau in 90 patients with FTD, obtained through a genetic epidemiological study performed in the Netherlands since January 1994. Our analysis revealed a mutation in the *tau* gene in 17.8% of patients with FTD and in 40.5% of patients with a positive family history for dementia. Although we excluded secondary cases to avoid referral

Table 3

Mutations Identified in the Tau Gene

Exon	Nucleotide Change	Amino Acid Change	No. of Cases	No. with Positive Family History
9	G→T	G272V	2	2
3′ exon 9	+33 G→A	...	1	1
10	C→T	P301L	11	11
10	ΔAAG	ΔK280	1	0

NOTE—Positions are numbered according to the longest human brain tau isoform (441 amino acids).

bias resulting from familial clustering, the 37 nuclear families have been reduced to 30 independent families by use of extensive genealogical studies and haplotype analysis. In seven (23.3%) of these families, a mutation in *tau* was detected.

Three distinct missense mutations, one 3-bp deletion, and one mutation in the intron after exon 9 were found. No nonsense mutations were found and none of the previously described intronic mutations in the predicted stem loop after exon 10 were found (Hutton et al. 1998; Spillantini et al. 1998*b*). No large insertions or deletions were detected.

In samples of adult human brain, six tau isoforms were expressed from a total of 11 exons (Goedert et al. 1989*a*; Andreadis et al. 1992). In this study, we analyzed all 11 coding exons. We used SSCP analysis for mutation detection and, although we used different experimental conditions, this method might detect effectively only 80%–90% of mutations present. Furthermore, additional sequences, such as coding exons that are expressed in the peripheral nervous system, or regulatory elements, such as the promoter region of *tau*, were not tested in this study. That we did not find mutations in ~60% of the familial cases, and that we did not find a mutation in patients from family HFTD III, which is linked to chromosome 17 (Heutink et al. 1997), clearly indicate that the present findings may constitute an underestimate of the percentage of mutations in the *tau* gene in cases of FTD.

We therefore currently are extending our mutational analysis to the noncoding region of the *tau* gene. For many familial cases, we could not determine whether they were linked to chromosome 17, because additional family members were not available. We therefore cannot exclude locus heterogeneity for FTD.

Additional evidence that the G272V, P301L, and R406W mutations are pathogenic comes from the observation that all three mutations segregated with the clinical phenotype in families HFTD II, HFTD I (Hutton et al. 1998), and HFTD IV, respectively. Furthermore, the P301 substitution occurs in a highly conserved region of the tau protein sequence. A proline residue is present at the equivalent position in all species from which tau has been sequenced. The G272V mutation in exon 9 also affects a highly conserved residue within the microtubule-binding domain. Within the imperfect repeat sequences that make up the four microtubule-binding domains, the G272V and P301L mutations affect positions that are separated by only one residue. Thus, for P301L, the invariant PGGG motif in the binding repeat becomes LGGG, and for G272V, it becomes PGVG. In contrast to the P301L mutation (exon 10), the G272V mutation (exon 9) affects all tau isoforms. Analysis of tau filaments extracted from HFTD 1 brain samples has revealed that the filaments are narrow, twisted ribbons that consist mainly of four-repeat tau isoforms, which is consistent with the P301L mutation only affecting four-repeat tau isoforms (Spillantini 1998*a*). The R406W missense mutation in exon 13 alters a highly conserved residue near the C-terminus, outside the microtubule-binding repeats, in close proximity to residues S396 and S404, that are phosphorylated in hyperphosphorylated filaments (Goedert 1993).

Experimental confirmation that the mutations are pathogenic has been obtained by means of functional studies of microtubule assembly. All three missense mutations reduce the ability of tau to promote microtubule assembly in vitro, with the P301L mutation having the strongest effect (Hasegawa et al. 1999). In the present study, we compared the effects of the P301L mutation and of the ΔK280 deletion mutation on microtubule assembly. Tau protein with the ΔK280 mutation showed a strongly reduced ability to promote microtubule assembly. This effect was much larger than for the P301L mutation. The ΔK280 mutation is located in the exon 10 "linker region," between two microtubule-binding repeats that contain the following amino acids, starting at amino acid 274, using the numbering for the longest tau isoform: KVQIINKKLD. The deletion removes either the second or the last lysine residue at position 280 or 281. Two other studies have used site-directed mutagenesis to mutate the K280 and/or K281 residues (Goode and Feinstein 1994; Trinczek et al. 1995). By use of microtubule binding and assembly assays, it was shown that the mutated proteins had a 3.6 times lower affinity for microtubules than the wild-type tau. In addition, the dynamic instability of microtubules was increased strongly, demonstrating that these lysine residues are critical for tau function.

The patient with the ΔK280 mutation has a negative family history of dementia, but his father was diagnosed with Parkinson disease. Clark et al. (1998) recently have reported a mutation that introduces an additional lysine residue (N279K), directly adjacent to K280, in a family with pallido-ponto-nigral degeneration. Characteristic of the clinical phenotype in this family is the rapidly progressive parkinsonism. Unfortunately, the limited clinical and genetic data available for the patient with

the ΔK280 mutation and other family members do not permit us to determine the significance of these similarities.

We could not determine whether the intronic mutation after exon 9 at position +33 segregates with the clinical phenotype, since no other family members were available. The mutation may affect the alternative splicing pattern of tau, although the mechanism is not yet clear. Exon 9 itself is not spliced alternatively, and no potential stem-loop structure was observed after exon 9. No sample of brain material was available from the patient, which precluded experiments to determine whether the mutation causes a change in tau mRNA transcripts.

A possible explanation for the effect of the mutation could come from two recent reports implicating multiple copies of short intronic (A/U)GGG repeats in splicing efficiency (Sirand-Pugnet et al. 1995; Cogan et al. 1997). The disruption of these short motifs reduces splicing efficiency. In the ±200 bp of intronic sequence currently available, eight (A/U)GGG motifs were found. The +33 G→A base change disrupts the first of these motifs. The expected effect would be inhibition of splicing. We are currently in the process of obtaining additional intronic sequences, and we hope this will enable us to design an assay for testing the significance of this mutation in vitro.

How can the mutations that have been found so far provide an explanation of the clinical phenotype and neurodegeneration found in patients? At first sight, the missense and deletion mutations, on one hand, and the intronic mutations, on the other hand, appear to produce opposite effects on tau function, whereas the clinical phenotype of patients with these different types of mutations is highly similar (Foster et al. 1997). Both the missense and deletion mutations reduce the ability of tau to promote microtubule assembly, which is the product of microtubule nucleation and/or growth. The intronic mutations lead to increased levels of four-repeat tau isoforms (Hutton et al. 1998; Spillantini et al. 1998*b*), which could lead to an increase in microtubule assembly. It is well established that four-repeat tau isoforms are better at promoting microtubule assembly than are isoforms with three repeats (Goedert and Jakes 1990).

There is some evidence to suggest that tau may nucleate microtubules in nerve cells (Bré and Karsenti 1990; Hirokawa 1994). A possible explanation of why mutations with opposite effects do not seem to influence clinical phenotype might be that a reduced ability to promote microtubule assembly, resulting from the missense and deletion mutations, could lead to an excess in free, unbound cytoplasmic tau. An increase in four-repeat tau isoforms, resulting from the intronic mutations, similarly leads to an excess of four-repeat tau over available binding sites on microtubules, because three- and four-repeat tau isoforms may bind to different sites on microtubules, as has been suggested by Goode and Feinstein (1994). A reduced ability of tau molecules to interact with microtubules thus could be the primary defect resulting from the different mutations in tau. This may in turn lead to the hyperphosphorylation of tau, which could reinforce the primary effect of the mutations. It is well known that hyperphosphorylated tau from samples of brains affected by Alzheimer disease is unable to bind microtubules or to promote microtubule assembly (Brambiett et al. 1993; Yoshida and Ihara 1993). Over time, perhaps in conjunction with other factors (Goedert et al. 1996), hyperphosphorylated tau will then assemble into filaments. In addition to effects on microtubule assembly and microtubule binding, mutations in tau may also have additional, direct effects on phosphorylation of tau and may favor its assembly into filaments.

In this view, the formation of tau filaments is the gain of the toxic function that is believed to underlie autosomal dominantly inherited, late-onset neurodegenerative diseases (Goedert et al. 1998). Future studies will show whether this view is correct or whether tau filament formation is only a by-product of the underlying pathogenic process. It will be important to understand how mutations in *tau* lead to neurodegeneration, because it is already clear that the *tau* gene is a major locus of inherited dementing disease. The discovery of mutations in the *tau* gene will advance the study of dementia in general, since Alzheimer disease, the most common dementing illness, is characterized by an abundant *tau* pathology whose presence correlates with the degree of cognitive impairment.

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Electronic-Database Information

Accession numbers and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for FTDP-17 [MIM 601630] and microtubule binding–associated protein tau [MIM 157140])

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Chapter 5.5.3

Mutation-dependent aggregation of tau protein and its selective depletion from the soluble fraction in brain of P301L FTDP-17 patients

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Mutation-dependent aggregation of tau protein and its selective depletion from the soluble fraction in brain of P301L FTDP-17 patients

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Mutations in the gene for the microtubule-associated protein tau are associated with frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). In this study we compared the presence of the P301L mutated tau protein from brain material of patients with that of the normal 4-repeat, using polyclonal antibodies specific for the P301L point mutation and its normal counterpart. We determined the relative ratio of mutated versus normal tau protein in the sarkosyl-soluble and -insoluble protein fractions from several brain regions. Although mutated and normal tau proteins are both present in the sarkosyl-insoluble deposits, quantitative analysis showed that the mutated protein is the major component. In the sarkosyl-soluble fraction of frontal and temporal cortex the overall ratio of 3-repeat versus 4-repeat tau isoforms is unchanged but there is a dramatic depletion of mutant tau protein. Furthermore, we observed an increase in tau-immunoreactive cleavage products with the P301L antibody, suggesting that the mutant protein is partly resistant to degradation and this is confirmed by pulse–chase experiments. This is the first direct evidence using patient material that shows a selective aggregation of mutant tau protein resulting in sarkosyl-insoluble deposits and the specific depletion of mutated tau protein in the soluble fraction.

INTRODUCTION

The term frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), covers a number of neurodegenerative syndromes with diverse but overlapping clinical and neuropathological features. Early behavioral changes, later accompanied by cognitive and motor disturbances, and often by atrophy in the frontal and temporal lobes on neuroimaging, are characteristic for this condition (1,2). Pathological hallmarks of most FTDP-17 brains are insoluble filamentous aggregates of hyperphosphorylated protein tau, in neurons or in both neurons and glial cells, similar to those found in other neurodegenerative disorders such as Alzheimer's disease (AD), Pick' s disease, progressive supranuclear palsy, corticobasal degeneration and amyothrophic lateral sclerosis (ALS), Parkinsonism-dementia complex of Guam (3).

Genetic analysis of FTDP-17 has revealed that the disorder is caused by mutations in the gene for the microtubule-associated protein tau (4–6). The tau protein is encoded by a single gene but, due to differential RNA splicing, is expressed in the adult human brain in six isoforms. These isoforms differ by the presence of three or four imperfect repeats in the C-terminus, which are part of the microtubule-binding region and by the presence or absence of two N-terminal inserts of 29 and 58 amino acids (7).

The tau protein regulates the dynamic stability of the neuronal cytoskeleton and plays an important role in neuronal differentiation and axonal development (8) through its ability to promote microtubule assembly. Moreover, tau protein is involved in positioning of cell organelles, axonal transport and the maintenance of neuron polarity (9–12).

The mutations identified in the *tau* gene are localized both in the coding and non-coding sequences of the gene (13). The intronic mutations are all located close to the 5′ splice-donor site of the intron following exon 10. In addition, several mutations within exon 10 have been shown to cause a shift in the normal splicing ratio of transcripts containing exon 10, mostly resulting in an increased ratio of 4-repeat (4R) with respect to 3-repeat (3R) tau (4,6,14–16).

The missense mutations and a small deletion are localized in the C-terminal part of the protein containing the microtubule-binding domains and are likely to alter tau's interactions with tubulin. The mutations in exon 10 only affect the 4R tau isoforms, but other mutations affect both 3R and 4R tau isoforms.

The P301L mutation in FTDP-17 kindreds has been most extensively described (4,17–21). Perinuclear deposits of hyperphosphorylated tau are seen in neurons, glial cells and neurites of frontal and temporal cortex, hippocampal formation and substantia nigra in brains of P301L patients. The deposits consist of slender twisted filaments 15 nm wide with variable

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periodicity and a few straight filaments (22). Tau extracted from these filaments appears on immunoblot as two major bands of 64 and 68 kDa and a minor band of 72 kDa, that after alkaline phosphatase treatment can be resolved in two 4R (4R, 4R + 28 amino acids) and one 3R tau isoforms (3R + 28 amino acids).

The proline residue at codon 301 is part of a highly evolutionary conserved PGGG motif that is found in all microtubule-binding repeats and is located in the microtubule–binding repeat specific for 4R tau. Several studies (15,16,23,24) have been performed to investigate the possible effect of the tau mutations on microtubule polymerization and binding and the results indeed showed impairment in these parameters.

Tau filaments, similar to those found in brains of patients, can be generated *in vitro* as well. 4R tau aggregates more readily than 3R tau and, compared with this, tau with missense mutations aggregates even faster. Results from three independent studies demonstrate that the P301L mutation has a high potential for fibril formation (25–27). Until now, however, it has not been shown that the P301L mutated tau protein has these properties *in vivo*.

For the present study, we developed specific rabbit polyclonal antibodies raised against the P301L point mutation and its normal counterpart and showed that although both normal 4R and mutated P301L tau are present in the insoluble deposits of the cerebral cortex, the main component of these deposits is the mutated tau protein. Conversely, in the soluble fraction we observed a strong reduction in the level of mutated P301L protein, especially in the frontal cortex. These findings suggest that the low level of mutated protein in the soluble fraction is due to a selective depletion, resulting from the selective aggregation of mutant protein in the sarkosyl-insoluble deposits. In addition, we observed an increase of tau-immunoreactive cleavage products for the mutant protein compared with the normal protein, strongly suggesting that an inappropriate digestion of the mutated protein plays an important role in the pathogenesis of FTDP-17 as well.

RESULTS

Characterization of the antibodies tau-P301 and tau-P301L

To determine the specificity of tau-P301 and tau-P301L antibodies, transient and stable transfection experiments were performed using COS and PC12 cell lines. Immunocytochemical (Fig. 1) and immunoblot (Fig. 2A and B) analyses showed that the tau-P301 and tau-P301L antibodies specifically recognize the normal 4R tau or the P301L mutant protein, respectively.

In order to compare the strength of the tau-P301 and tau-P301L antibodies, both normal and mutant P301L tau (longest isoform), were synthesized by *in vitro* transcription and translation in the presence of [^{35}S]methionine. Identical amounts of both tau proteins were loaded on SDS–polyacrylamide gel, blotted (Fig. 2C) and incubated with tau-P301 and tau-P301L antibodies (using the same dilutions), respectively (Fig. 2D). Quantitative analysis showed that the ratio tau-P301L:tau-P301 was 0.86; thus, no significant difference was found in the binding of the two antibodies to their respective epitopes, making them suitable for quantitative western blots studies.

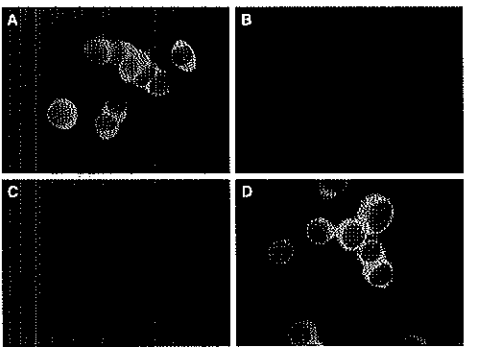


Figure 1. PC12 stably transfected cells with normal 4R (A and C) and mutant P301L tau (B and D) were stained with tau-P301 antibody (A and B) and tau-P301L antibody (C and D).

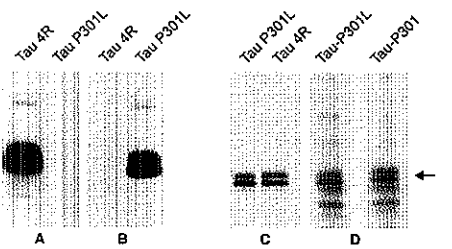


Figure 2. Immunoblot of COS cells transfected with normal 4R and mutated P301L tau and incubated with tau-P301 (A) and tau-P301L (B) antibodies. (C) Autoradiograph of SDS–PAGE gel showing P301L mutated and normal 4R tau proteins synthesized by *in vitro* transcription and translation in the presence of [^{35}S]methionine. The two bands seen in each lane might be the result of an additional translation initiation due to the presence of a second methionine 10 amino acids downstream from the first. (D) Immunoblot of *in vitro* synthesized P301L mutated and normal 4R tau proteins, detected with tau-P301L and tauP301 antibodies, respectively. The bands indicated by an arrow were quantified to compare the strength of both antibodies.

Histochemical analysis of mutated and normal tau proteins

In patients carrying the P301L mutation, the tau-P301 and the tau-P301L antibodies gave a similar staining patterns in brain slides from the cerebral cortex and the hippocampus. They recognized especially the aggregates in the superficial layers 2–3 and deep layer 6 of the temporal cortex (Fig. 3A) and to a lesser extent aggregates in the frontal and entorhinal cortex. These aggregates were mainly located in the perinuclear region, cell body and sometimes extending to the apical dendrites of neurons. In one patient occasionally a diffuse staining with both antibodies was observed in the axons. Tau-

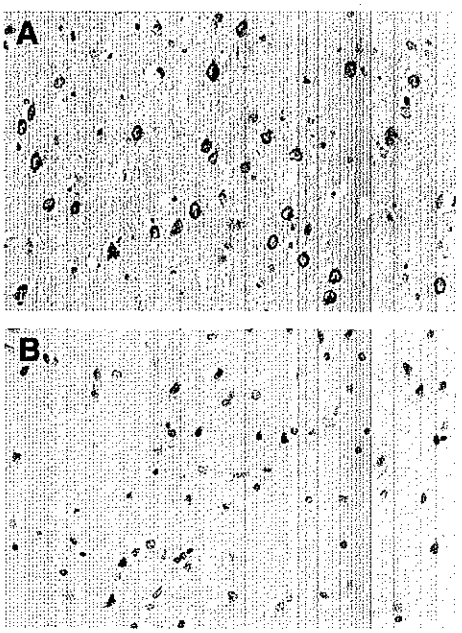


Figure 3. Sections of the temporal cortex of P301L patient 3 (A) and an AD patient (B) stained with tau-P301L antibody. Magnification, ×40.

P301L antibody did not show any signal in AD brain (Fig. 3B), non-demented control and R406W cases.

Biochemical analysis of mutated and normal tau proteins

Sarkosyl-insoluble tau, isolated from frontal cortex, temporal cortex and cerebellum of three patients carrying the P301L mutation, one AD patient and one disinhibition dementia Parkinsonism amyotrophy complex (DDPAC) patient, was run on an SDS–PAGE gel and immunoblotted with the antibodies H-7, tau-P301 and tau-P301L.

With H-7 and tau-P301 antibodies, three major bands (60, 64 and 68 kDa) were seen in the AD patient and two bands (64 and 68 kDa) in the DDPAC patient. These brains did not show any signal with the tau-P301L antibody, as expected (Fig. 4A).

In the material extracted from frontal cortex of the P301L patients, all three antibodies recognized two major bands of 64 and 68 kDa and a minor band of 72 kDa, with the 68 kDa band being the strongest (Fig. 4A). After alkaline phosphatase treatment of the material from P301L patients and staining with H-7 antibody, the insoluble tau was resolved into four bands corresponding to the 4R, 3R + 29 amino acids, 4R + 29 amino acids and 4R + 58 amino acids isoforms (Fig. 4B). The bands corresponding to the 4R and to the 4R + 29 amino acids appeared to be the strongest.

Quantitative analysis was used to determine the relative amount of mutated tau-P301L protein in the insoluble deposits for all the three patients with the P301L mutation (Fig. 4C). The P301L mutated tau protein was found to be on average four times more abundant than the normal 4R tau.

These results show that in the sarkosyl-insoluble deposits of the frontal cortex both normal 4R and mutated P301L tau protein are present, but that 4R protein consists mainly of mutated P301L protein.

Similar experiments performed on the sarkosyl-insoluble tau isolated from the temporal cortex showed that also in tau deposits of the temporal lobe the mutated tau is the major 4R tau component. The sarkosyl-insoluble fraction from the relatively unaffected cerebellum of P301L patients did not show the presence of hyperphosphorylated tau protein, as expected (data not shown).

Sarkosyl-soluble tau from the frontal, temporal cortex and cerebellum of the three P301L patients, stained with the H-7 antibody, showed a pattern similar to that of the control case (Fig. 5): six bands aligned with the six isoforms from the recombinant tau. Staining with tau-P301 and tau-P301L antibodies (Fig. 6A–C) resolved this sarkosyl-soluble dephosphorylated tau into two major bands corresponding to 4R and 4R + 29 amino acids isoforms. A quantitative analysis was performed to compare the 3R:4R ratio in the soluble fractions among the P301L cases and the age-matched control. No significant differences were observed in all cases, the ratio was ~1 in the frontal cortex. A single patient showed a decrease of 20% for 4R tau, similar to what has been described previously (16). The cerebellum of the FTDP-17 cases and the control contained more 4R tau than 3R, again in agreement with Hong *et al.* (16).

The same experiment was repeated to determine the ratio between mutated P301L and normal 4R tau (Fig. 6D) and a striking decrease of mutated P301L protein was observed in the soluble fraction from the frontal cortex where the amount of the normal 4R tau was on average 5-fold higher than the mutated tau. In the temporal cortex the difference was less strong, showing a 2- to 5-fold decrease of mutated P301L tau compared with the normal 4R. In the non-affected area of the cerebellum a 2-fold decrease was observed for the P301L protein with respect to the normal 4R tau.

Below 45 kDa, two bands were detected with both the tau-P301 and tau-P301L antibodies but their intensity was significantly stronger with the tau-P301L antibody. Since we carefully matched the total amount of protein loaded on the gel for each sample and we have showed that both antibodies have similar strength, it is unlikely that the differences in the intensity of the two bands seen with the two antibodies are caused by proteolysis in the experimental procedure, suggesting therefore an alteration of the proteolytic processing of the mutated P301L protein compared with the normal protein.

Therefore, we performed pulse–chase experiments and determined the half-life of normal 4R tau and its mutant P301L counterpart in transiently transfected COS cells. Newly synthesized tau proteins were harvested at different time points (0, 6, 10 and 22 h) and immunoprecipitated with H-7 monoclonal antibody. After 22 h only little amounts of normal 4R (4.9 ± 2.4%) and P301L (6.6 ± 3%) tau proteins were present. However, after 10 h of chase a significant delay in the degradation of P301L tau (40 ± 13%) compared with the normal 4R (67 ± 1.75%) was observed (Fig. 7).

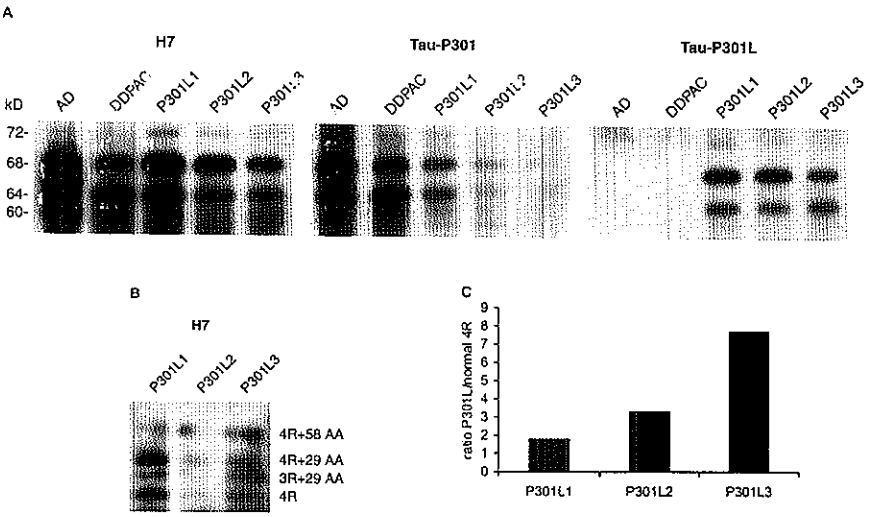


Figure 4. (A) Immunoblot of sarkosyl-insoluble tau from the frontal cortex of one AD, one DDPAC and three FTDP-17 patients carrying the P301L mutation (P301L1, P301L2 and P301L3), using the phosphorylation independent antibody H-7, the tau-P301 and the tau-P301L antibodies. (B) Immunoblot of sarkosyl-insoluble tau from the frontal cortex of the three P301L patients after alkaline phosphatase treatment, stained with the phosphorylation-independent antibody H-7. (C) Ratio between the relative amounts of mutated P301L protein and normal 4R tau in the sarkosyl-insoluble deposits from the frontal lobe.

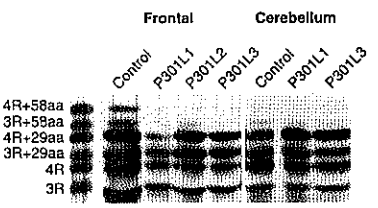


Figure 5. Quantitative immunoblot of sarkosyl-soluble tau from frontal cortex of one control and three P301L patients and cerebellum from two P301L patients and one control. Sarkosyl-soluble tau was dephosphorylated and stained with the H-7 antibody. Six bands were obtained that aligned with the six isoforms of the recombinant tau.

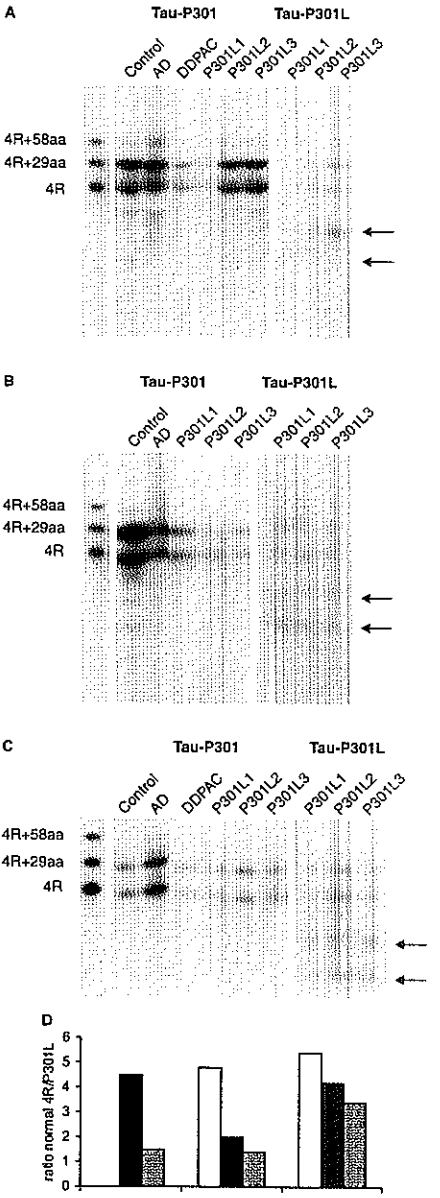
DISCUSSION

The mutations found in the gene for the microtubule-associated protein tau in FTDP-17 patients have provided us with new opportunities to study the role of tau protein in neurodegenerative disorders characterized by tau pathology. Several *in vitro* studies have demonstrated that most missense mutations result in impaired binding and microtubule polymerization capacities compared with the normal 4R protein (15–17,23,24), suggesting a possible role for mutated tau protein in the neurodegeneration process through a progressive disruption of microtubule function and stability. However, in a recent study (24) cells transiently transfected with wild-type or mutated tau constructs could not be distinguished in terms of tau-decorated microtubule networks and bundles, suggesting that mutations do not have a large and immediate effect on the interactions between tau and microtubules.

More recently, *in vitro* studies demonstrated that most missense mutations also increase tau's potential for the formation of fibrils, both at the nucleation and the elongation phases and this 'gain-of-function' of mutant tau might explain the observed aggregation of tau protein in brain.

For a better understanding of the role of the mutated protein in the pathogenesis of the disease we studied the localization and levels of the P301L and normal 4R tau proteins in brain material from patients with P301L mutation using rabbit polyclonal antibodies specifically directed against the P301L mutation and its normal 4R counterpart.

Our immunohistochemistry experiments clearly show the presence of both mutated and normal 4R tau protein in the perinuclear deposits in the affected areas of frontal and temporal cortex and hippocampal formations. No clear differences were observed in the cellular localization of the mutated P301L protein versus the normal 4R tau. The diffuse staining seen occasionally in the axons with both antibodies, suggests that both normal and mutated protein are present in the axons. This finding suggests that the P301L mutation does not result in the absence of mutated protein from the axon, which can be



explained by the observation that tau mRNA is selectively translated in the axons of neurons. The fact that the axonal staining is seen only in one patient might reflect the limits of using these antibodies on paraffin-embedded material.

In our biochemical experiments, we show that the aggregates, in patients with the P301L mutation, mainly consist of mutant 4R tau and that only small amounts of normal 4R and 3R proteins are present. This preferential incorporation of mutated protein is, however, not reflected in the overall ratio of 3R:4R tau protein in the soluble fraction of frontal and temporal lobe and cerebellum. The 3R:4R tau ratio remained unchanged in two of the P301L patients with respect to the age-matched control and one patient showed only a 20% decrease of 4R tau. FTDP-17 is an autosomal dominant disorder and, therefore, the P301L protein in patients is produced by a single allele. Assuming that the normal ratio of 3R:4R tau is −1, a 2-fold change in ratio of 3R:4R protein would be expected if all P301L proteins were effectively incorporated in aggregates or selectively degraded. The fact that this change in 3R:4R ratio is not observed suggests that upregulation of the expression level of the normal 4R tau isoform might compensate for the depletion of mutant 4R tau. However, to determine whether our finding is a general finding for the P301L mutation more patient material should be investigated.

Using the tau-P301L and tau-P301 antibodies, we demonstrate that in the soluble fraction the 4R tau mainly consists of normal protein and the P301L protein is almost not present. The most notable depletion of the mutant P301L protein with respect to the normal 4R protein was observed in the frontal lobe (~5-fold) and temporal lobe (on average 3-fold), the most affected areas of the brain. In cerebellum, where no tau aggregates were observed, the depletion of mutant protein was the smallest (2-fold).

The observation that P301L protein is selectively trapped in the insoluble deposits of the frontal and temporal cortices and the finding that tau protein with the P301L mutation has a high potential to form fibrils *in vitro*, provides an attractive explanation for the formation of the characteristic tau aggregates in brains of patients. However, we then would expect that during the period of 40–60 years before the death of the patient the selective trapping of the mutated protein from the soluble tau fraction into insoluble aggregates would lead to extensive deposit formation in all neurons. Instead, insoluble tau aggregates are visible only in a small subset of neurons and in specific areas of the brain. So other factors must be involved as well.

Our finding that the immunoblots of the soluble tau material show two bands of <45 kDa might point towards an alternative explanation. These two bands are strongly stained with the

Figure 6. Quantitative immunoblot of sarkosyl-soluble tau from the frontal cortex (A), the temporal cortex (B) and the cerebellum (C) of one control case and three P301L patients, using the tau-P301 and tau-P301L antibodies after alkaline phosphatase treatment. The three isoforms containing 4R + 0, + 29 and + 58 amino acids, respectively, aligned with the corresponding isoforms of the recombinant tau. The two intermediate cleavage products, <45 kDa, are indicated by arrows. (D). Ratio between normal 4R and mutated P301L protein in the soluble fraction from frontal cortex, temporal cortex and cerebellum. The amount of mutated P301L tau in the soluble fraction of frontal cortex in P301L1 patient was below detection level.

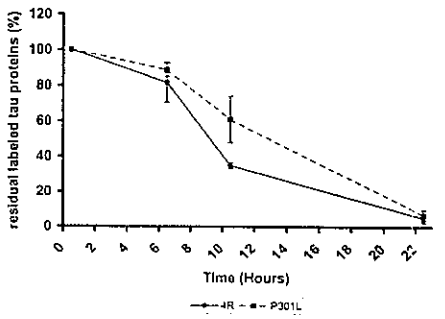


Figure 7. Pulse–chase experiments on COS cells transfected with normal 4R and P301L tau. Cells labeled with [^{35}S]methionine were incubated in unlabeled chase medium for intervals up to 22 h and harvested at time points indicated. NT, non-transfected COS cells used as negative control. The amount of residual ^{35}S-labeled 4R and P301L tau proteins in COS cells are expressed as percentage of the label measured immediately after pulse labeling (0 h).

antibody specific for the point mutation and only weakly with the tau-P301 that recognizes the normal 4R. They are present in the frontal and temporal cortex as well as in the cerebellum, an area that does not show any deposits. These bands may be intermediate fragments generated during the normal proteolysis of the mutated protein. Tau is sensitive to calpain and caspase proteolysis in neurons (28), resulting in the generation of multiple fragments, the major fragment being 42 kDa in size. The relative increase in mutant tau positive cleavage products might suggest that the mutant protein is more resistant to further calpain degradation with respect to the normal protein and this resistance might cause accumulation of intermediate proteolysis products. Yen *et al.* (29), using an *in vitro* assay, indeed reported that the P301L protein is more resistant to calpain degradation than the normal 4R tau. Our pulse–chase experiment showed that there is a delay in the degradation of mutated P301L protein in COS cells. Although our data are based on overexpression studies, the finding that there is a delay in the degradation of the mutated P301L tau protein compared with the normal 4R under the same experimental conditions suggests a potential mechanism by which the mutated protein accumulates in the cytoplasm at higher concentrations.

It is tempting to speculate that due to the presence of mutated protein and the accumulation of intermediate cleavage products, cells are more vulnerable to stress agents associated with aging, which in time might trigger alterations and modifications in the proteolytic degradation of the mutated tau versus the normal protein. Over time the combined effects of reduced microtubule assemblage and accelerated filament formation characteristic of the P301L mutation might lead to an increase of unbound cytosolic mutated P301L in the form of aggregates.

The question arises of whether for FTDP-17, as for many other neurodegenerative disorders such as AD, prion diseases, ALS and the polyglutamine repeat disorders, the aggregates are the pathogenic agents or simply markers of the cell's demise. For polyglutamine disorders evidence is emerging that inhibiting the aggregation of the mutated protein will not prevent disease (30) but instead accelerates neurodegeneration. Moreover, in a recent paper, Passani *et al.* (31) observed that modification of mutant huntingtin in target neurons promotes an abnormal interaction with one or all of huntingtin's WW domain partners, with toxic consequences, demonstrating that aberrant protein interactions play an important role in the pathogenesis of Huntington's disease.

In this study we presented evidence that mutant tau protein not only is deposited in insoluble aggregates but must for the major part be partially digested by the cell. Detailed research into the processes that result from the presence of mutated tau, including factors that are involved in tau proteolysis and the formation of aggregates, will help us to understand the critical events that lead to neurodegeneration.

MATERIALS AND METHODS

Patient materials

Paraffin-embedded sections of frontal, temporal and parietal cortices, hippocampus and substantia nigra from three FTDP-17 patients with P301L mutation, (64, 66 and 73 years) (18,22,32), two AD, two non-demented controls and one FTDP-17 patient with an R406W mutation (18) were used for immunohistochemistry. Fresh frozen tissue from frontal and temporal cortices and cerebellum of three P301L patients (60, 63 and 73 years), one AD case, one DDPAC (E10 + 14 intronic mutation in tau) (4) and 1 non-demented control were used for biochemical studies. The ages of the non-demented patients, the AD, DDPAC and R406W FTD patients were matched with the age of the P301L patients.

Antibodies

The following anti-tau antibodies were used: mouse monoclonal H-7 and AT8, rabbit polyclonal tau-P301 and tau-P301L. H-7 is a phosphorylation-independent antibody which epitope maps at amino acids 157–163 (33) (Innogenetics, Gent, Belgium). AT8 recognizes human tau isoforms when phosphorylated at Ser202 and Thr205 (34) (Innogenetics). Two rabbit polyclonal antibodies were generated in our laboratory using synthetic peptides encoding amino acids 291–305, numbered according to the longest tau isoform: CGSKDNIKHVPGGGS, for tau-P301 and CGSKDNIKHVLGGGS for tau-P301L.

Immunohistochemistry

For immunohistochemistry experiments paraffin sections were incubated with the polyclonal tau-P301 and tau-P301L antibodies (1:2000 dilution). After endogenous peroxidase inhibition (35), the sections were subjected to antigen retrieval treatment using a microwave oven. (35). Primary antibodies were incubated at 4°C overnight and visualized with an indirect immunoperoxidase technique utilizing the Histostain–Plus (Zymed, San Francisco, CA).

As a test for labeling specificity, primary antibodies were absorbed with their respective peptides as described by Hoogeveen *et al.* (36). Background labeling was negligible. Staining with the AT8 antibody was carried out as described (22).

Plasmids and constructs

Site-directed mutagenesis on a cDNA clone expressing the longest tau isoform was used to change P301 to a leucine in the longest tau isoform, with the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenesis was performed according to the manufacturer's instructions. DNA was isolated from the wild-type clone and its mutant, verified by sequencing using the BigDye Terminator Cycle sequencing kit (PE Biosystems, Foster City, CA) on an ABI 377 automated sequencer (PE Biosystems) and the tau cDNA was sub-cloned into CMV, pCDNA3 (Invitrogen, Carlsbad, CA) and pTRE vectors (Clontech, Palo Alto, CA).

In vitro transcription and translation

The longest tau isoform and its mutant P301L cloned in pCDNA3, were synthesized by *in vitro* transcription and translation in presence of [^{35}S]methionine (Amersham Pharmacia Biotech, Little Chalfont, UK) using the TNT Coupled Reticulocyte Lysate system (Promega, Madison, WI) for 90 min at 30°C. After adding 2× sample buffer, the samples were run on 10% polyacrylamide gel, dried and exposed to film.

Cell culture and transfection

COS cells were cultured in Dulbecco's minimal Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) (Clontech), 100 IU/ml penicillin and 100 µg/ml streptomycin (all from Gibco BRL, Bethesda, MD) and were kept at 37°C in 5% CO_2. PC12 cells were grown in DMEM supplemented with10% (v/v) horse serum (Sigma, St Louis, MO) and 5% (v/v) FCS (Clontech), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco BRL), and kept in 10% CO_2. COS cells were transiently transfected with pCDNA3 constructs mentioned above, using Lipofectamine reagent (Gibco BRL) according to the manufacturer's recommendations. PC12 cells were stably transfected with the wild-type and P301L tau cloned in pTRE vectors using the Tet-On Gene expression system (Clontech) (37).

Immunofluorescence

Cells were rinsed twice in cold phosphate-buffered saline (PBS), fixed and permeabilized, essentially as described by Preuss *et al.* (38). The fixed cells were then incubated with tau-P301 and tau-P301L antibodies (1:100 dilution) for 1 h at room temperature and after washing in PBS with 0.5% bovine serum albumin and 0.15% glycine, were incubated with secondary fluorescein-conjugated goat anti-rabbit antibody (1:100 dilution; Sigma). Cells were mounted in Vectashield mounting media (Vector, Burlingame, CA) and examined with a Leica DMXRA fluorescence microscope equipped with digital camera.

Gel electrophoresis and immunoblotting

Homogenates from transfected cells were prepared by lysing subconfluent cells on ice in lysis buffer (50 mM Tris–Cl pH 7.4, 1% Nonidet P-40, 1 mM $MgCl_2$, 5 mM EGTA, 5 mM dithiothreitol, 120 mM NaCl, 100 µM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin). Extracts were centrifuged immediately at 13 000 *g* for 10 min and the supernatants were used directly for SDS–PAGE. No cross-reactivity was observed for tau-P301 antibody. A faint band was visible in the tau 4R lane after incubation with tau-P301L antibody. However, quantitative analysis showed that the band intensity was <1% compared with the band in the tau P301L lane.

Sarkosyl-insoluble tau from brain was extracted and dephosphorylated as previously described (39). Samples were run on 10% SDS–PAGE and electrophoretically transferred to a nitrocellulose membrane (Scheicher & Schuell, Dassell, Germany). Residual protein-binding sites were blocked by incubation in low fat milk for 1 h at room temperature. After 30 min of washing at room temperature in PBS containing 0.1% Tween 20, the first antibody (1:1000 and 1:2000 dilution for the polyclonal and monoclonal H-7 antibodies, respectively) was incubated for 1 h at room temperature or overnight at 4°C in PBS + 0.1% Tween 20. Following 30 min of washing in PBS + 0.1% Tween 20 at room temperature, the nitrocellulose membrane was incubated 1 h at room temperatuare with the appropriate secondary antibody conjugated with horseradish peroxidase and the reaction products were visualized by using the ECL kit (Amersham Pharmacia Biotech).

To determine the isoform composition of soluble tau present both in the affected and unaffected areas, the sarkosyl-soluble tau was concentrated ~10× using Centricon devices (Millipore, Bedford, MA) and treated with protease inhibitor (Complete; Boehringer, Mannheim, Germany). Protein concentration was determined by using BCA protein assay (Pierce, Rockford, IL). After dephosphorylation, equal amounts of protein for each patient were resolved by 10% SDS–PAGE and the antibodies tau P301, tau-P301L and H-7 were used as described above.

For quantitative immunoblot analysis Protein A labeled with ^{125}I (Amersham Pharmacia Biotech) was used. The radiolabeled tau bands were analyzed and quantified using Image master 1D elite software (Amersham Pharmacia Biotech).

[^{35}S]methionine pulse–chase experiments

COS cells transiently transfected with wild-type and P301L tau, were rinsed in PBS and incubated for 3 h in 5 ml of Met–Leu-free DMEM containing 200 µCi of [^{35}S]methionine-*trans*-labeled methionine (ICN, Irvine, CA). Cells were then rinsed with 5 ml of PBS and incubated for chase intervals of 0, 6, 10 and 22 h in 10 ml of non-radioactive DMEM supplemented with 10% FCS. Cells lysates were precipitated with H-7 monoclonal antibody overnight at 4°C. The antibody–antigen complex was extracted from the lysate by incubating with Protein G–Sepharose (Amersham Pharmacia Biotech) for 1 h at 4°C, followed by centrifugation for 1 min at 10 000 *g*. Protein G–Sepharose pellets were suspended in lysis buffer and rinsed three times. After adding sample buffer the sample was then electrophoresed in 10% polyacrylamide gel and vacuum-dried. Radiolabeled tau bands were visualized and quantified by Image Master 1D elite software (Amersham Pharmacia Biotech).

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Chapter 6

Overview of Cellular and Animal Models

Cell systems.

6.1 Expression of *tau* mutants in cultured cells.

Cultured cells transfected with mutant *tau* have been examined to determine whether presence of abnormal tau affects cytoskeletal integrity, cell shape, and the state of tau phosphorylation. Human SHSY5Y neuroblastoma or CHO cells transiently transfected with 4 or 3 R wild type *tau* and corresponding mutant *tau* P301L, V337M, and R406W, respectively, were indistinguishable in their phenotype (225). In cells with low levels of tau expression, tau co-distributed with tubulin as a microtubule network, and in cells with high expression tau protein was localized with microtubule bundles.

Similar results were obtained from studies of CHO cells stably transfected with 4R(-2-3) wild type and G272V, P301L and R406W mutants (226). Only when the transfected cells were treated with cytochalasin B, an actin filaments disrupting agent, or colcemide, a microtubule depolymerizing drug, it was possible to distinguish mutant from wild type transfected cells (225, 226). The R406W mutation was the least effective in reducing microtubule stability, followed by V337M, G272V, and P301L.

In contrast, disruption of microtubule networks was observed in COS-7 cells that express three isoforms of tau, 3R(-2-3), 4R(-2-3), 4R(+2+3), with the V337M mutation (227). In particular the expression of the V337M 3R(-2-3) isoforms caused more disruption of the microtubule networks than the other V337M isoforms.

The discrepancy in phenotypic changes observed in these transfected cells studies may be due to differences in cell type and vector used in the transfection studies, expression level of tau and procedures in cell fixation.

More dramatic changes in cytoskeleton integrity are observed in two recent studies. Nagiec *et al.* (228) explored the effect of expression of wild type and mutant EGFP-*tau* in HEK293 cells after transient transfection. The results show that much of the expressed tau colocalizes with tubulin in microtubules as expected, although fixation reduces the amount of tau on microtubules. EGFP-tau seems to associate on interphase microtubule as well, including the microtubule-organizing center. More of the wild type tau than mutant tau remains bound to microtubules in living and fractionated cells. More mutant tau was seen in the cytoplasm as compared to wild type in transfected cells. The P301L mutation showed the most dramatic increase in unbound tau in the intact cells.

Lu *et al.* (229) used an elegant approach to detect wild type and mutant tau in living cells with fusion constructs that fluoresce at different wavelengths. Tau with missense mutations, P301L, V337M and R406W distributed with microtubules when expressed alone but were significantly dissociated from microtubules and appeared diffuse in the cytoplasm when coexpressed with wild type tau. These *in vivo* data are consistent with the reduced binding properties of these mutant isoforms *in vitro* but suggest a more complex regulation that includes competition among the tau isoforms for binding to microtubules. The size of both the free and bound pools of tau depends on competition among the isoforms. Within a certain concentration range, the isoforms that predominates in the free pool depends more on the presence of competing isoforms than on concentration effects. Because the mutations that cause FTDP-17 are present in the heterozygous condition, indeed both the wild type and mutant isoforms will be present in affected neurons, thus altering the competitive kinetic interactions with the microtubule.

6.2 Cell systems that show fibrillar aggregates.

Tau protein can assemble into various types or filaments under *in vitro* conditions. However, until recently it has not been possible to produce tau aggregates in intact cells even after massively over-expressing wild type tau in cultured neuronal and non neuronal cells (108, 230, 231).

To investigate the biological consequences of FTDP-17 tau mutants and assess their ability to form aggregates, stable transfected CHO cells were recently engineered that express tau harboring one (ΔK280) or several topographically distinct FTDP-17 missense mutations (232). It was shown that different tau mutants produced distinct pathological phenotypes. For example, ΔK280, but not other single tau mutants (V337M, P301L, R406W) developed insoluble amorphous and fibrillar aggregates, whereas a triple *tau* mutant (VPR) containing V337M, P301L and R406W substitutions also formed similar aggregates. The aggregates increased in size over time in culture and could be detected by indirect immunofluorescence, transmission and immuno EM. Moreover, the presence of tau aggregates correlates with mutant tau proteins that remains insoluble in nonionic detergents such as Triton X-100. However the tau-positive aggregates were not detected by histochemical dye such as Thioflavin S, lacking therefore the fibrillary nature of classic tau tangles. Furthermore the filaments detected in the aggregates were not identical to authentic PHFs in AD or tau filaments in tangles of FTDP-17 patients. This is not surprising because tau tangles might

develop over a long period of time and they are found in postmitotic neurons and non-dividing glial cells.
Nevertheless the clear limitations the CHO cell mutant tau model might prove to be useful in studies to further elucidate mechanisms leading to the formation of tau pathology.

The first time that tau aggregation has been demonstrated experimentally *in vivo* was in the ABC (anterior bulbar cells) neurons of central nervous system of a lower vertebrate, the sea lamprey (233, 234). ABC neurons have been morphologically characterized and studied in detail on a single-cell level (235-238), and they can be microinjected *in situ* with drugs for chronic experiments. Because the expressing neurons can be injected and maintained *in situ*, the lamprey ABC system offers the opportunity to study the effect of exogenous proteins in identified mature neurons in a normal environment. ABCs cells were microinjected with vectors expressing two full-length tau isoforms and deletion mutants expressing the N- or C-terminal halves of tau in intact lampreys.
Heavy tau accumulation was observed in some of the injected cells, accompanied by somatodendritic accumulation of immunopositive tau in filaments of 10- to 15-nm. The filaments containing tau were clearly different from PHFs, although they resembled in some respects the straight tau filaments seen in AD brain and some of the tau filaments generated *in vitro*. Such changes were not seen with the over-expression of *tau* deletion mutants.
The results obtained in this study suggest that tau accumulation may be followed by the formation of condensed intracellular accumulation of phosphorylated tau, the development of extracellular tau deposits, and cellular degeneration. The system therefore proved to be an excellent *in situ* model of cellular mechanisms underlying the development of the cytoskeletal pathology seen in neurodegenerative conditions.

Mouse models
Several transgenic mice have been generated with the hope of recapitulating the most important aspects of the neurodegenerative pathway. The issue has been indirectly addressed in the transgenic mouse model of Aβ amyloid deposition, which is based on the expression of mutated amyloid precursor protein (239-241).
Although some staining for hyperphosphorylated tau has been described in nerve cells processes around Aβ deposits in transgenic mice expressing mutated APP,

no somatodendritic staining of hyperphosphorylated tau was described in these mice.
More recently, several animal models of tau pathology were produced by over-expressing human tau proteins.

6.3 Mice expressing 4R tau.

In mouse brain only tau isoforms containing all four microtubule-binding domains are produced, as opposed to the approximately 1:1 ratio of 4R to 3R in adult human brain.
The first study expressing human tau protein in transgenic mice was published in 1995, and described the expression of the longest human brain tau isoform 4R (2N) under the control of the Thy-1 promoter (57). Transgenic mice showed strong somatodendritic and axonal staining for hyperphosphorylated tau in a subpopulation of nerve cells suggestive of pretangle tau pathology but they did not develop filamentous tau inclusions and were phenotypically normal. The level of expression of human tau protein was modest, ~10% above that of non-transgenic animals.
Subsequent attempts, also using the same promoter and human 4R cDNA trangenes, produced much higher levels of human tau expression in mice, between 1,5 (242, 243) and 6 times (244) the total mouse brain tau. But even with this range of over accumulation of human 4R tau, no NFTs were reproduced. Moreover, although FTDP-17 is seen primarily as a disease of the cortex with involvement of the basal ganglia and substantia nigra, much of the severe pathology in these mice is associated with the spinal cord. The prominent phenotype is motor neuron dysfunction and muscle weakness (242, 244). Inclusions in neurons of both the brain and spinal cord are seen and they apparently consist of tau, phosphorylated at some of the same epitopes involved in human disease, neurofilaments, mitochondria and vesicles, suggesting impairment in axonal transport.

6.4 Mice expressing the human *tau* gene.

A different approach was used to produce mice expressing all six tau isoforms using a transgene containing the entire human *tau* gene (243). The resulting transgenic mice showed a more realistic picture of the situation in the human brain. *Tau* expression levels were increased by only 2,5-3,5 fold and no somatodendritic accumulation of human tau and much less tau-containing tangles were observed in these animals.

6.5 Mice expressing 3R tau.

Although the FTDP-17 splice variants appear to yield an increase in 4R *tau*, two groups have examined the consequences of the opposite shift in tau isoforms accumulation: selective increases in the 3R tau isoform (245, 246). The rationale here was perhaps mindful that in several examples of other disorders tau filaments are apparently enriched in 3R tau.

The first transgenic animal expressing the shortest tau isoform (3R) was described by Brion *et al.* (245). They used the mouse *3-hydroxy-methyl-glutaryl CoA reductase* promoter. Axonal and somatodendritic staining for hyperphosphorylated tau was observed in neurons and in some astrocytes. However, antibodies that are entirely specific for PHFs tau, such as AT100 and AP422 (128, 129, 247) failed to show any staining in mouse transgenic brain, a finding in agreement with the lack of tau filaments.

Ishihara *et al.* reported a transgenic mouse expressing the 3 R tau isoform under the control of the mouse *prion* promoter, with 5-15 times the normal level of *tau* (246). Age-dependent increases in insoluble tau, motor neuron degeneration, and, in the mouse lines with the highest level of expression, death by three months were observed. Argyrophilic tau positive inclusions were present in the brain and spinal cord. However, although these inclusions were silver positive, they lacked the fibrillary nature of classic tau tangle since they were Thioflavin S negative.

Therefore, notwithstanding the careful work in these examples of expression of tau isoforms in mice, the relevance of these models to the disease mechanism in tauopathies such as FTDP-17 is unclear. Certainly it cannot be concluded that too much tau is detrimental to neurons, but neither the characteristic pathology nor neuronal selectivity of the human disease has been mimicked very faithfully.

6.6 Mice expressing wild type tau and P301L FTDP-17 mutation.

A more promising example has emerged from the construction of mice expressing a P301L *tau* transgene (248). Using a mouse prion protein promoter linked to wild type or P301L *tau* cDNA, mouse lines were established, where mutant human *tau* accumulate to levels equivalent to the endogenous *tau* in hemizygotic animals. These animals developed NTFs and Pick-body-like lesions in many regions of the brain, and displayed pretangles in the cortex, hippocampus and basal ganglia. Importantly, development of NTFs was gene-dose and age

dependent, with tau accumulation in the brain. These phenotypic changes, presumably due to progressive tau filament accumulation, begin to occur at approximately 4,5 months for homozygotes and 6,5 months in hemizygotic animals. These neuropathological features were associated with the development of both behavioral and motor deficits.

These animals therefore highlight the relevance of *tau* mutations in disease progression. They express considerably lower levels of *tau* compared to earlier transgenic animals, yet P301L mice develop NTFs, which do not occur in the higher expressing wild type *tau* transgenics, in cells that are affected by the disease.

Chapter 7

Discussion

The relevance of tau: from Cinderella to princess of neurodegeneration

The first description of fronto temporal dementia in the neurological field appeared in 1892 (167). However, for many decades the disorder was largely ignored in the shadow of the much more common Alzheimer's disease. Only in the 1980th a group of neurologists began to focus their attention on FTD, and in 1994 the Lund and Manchester groups defined the first clinical and neuropathological criteria for this particular disorder (8).

Nevertheless it was still difficult to classify other apparently different syndromes, showing some clinical or pathological overlap with FTD. Among them are semantic dementia, Pick's disease, FTD with motor neuron disease and primary progressive aphasia. The pathological similarities suggested that these syndromes might represent different phenotypic variations of a common pathology.

A major clue was provided by genetic studies. Linkage to chromosome 17q21-22 was found for several families with a remarkable variety of clinical and pathological phenotypes and even more remarkable names, that emphasize their particular clinical or pathological features like disinhibition-dementia-parkinsonism-amyotrophic complex (DDPAC), pallido-ponto-nigral degeneration (PPND), familial progressive subcortical gliosis (FPSG), familial parkinsonism with dementia, familial multisystem tauopathy and others.

As described in chapter **4.1** we reported linkage to chromosome 17q21-22 in three Dutch families with hereditary FTD. These families showed strong clinical and pathological similarities, with the selective frontotemporal atrophy as unifying pathological feature. However, there were also some clinical differences between the families. The mean age of onset in Family III (63.4 years) was significantly higher ($p < 0.001$) than in the two other families (50.4 years in Family I, and 46.5 years in Family II). Disinhibition was the presenting clinical symptom in families I and II, but in patients of Family III, loss of initiative was the first symptom. All families generated positive lod scores with several markers from 17q21-22 region. The clinical heterogeneity of family III compared with families I and II was not reflected in the linkage results. Further support that family III was indeed linked to chromosome 17q21 came from the fact that all 5 patients shared a common haplotype for more than 20 cM on chromosome 17q21-22 (see chapters **4.1** and **4.4.1** in this thesis). The three families did not share a common disease haplotype suggesting therefore that independent mutations might have been responsible for the disease.

Immunohistochemical studies performed on brain specimens from different regions of patients from all families were negative for tau or ubiquitin staining.

However, in subsequent studies we contradicted these previous results. In both families I and II phosphorylation-dependent and –independent anti-tau antibodies stained in fact numerous intracytoplasmic deposits in neurons, glial cells and neuritis in several brain regions including the hippocampal formation, neocortex and substantia nigra (187). The antibody against ubiquitin showed staining of numerous neurons and dystrophic neurites (249). Ubiquitin inclusions but no tau deposits were observed in neurons of frontal and temporal cortex in brains of patients of family III (250). A wrong choice of tau antibodies and misinterpretation of data might account for the negative results previously reported for families I and II.
The heterogeneity between families is extended to a certain degree at the genetic level. So far tau mutations have been detected only in families I and II, but not yet in family III.
The forum held in Ann Arbor in 1996 brought together several investigators to compare the clinical and neuropathological features of the progressive neurodegenerative disorders linked to chromosome 17 and determine whether they shared common elements (26). The group identified 13 kindreds with sufficient evidence for linkage, finding a common critical region for all families of 2 cM. Although some clinical and neuropathological differences within and between kindreds were evident, many features were found in common among affected individuals. Tau positive inclusions were found in five kindreds, not including at that time Dutch families I and II. Dominant features of this hereditary disorder consisted of a frontotemporal dementia with parkinsonism, therefore for the sake of simplicity the new syndrome was named FTDP-17 (26). The name chosen was probably as colourful as the previous ones, but with an immediate effect.

The ultimate goal of the meeting was to identify the gene responsible for the 17-linked syndrome. Thus, at the end of 1996, in our lab we started a positional cloning project in collaboration with groups from USA and Sweden.
As described before, the gene for the microtubule associated protein tau was an obvious candidate, however, some practical considerations made us decide not to pursue it immediately; several others groups in fact, previously failed in identify tau mutations in selected FTDP-17 families. Therefore, we first concentrated our effort in defining the smallest genomic region shared by all affected individuals. By investigating recombination events in Dutch family I and III we were able to narrow down the critical region to 0.5 cM (see chapter **4.2** in this thesis). Because

we were not able to map the tau gene in this interval, we then assumed that it was located outside our smallest critical region.

Generation of high-resolution genomic maps is the first step toward identification and localization of candidate genes. Compared to the past, at the time we started the collaborative effort to identify the gene responsible for FTDP-17, positional cloning of disease genes have been speed up. Many new techniques have in fact been developed to screen rapidly large regions of the genome for genes, and the Human Genome Mapping Project has led to a large collection of ESTs in the databases and to the genomic sequence of parts of several chromosomes. Taking advantage of both these developments, we built a high-resolution contig of the candidate region and employed several different techniques to generate a transcript map of the FTDP-17 critical region, identifying seven complete novel transcripts. Comparing the methods used, we found that for this specific region of chromosome 17q, the major part of the data was available in the public databases, among them the Human Transcript Map that holds information on the chromosomal position of 30.000 genes and ESTs (251), allowing to rapidly identify ESTs within a specific genetic region. As even more data sequence accumulates rapidly and with the Human Genome Project being more or less finished (252, 253), in the near future the positional candidate approach will become very powerful. The screening of libraries and other time consuming laboratory techniques will become less and less popular as cloning *in silico* will be the tool of choice. Computer-based methods will replace hybridisation ovens, filters, and messy radioactive solutions for library screening and cloning.

Although our positional cloning efforts generated a high-resolution genomic map of the FTDP-17 critical region, used as a template for large-scale sequencing of the chromosome 17, and a detailed transcript map, however, they did not lead us to identify the gene responsible. Several genes were in fact considered as potential candidate for their possible role in neurodegenerative disorders, but we could not detect any mutation segregating with the disease.

As described before in chapter **4.3**, the change of diagnosis of the affected individual that allowed us to narrow down our critical region immediately prompted us to investigate the *tau* gene. Considering the complex clinical picture that characterizes FTDP-17, a change of diagnosis is not an atypical situation. In living patients the diagnosis can only be possible or probable FTD, it can become definite FTD only post-mortem, after autopsy confirmation. Therefore it is essential to have a strict but independent collaboration between clinicians and laboratory investigators in order to design a flexible strategy that can allow to

promptly focus attention and energies to other directions without too many delays.
It took about four years from the original linkage report to the isolation of the gene itself. The time span between localization and identification of the gene responsible for FTDP-17 is considerably brief in respect to other neurodegenerative disorders, like for example Huntington's disease (254).

Mutations in FTDP-17 link tau to neurodegeneration.
The identification of missense and splice mutations for FTDP-17 found by us and others in the gene for the tau protein finally linked tau dysfunction to neurodegeneration (see chapter **5.1** in this thesis). For the first time there was direct evidence that tau played a central role in neurodegeneration.
As genetic and functional studies outlined, *tau* mutations fall in two categories:

- Mutations characterized by a reduced microtubule interaction and an enhanced capability to aggregate: mostly missense mutations.
- Mutations whose primary effects are at RNA level, resulting in the overproduction of 4R tau: mostly intronic mutations.

However, the distinction is not so sharp; four missense mutations in exon 10 deviate in fact from this scheme (N279K, L284L, S305N, and S305S). They increase splicing-in of exon 10, like the intronic mutations. In addition, the ΔK280 mutation seems to have the opposite effect resulting in a reduction of exon 10+ transcripts (188)
Missense mutations reduce the binding of tau to microtubules and decrease the ability of tau to promote microtubules stability and assembly in *in vitro* experiments. With the exception of the ΔK280 mutation, these effects are not observed with the tau missense mutations that directly are involved in exon 10 splicing (149, 188, 199). The partial loss of function of mutant tau could cause a variety of toxic effects within affected cells for example it might affect axonal transport.
Expression of tau missense mutations in several cell systems caused, to varying degrees, disorganized microtubule morphology, defects in microtubule assembly and microtubule instability. However, many of the mutations had only a modest or no effect on microtubule binding and/or function. More dramatic changes in cytoskeleton integrity are observed in two recent studies (see section **6.1** in this thesis). Two independent groups showed that tau protein with missense mutations distributed with microtubules when expressed alone, but was significantly dissociated from microtubules and appeared diffuse in the cytoplasm when

coexpressed with wild type tau. These *in vivo* data are consistent with the reduced binding properties of these mutant isoforms *in vitro*.
There is disagreement as to which missense mutation is most deleterious in altering the function of tau, and which phase of microtubule assembly is most affected. We reported that the ΔK280 mutation exhibit the most dramatic effect in microtubule binding (see chapter **5.5.2** in this thesis). Three studies showed P301L tau to be less efficient than R406W tau in promotion of microtubule assembly (192, 209, 210). In contrast, in another study, Hong and colleagues obtained opposite results (200). There are also discrepancies as to the extent of reduction of microtubule polymerization by different mutations (from as little as 15% to 50 % reduction has been reported). The variability shown in the different studies likely reflects methodological differences in the performance of the assays, purity of tau used and freshness of the tubulin preparations in the microtubule assembly experiments. Nevertheless, the different results obtained by different groups might undermine the validity of the assay and question its biological relevance, especially considering the case of the ΔK280 mutation. According to the microtubule binding experiment this should be one of the most severe mutations; it has in fact the most drastic effect on microtubule binding. By exon trapping experiments however, it also proves to determine a shift toward 3R in the 3R/4R ratio due to the disruption of a splicing enhancer element in exon 10.

The reduced binding of tau mutants to microtubule, however, might result in an excess of mutated unbound tau that would be then available for hyperphosphorylation and/or assembly into filaments, leading to aggregates formation as final event. Many studies in fact showed that several of the missense mutations enhance heparin-induced tau filament formation in *in vitro* experiments compared to wild type tau. The partial loss of function could therefore lead to a gain of toxic function.
The same gain of toxic function might well appeal for the intronic mutations and the missense mutations that cause a shift in the isoforms expression pattern determining an overproduction of 4R, compared to the normal situation where 3R and 4R are present roughly in equal amounts.
4R tau isoforms generally bind and stimulate microtubules better than 3R forms; therefore the shift to 4R forms could override any mutation that weakens the binding. In addition overproduction of 4R isoforms may result in an excess of tau over available binding sites on microtubules. It has been suggested that 3R and 4R tau isoforms bind to different sites to microtubules (68, 70) and it is possible that a specific ratio of tau isoforms is necessary for normal microtubule function.

However, the results of *in vivo* coexpression experiments with 3R and 4R tau performed by Lu *et al.* (229) show that the above theory is to some extent oversimplified. The authors found that when 3R and 4R tau were expressed together in the same cell, 3R tau was dissociated from the microtubules demonstrating that the two isoforms compete for the same binding sites on microtubules. The size of both the free and bound pools of tau depends on the competition among the isoforms.
Splice site mutations, which increase the amount of 4R tau relative to 3R, lead to inclusions consisting of 4R tau. Paradoxically the shift of expression to 4R tau, which dissociates 3R tau from microtubules, results in inclusions made from 4R. A possible resolution to this apparent contradiction might be that microtubules, in an attempt to maintain a controlled distribution of tau isoforms when the shift of expression to 4R tau occurs, actively seed the formation of tau self-assembly. In any case further investigation is needed to elucidate the matter.

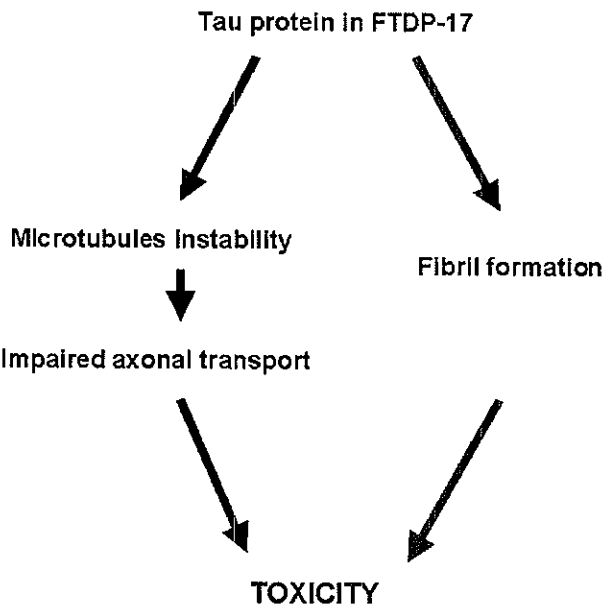

Figure 11: Simplified pathogenic mechanism of *tau* mutations in FTDP-17. On the pathway shown on the left decreased microtubule binding would lead to toxicity through axonal transport. The scheme shown instead on the right side indicates fibril formation as key feature in neurodegeneration.

Aggregates: first actors or secondary players?

Besides aggregates, neuronal loss is another important characteristic neuropathological feature of FTDP-17. While we can predict the biochemical and structural characteristics of the tau aggregates in FTDP-17 based on our knowledge of mutated tau proteins, the exact role of the aggregates still remains to be determined.

Do aggregates lead directly to neurodegeneration or are they only a secondary event with no pathogenic role? And what is their relation with neuronal loss?

Assuming that the aggregates are harmful, we need to hypothesize a series of events mediated by their presence. They might trap essential molecules in the filaments, for example several protein kinases and other not yet identified substances. Because they occupy vital spaces they might prevent axonal and dendritic transport and disrupt intracellular compartments essential for ordered metabolism.

Although an accurate quantitative analysis has not yet been performed, the aggregates in FTDP-17 are not as frequent as in Alzheimer's disease, however, the neuronal loss is very severe. In this respect it becomes difficult to attribute a central role to aggregates. It might be argued that once tangles-bearing cells die, tangles are rapidly removed by microglia cells for example. However, it is well known that tangles are extremely resistant to proteases, even to proteinase K (255) and it is apparent that neurons continue to survive and express a variety of gene products for some currently undefined period of time after NFTs appear in their cytoplasm (256 298, 257 299, 258 300, 259 301). Results from studies conducted on CA1 neurons bearing aggregates in Alzheimer's disease point out that these cells might live even more than decades (260). A possible explanation for the neuronal loss might be that most neurons are lost through a non-aggregate pathway.

The outstanding progress that recently characterized the field of polyglutamine diseases and contributed a great deal to our understanding of their pathogenic mechanisms, might give us some insights for other neurodegenerative disorders.

Abnormal filamentous inclusions within nerve cells are a characteristic shared also by polyglutamine disorders. An increasing number of different hereditary neurodegenerative diseases resulting from expanded glutamine repeats in otherwise unrelated proteins have been identified (261-263). The mutant proteins form nuclear aggregates termed neuronal inclusions (NIs) that also contain ubiquitin. Because of the clinical correlation between aggregates formation in specific neurons and their degeneration in affected individuals, in the past it has been widely accepted that polyQ containing NIs inclusions themselves are toxic

(264). The ubiquitinated mutant protein could represent a blocked intermediate in the degradation pathway used by the wild type protein or alternatively, ubiquitination could represent an attempt by the cell to degrade a protein whose turnover by the usual methods has been compromised by the polyQ expansion. Recent studies, however, strongly suggest that the toxicity that eventually leads to cell death is likely to occur via soluble polyQ expanded protein in the nucleus. Expression of polyQ streches can cause caspase activation (265, 266). Triggering a caspase cascade could lead to the activation of a protease that would act on a polyQ-containing protein to lead to the production of the toxic fragment. This fragment could be either the polyq-containing domain or some other part of the protein. Ubiquitination of the protein may not be required for toxicity. If they are relevant at all, the nuclear inclusions are likely to be protective, not toxic. They might in fact function as a storage compartment that protects the cell by segregating the toxic polyQ protein until they can be properly degraded. By overexpressing a dominant-negative ubiquitin-conjugating enzyme and presumably altering the ubiquitination and clearance of mutant huntigtin in cell culture, Saudou *et al.* (267) were able to inhibit NIs formation while accelarating cell death. Similarly, when crossing the SCA1 transgenic mice with mice lacking an E3 ubiquitin-protein ligase (Ube3A) Cummings *et al.* (268) observed that Purkinje cells from the SCA1/Ube3A mice had significantly fewer NIs than SCA1 littermates but markedly worse SCA1 pathology. These data suggest that impaired proteosomal degradation of mutant polyglutamine proteins may contribute to pathogenesis and more importantly, the mutant protein may be more toxic when is not properly ubiquitinated, turned-over or possibly sequestered to an NI.

By analogy with this scheme the following can be postulated. In FTDP-17 a truncated form of tau may have a profound effect on cell viability via certain routes and at the same time it might form cytoplasmic aggregates. Perhaps in the neurons such toxic fragments are constantly generated and efficiently removed. At a certain age however with the slowing down of the intracellular degradation machinery, the fragments may stay longer in the affected cells and reach sufficient levels to exhibit intracellular toxicity. The neurons taking advantage of the high aggregation potential displayed by the truncated tau fragments might try to segregate them, becoming therefore aggregates-bearing and likely long survival neurons. In this scenario failure in segregating the tau fragments in aggregates might have catastrophic consequences condemning the cells to a sure death.

The recent findings of Wittmann *et al.* (269) strongly support this hypothesis. The authors created a genetic model of a tau related disorder by expressing wild-type and mutant forms of human *tau* in the fruit fly drosophila. Transgenic flies undergo brain neuron degeneration and present most of the key features of the human disorders: adult onset, progressive degeneration, early death, enhanced toxicity of mutant tau, accumulation of abnormal tau and relative anatomic selectivity. The results suggest that the fruit flies with human tau mimic the tau-induced damage observed in FTDP-17 and other tau related disorders. Notably, however, no tangles were present.

Because of the absence of tangles it might be argued that flies are not a good model for human dementias. However, considering especially the short life span if the fly, it is more likely that the mechanism of neurotoxicity relies on defective but soluble tau.

Our findings also point to this direction (see chapter **5.5.3** in this thesis). For a better understanding of the role of the mutated protein in the pathogenesis of the disease we studied the localization and levels of the P301L and normal 4R tau proteins in brain material from patients with P301L mutation using rabbit polyclonal antibodies specifically directed against the P301L mutation and its normal 4R counterpart.

In the immunoblots of the soluble tau material we observed two bands smaller then 45 kD beside the normal and mutated full-length proteins. These two bands are strongly stained with the antibody specific for the point mutation and only weakly with the antibody that recognizes the normal 4R tau. They are present in the frontal and temporal cortex as well as in the cerebellum, an area that does not show any deposits. These bands may be intermediate fragments generated during the normal proteolysis of the mutated protein.

Tau is susceptible to both calpain and caspase degradation in mature neurons (270), resulting in the generation of multiple fragments, the major fragment being 42 kD in size. It has been shown that in cerebellar granule-neuron apoptosis, tau is also degraded and that is partially sensitive to caspase and calpain inhibitors. In fact the combination of inhibitors of both proteases provides the best protection.

The relative increase in mutant tau positive cleavage products might suggest that the mutant protein is more resistant to further calpain and/or caspase degradation with respect to the normal protein and this resistance might cause accumulation of intermediate proteolysis products. Using an *in vitro* assay, it was indeed reported that many of the tau mutated proteins, and among them the P301L, are more resistant to calpain degradation compared with the normal 4R tau (186, 271).

Alteration in proteolytic processing by FTDP-17 mutations is also shown in a CHO cell system by Vogelsberg-Ragaglia *et al.* (232). Upon investigation of the banding pattern of proteolytic tau fragments it was found consistent difference among wild type proteins and some of FTDP-17 mutated tau. The alterations might suggest a conformational change induced by the mutations that modify the proteolysis of these proteins.

These intermediate tau proteolytic products both from mutant and wild type protein might be deleterious for the neurons in several ways. They might kill neurons by crippling their transport system, perhaps by wrong interactions with other proteins involved in ferrying life-sustaining molecules to nerve endings or they might sequester in the aggregates proteins with which normally tau interacts. Alternatively they might alter a signaling cascade that leads to apoptosis or oxidative stress for example. In this respect it becomes important to put effort in individual proteins that interact with tau using for example two- hybrids systems studies.

Recently Nucifora *et al* reported that expanded polyglutamine may exert toxic effects within cells by sequestering proteins containing short polyglutamine stretches, such as CBP, the transcriptional coactivator CREB binding protein, away from their critical sites of action (272). In this model, it is not the inclusions or aggregation that are directly toxic, but rather the indirect effect on other proteins such as CBP. These results are consistent with the emerging data linking transcription abnormalities with polyglutamine pathogenesis and suggest a unifying mechanism of cellular toxicity for glutamine repeat diseaes. Mc.Campbell *et al* recently showed that the androgen receptor with an expanded polyglutamine repeat, which is the cause of spinal and bulbar muscular atrophy (SBMA), interacts with CBP, leading to cell toxicity (273). It has also recently been shown that atrophin-1 and other polyglutamine proteins associate with TAF $_{II}$ 130 and can interfere with CREB-mediated transcription (274). We cannot exclude that such mechanism play a role in the pathogenesis of FTDP-17 as well. Aberrant interactions might take place between mutated or abnormally folded tau and other cellular proteins. Therefore further investigation in this direction becomes essential.

Apoptotic cell death has been implicated as a major mechanism in many neurodegenerative disorders. However, his role is always been controversial. Neuropathological studies in Alzheimer and Parkinson's brains point towards a disturbed balance of pro- and anti-apoptotic proteins indicating the presence of a proapoptotic environment (275-280). Another argument in favour of apoptotic cell death is the elevated number of cells with DNA fragmentation in Alzheimer's

brains compared to age-matched controls as detected by TUNEL experiments. However, considering the slow and progressive course of the disease, if all the neurons that present evidence of DNA cleavage underwent apoptosis, neuronal loss would be rapid and would contract the course of the disease to weeks or months. Nevertheless the significantly increased incidence of cells with DNA fragmentation compared to controls might indicated that Alzheimer's neurons are more vulnerable to pathogenic factors.

The role of caspase has been investigated in relation to mitochondrial dysfunction in many neurodegenerative disorders, among them Parkinson's disease. Parkinson's is a common progressive neurodegenerative disorder caused by the loss of dopaminergic neurons in the substantia nigra (281). Mutations in α-synuclein have been identified in autosomal dominant Parkinson but the mechanism by which dopaminergic neural cell death occurs remains unknown (282).

Recently, it has been reported that mutant α-synuclein decreases proteosome activity, leading to increased sensitivity to mitochondria-dependent apoptosis as demonstrated by activation of caspase 3 and 9 (283). Proteosomal function is known to be impaired in the substantia nigra in sporadic Parkinson's cases, suggesting that dysregulation of the ubiquitin-proteosome pathway contributes to the pathogenesis of not only familiar cases but also sporadic PD. Proteosome inhibition results in accumulation of molecules normally degraded by the ubiquitin-proteosome pathway such as p53, NFkB and bax. These molecules appear to participate in apoptosis signalling.

Mitochondrial dysfunction might be also an active player in Alzheimer's disease. Mutations in presenilin 1 and 2 increase the vulnerability of neuronal cells to mitochondrial toxins (284-286). What might be the relevance of mitochondrial dysfunction and apoptosis with respect to the neuronal loss that characterises FTDP-17 has yet to be determined. To date not many studies have been performed on this direction. Therefore we can only indirectly speculate on their importance in FTDP-17 by discussing what is known in other neurodegenerative disorders.

Moreover, in addition to caspase, calpain appears to assist in the degradation of key cellular proteins and, thus, can be viewed as having an auxiliary and augmentative role in the transduction of neuronal apoptosis.

The recent finding that the truncated form of p35, p25 accumulates in neurons of Alzheimer's patients (287) prompted many investigators to reevaluate the role of calpain in neurodegeneration. P35 is the neuron-specific activator of cdk5, a small serine/threonine kinase with tau as substrate. The cdk5/p35 complex is required

for proper development of the mammalian central nervous system but has important functions in the mature brain as well. In cultured primary cortical neurons, excitotoxins, apoxic stress and calcium influx activate calpain, thereby inducing the production of p25 by cleavage of p35. These some findings are replicated in brain lysates (288).

By cleaving p35, calpain alters the properties of cdk5 such that the cdk5/p25 causes collapse of the cytoskeleton and cell death. Cdk5/p25 activity is detrimental to neurons. Unlikely p35, p25 is not readily degraded and its binding with cdk5 constitutively activates cdk5, changes its cellular location and alters its substrate specificity. The cdk5/p25 complex hyperphosphorylates tau *in vivo* reducing therefore tau's ability to associate with microtubules. Expression of cdk5/p25 in cultured primary neurons induces cytoskeletal disruption and morphological degeneration, a probable consequence of tau hyperphosphorylation.

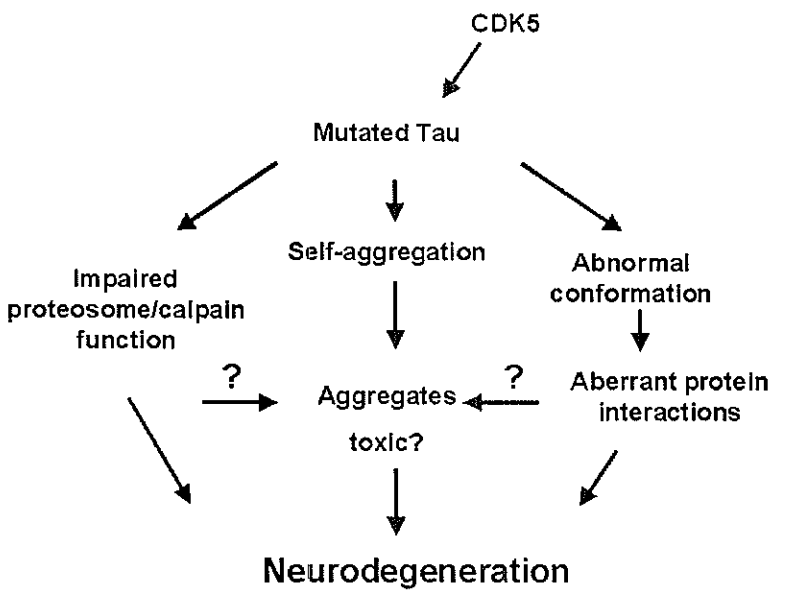

Figure 12. Simplified model for neurodegeneration in FTDP-17. Three possible mechanisms are depicted: impaired proteosome or calpain function,aberrant protein interactions and self-aggregation might lead to neurodegeneration. One mechanism does

not necessarily exclude the others. On the left hand abnormal proteolitic cleavage of the mutated and normal protein due to impaired proteosome/calpain activity would generate toxic fragment. These fragments may exert toxicity by abnormal protein interactions. Segregation of these fragments in aggregates may protect the cell from toxicity. On the pathway shown on the centre mutated and normal tau would form insoluble aggregates. In this scheme the insoluble aggregates would be protease resistant and they would be the cause of toxicity. On the right hand the mechanism proposed is based on aberrant protein interactions between tau and important cellular components, due to tau mutations and/or abnormal tau conformation. The cdk5/p25 complex hyperphosphorylates tau *in vivo* reducing therefore tau's ability to associate with microtubules leading to cytoskeletal disruption and morphological degeneration.

Calcium homeostasis is a convergence point of various aetiological factors relevant to the pathogenesis of neurodegenerative disorders. In particular loss of calcium homeostasis has been implicated in causing tau hypersphorylation and neuronal death.

Recently Furukawa *et al.* (289) observed in a cell system that tau mutations modulate neuronal apoptotic cell death probably by disrupting the intracellular calcium homeostasis. However definite evidence of apoptosis was not demonstrated in this study. Further investigation is needed to determine if apoptosis plays a central role in neurodegeneration and in FTDP-17 in particular.

Importance of tau mutations in other tauopathies.

Thanks to the mutations that we and others found in the *tau* gene, tau could finally earn the deserved "place in the sun". But the consequences of this major breakthrough in the field of dementia go beyond FTDP-17 itself. The clinical and neuropathological phenotype of individuals with FTDP-17 mutations is extremely complex and heterogeneous ranging from Pick's disease, Alzheimer's disease, corticobasal degeneration, progressive superior palsy to a multisystem neurodegeneration.

Some *tau* gene mutations cause a phenotype relatively similar to some of these disorders. The G389R mutation for example causes typically a phenotype reminiscent of Pick's disease. The phenotype due to the N279K mutation instead resembles PSP with superimposed dementia. However, there are several clinical and pathological descriptions of families with the same mutation and highly variable phenotypes.

Sometimes, even more puzzling, there is high variability in the same family. For instance the S305S mutation leads to a clinical and neuropathological picture typical of PSP in one individual and produces a clinical picture of pre-senile dementia in two other individuals from the same family (199). Another example is the P301S mutation. Two families have been described with a very early onset. One family showed epileptic seizures and euphoric moods (197). In the other family hallucinations and delusions have been reported with one individual presented clinically with FTD while his son presented clinically with CBD (196). These reports indicate a clear overlap between the various tau-related disorders and point toward a common pathological mechanism with tau playing a pivotal role.

We could speculate on an active role of genetic and epigenetic factors in determining the clinical and pathological differences seen in patients carrying the same mutation. Specific genetic and/or environmental modifiers might also account for the selective neuroglial vulnerability observed so far in FTDP-17 and in tauopathies in general. Currently the modifiers that might mediate the phenotype of specific mutations and influence the expression of the disease are not known.

These factors might modify the trafficking and compartmentalisation properties of tau and /or his aggregation properties. The development of animal models that closely resemble the human disease should facilitate the process.

The first application after the identification of the gene responsible was mutation analysis in our collection of 90 FTD patients identified in a genetic epidemiological study in the Netherlands (see chapter **5.5.2** in this thesis). The diagnostic criteria followed were clinical and neuroimaging findings defined by The Manchester and Lund groups in 1994 (8). In first instance we considered only patients with frontotemporal atrophy. Our analysis revealed a mutation in the *tau* gene in 17.8% of patients and in 40.5% of patients with a positive family history of dementia. No mutations were detected in sporadic cases.

To date four studies (including ours) have tried to determine the frequency of *tau* mutations in the general FTD population. The figures obtained clearly show the importance of the diagnostic criteria followed in ascertaining the patients. When the inclusion criteria are more stringent the method is more specific and sensitive resulting in a higher *tau* mutations frequency.

Poorkay *et al.* searched for *tau* mutations in a group of 101 patients who were ascertained with non Alzheimer's disease and non-vascular dementia and thought most likely to have FTD or some variety of FTD (290). Most of the patients had a

clinical diagnosis of typical FTD conforming to the guidelines of Neary *et al.* (10). Of these, 57 cases were familial and 44 were sporadic. Overall the frequency of *tau* mutations was low being 5.9% in the entire group. No mutations were found in the sporadic cases.

Houlden *et al.* assessed the genetic contribution of *tau* mutations to three patients series with non-Alzheimer's degenerative dementia (291). The groups included a community based dementia series from Minnesota, a referral series with clinicopathological tauopathy and a pathologically confirmed familial frontotemporal dementia from Manchester UK. Only in the stringently diagnosed Manchester frontotemporal dementia series, *tau* mutations were present in 13.6% of cases; in the clinical pathological referral series that used more general criteria, the frequency of mutation observed was low (3.6%). *Tau* mutations were non detected in the dementia series, suggesting that the occurrence of these mutations in dementia is generally rare.

Froelich Fabre *et al.* screened a clinic-based FTD population with approximately 35% of the patients with positive family history of dementia, with eight individuals coming from large kindreds with autosomal dominant inheritance pattern. No *tau* mutations were found (292).

The higher frequency of *tau* mutations in our FTD population reflects the stringency used in the inclusions criteria rather than the family clustering as suggested by Poorkay *et al.* We in fact excluded secondary cases to avoid referral bias resulting from familial clustering, and after extensive genealogical studies and haplotype analysis we were able to reduce the 37 nuclear families to 30 independent families. Thus the chance to find tau mutations greatly relies on the population investigated. In a second screening that we performed on a group of possible FTD patients, cases with only frontal or temporal atrophy were also considered. As expected the frequency of *tau* mutations was extremely low. However, the finding of mutation in a case with temporal atrophy without involvement of the frontal lobe, brought us to reconsider the very conservative criteria followed in the first screening.

The results from the above discussed studies have important practical implications. It is easy to predict in fact that unless there is a strong family history of dementia and tau-related neuropathological findings, screening patients with dementia for *tau* mutations will have a low yield of positive results. However, discovering a patient with *tau* mutation has important consequence to the family in providing genetic counselling. Therefore from a diagnostic point of view precise criteria have to be determined.

Not all the FTDP-17 families previously linked to 17q21-22 present mutations in the *tau* gene. The research gave so far negative results in at least three families including the Dutch family III described by us (2). It is likely that some mutations in the *tau* gene have remained undetected. Small mutations outside the coding region, for example intronic sequences outside the primers used to amplify, or unknown elements in the promoter cannot yet be ruled out. However, these families lack any obvious tau pathology like aggregates (250, 292); therefore an hypothetical mutation in the *tau* gene causing the disease in these families should be pathogenic through a different mechanism.

In my personal opinion a more likely that FTDP-17 is genetically heterogeneous and the disease in these families is caused by mutations in other genes. Considering the strong evidence for linkage for all the three families to 17q21-22 it is most likely that such genes have to be present in this candidate region.

In our positional cloning efforts we generated an accurate transcripts map of the region (183). Some of the genes were considered potential candidate because of their expression and predicted or known functions. These genes have been already sequenced in some of the above families with negative results. However, 17q21-22 is a highly gene dense region so further investigation is necessary.

Since the finding of the *tau* mutations for FTDP-17, without doubt, the tauopathies got a big booster. New pathological profiles are continuously added to their spectrum and more and more attention is given to tau, even when no *tau* mutations are found. An example is the case of frontal lobe dementia with novel tauopathy presented by Bigio *et al.* The authors discussed an "unique" cortical tau pathology named sporadic multiple system tauopathy with dementia (293).

Zhukareva *et al.* took a step further. They described novel sporadic and familial tauopathies with a unique mechanism of pathogenesis in sporadic cases with a subtype of FTD named dementia lacking distinctive histopatology (DLDH) and in a family with the FTD syndrome hereditary dysphasic disinhibition dementia (HDDD2) (294). This family showed linkage to the 17q21-22 locus. However, no *tau* mutations have been yet detected in this kindred or in the DLDH cases examined by the authors. After analyzing tau protein from different brain areas from the above cases the authors to everyone's surprise, found a selective loss of all six tau isoforms compared to controls and AD patients. In nine of the 14 sporadic cases the tau protein was almost entirely missing (about 10% of normal). The loss of protein is not reflected at mRNA level. Other mechanisms therefore must account for this particular phenomenon. Misregulation of translation and/or decreased stability of the mRNA are among the possible, however exotic,

explanations the authors suggested, after considering several potential models described in literature.
In their attempt to make a connection between the pathologic mechanisms underlying the sporadic cases and the familiar one, the authors in fact do not properly consider that HDDD2 is an autosomal dominant disorder. Even giving the benefit of the doubt that not technical procedures account for part of the results obtained, however, the scenario the authors proposed is extremely difficult to envision. It is highly unlikely that a mutation in one copy of the *tau* gene could influence the stability of mRNA or a protein made by the normal copy of the gene.

In conclusion the finding of mutations in the gene for the tau protein in FTD-17 was one of the most exciting moment in the continuously developing dementia field. Tau was finally catapulted to limelight. The importance to understand the mechanisms leading to neurodegeneration in FTDP-17 goes beyond the disease itself; it has in fact broader significance considering the role of tau in the several tau-related disorders. However, caution is necessary; an overenthusiastic attitude toward tau might be deleterious and misleading as well as the scepticism that for years in the past surrounded it.

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Summary

The work presented in this thesis describes how our group contributed to the continuously developing field of dementia, and to frontotemporal dementia in particular. Beside Alzheimer's disease, frontotemporal dementia (FTD) represents one of the common causes of early-onset progressive dementia. The disease usually begins insidiously in the fifth decade of life with behavioural, motor manifestations and subsequent development of cognitive impairment progressing to dementia. Approximately 35% of the cases have cellular inclusions that stain positively for the microtubule associated protein tau. Most cases of frontotemporal dementia are sporadic, with a proportion being inherited.

In the initial study we performed linkage analysis on three large families with early-onset progressive FTD (HFTD I, II, III), ascertained in a genetic-epidemiological study of FTD in the Netherlands. Previous genetic studies showed that other FTD families with similar clinical profiles were linked to 17q21-22. Therefore we selected 12 polymorphic markers from the CEPH/Généthon linkage map in the same chromosomal region. None of the individual families was powerful enough to provide significant evidence for linkage itself, but with the combined data of the three families, significant lod scores were obtained for the markers D17S932 ($Z = 4.0$ at $\theta = 0.0$) and D17S934 ($Z = 4.8$ at $\theta = 0.0$). Other families with a remarkable variety of clinical and pathological phenotypes were subsequently reported to be linked to 17q21-22. A consensus conference established that these families constituted a new distinct group with comparable clinical and neuropathological features. The disorder was named frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17). The linkage found in these families to chromosome 17q21-22 strongly suggested that a single locus was responsible for this new disorder. The gene for the microtubule associated protein tau was an obvious candidate for FTDP-17. It is localized within the disease critical region and hyperphosphorylated tau filaments are present in brains of affected individuals. However sequencing of the *tau* gene in some FTDP-17 families failed to identify any mutations segregating with the disease.

We formed an international consortium to characterize the FTDP-17 critical region and to find the gene responsible for the disease. A physical and transcript map was generated by assembling a contig of genomic clones and by positioning known genes and EST clusters on this physical map. We were able to localize 19 known genes and a number of ESTs on this chromosomal region. In addition seven novel transcripts were identified by exon trapping and their full length sequence was determined. A number of these genes were considered potential candidate genes for FTDP-17 because of their expression pattern and predicted role in the neurodegenerative disease process. These genes were sequenced in

several FTDP-17 families of the consortium. However, we were not able to identify any mutations segregating with the disease in the genes investigated. Therefore we extended the sequencing of the *tau* gene, the most obvious candidate, to a large number of families in the consortium and to_non-coding region in the gene. We detected three missense mutations: G272V, P301L and R406W, in exon 9, 10 and 13 respectively. In addition we observed three intronic mutations in a cluster of four nucleotides 13-16 bp 3' of the exon 10 5' splice site. The splice site mutations all destabilize a potential stem-loop structure involved in regulating the alternative splicing of exon 10. This causes a more frequent use of the 5'splice site and an increased level of *tau* transcripts that include exon 10. We failed to identify mutations in some FTDP-17 families of the consortium, including HFTD family III. The frequency of *tau* mutation was investigated in the general FTD population in the Netherlands ascertained in a genetic epidemiological study between January 1994 and June 1998. A mutation in the *tau* gene was found in 17.8% of patients with FTD and in 40.5% of patients with a positive family history of dementia. No mutations were detected in sporadic cases.

The identification of missense and splice mutations for FTDP-17 found by us and others in the gene for the tau protein finally linked tau dysfunction to neurodegeneration. For the first time there was direct evidence that tau plays a central role in neurodegeneration. The mechanism through which *tau* mutations lead to neurodegeneration is not yet fully understood. It still remains to be solved whether for FTDP-17, as for many other neurodegenerative disorders, the aggregates are the pathogenic agents or simply markers of the cell's demise. Therefore we further characterized the composition of the tau aggregates by developing specific rabbit polyclonal antibodies against the P301L point mutation and its normal counterpart. In biochemical experiments we determined the relative ratio of mutated versus normal tau protein in the sarkosyl-soluble and –insoluble protein fractions from several brain regions. We showed that, although both mutated and normal tau protein are present in the insoluble deposits of the cerebral cortex, the main component of these deposits is the mutated tau protein. In the soluble fraction we observed a strong reduction in the level of mutated P301L protein especially in the affected area. These findings strongly suggest that the low level of mutated protein in the soluble fraction is due to a selective depletion, resulting from the selective aggregation of mutant protein in the sarkosyl-insoluble deposits. Furthermore, we observed an increase of tau-imunoreactive cleavage products for the mutant protein compared with the normal one. Therefore it is possible that an alteration of the proteolytic process of the mutated P301L protein might plays a role as well in the pathogenesis of the disease.

Samenvatting

Dit proefschrift beschrijft hoe onze groep heeft bijgedragen aan het zich steeds ontwikkelende dementie-veld, in het bijzonder dat van de frontotemporale dementie. Frontotemporale dementie (FTD) is, naast de ziekte van Alzheimer, een van de grootste oorzaken van vroege progressieve dementie. De ziekte manifesteert zich gewoonlijk in het vijfde decennium van het leven met gedragsstoornissen en motorische afwijkingen, waarna cognitieve achteruitgang optreedt die overgaat in dementie. In ongeveer 35% van de gevallen worden cellulaire insluitsels gezien die aan te kleuren zijn met het microtubuli-geassocieerd eiwit tau. De meeste gevallen van frontotemporale dementie zijn sporadisch, en een gedeelte is erfelijk.

In de eerste studie hebben we koppelingsonderzoek uitgevoerd in drie grote families met vroeg optredende FTD (HFTD I, II, III), die gevonden waren in een genetisch-epidemiologisch onderzoek naar FTD in Nederland. Eerdere genetische studies hadden al aangetoond dat andere FTD families met soortgelijke klinische beelden gekoppeld waren aan 17q21-22. Daarom selecteerden we 12 polymorfe merkers uit de CEPH/Généthon koppelingskaart in dit chromosomale gebied. Geen van de families leverde afzonderlijk genoeg bewijs voor koppeling, maar met de gecombineerde data van de drie families werden significante lod scores verkregen voor de merkers D17S932 ($Z = 4.0$ bij $\theta = 0.0$) en D17S934 ($Z = 4.8$ bij $\theta = 0.0$). Vervolgens verschenen publicaties waarin andere families werden gekoppeld aan 17q36. Deze families vertonen een opvallende variatie aan klinische en pathologische fenotypes. In een consensus conferentie werd vastgesteld dat deze families een nieuwe aparte groep vormen met vergelijkbare klinische en neuropathologische kenmerken. Deze aandoening werd frontotemporale dementie met parkinsonisme gekoppeld aan chromosoom 17 (FTDP-17) genoemd. De koppeling met 17q21-22 in deze families duidt er sterk op dat een enkel locus verantwoordelijk is voor deze nieuwe aandoening. Het gen voor het micotubuli-geassocieerd eiwit tau was een duidelijk kandidaatgen voor FTDP-17. Het lag binnen het kritieke kandidaatgebied en hypergefosforyleerde tau vezels worden gevonden in hersenen van patiënten. Echter, sequeneren van het tau gen in een aantal FTDP-17 families leverde geen mutaties op die segregeerden met de aandoening.

We vormden toen een internationaal consortium om het FTDP-17 kritieke gebied te karakteriseren en het gen te vinden dat verantwoordelijk is voor deze ziekte. Een fysische en transcriptionele kaart werd gemaakt door een contig van genomische klonen samen te stellen en bekende genen en EST clusters daarin te plaatsen. We konden zo 19 bekende genen en een aantal ESTs in dit chromosomale gebied lokaliseren. Daarnaast werden zeven nieuwe transcripten geïdentificeerd met behulp van exon trapping, en hun volledige sequentie werd vastgesteld. Een aantal van deze genen werd beschouwd als potentieel

kandidaatgen voor FTDP-17 door hun expressiepatroon en voorspelde rol in het proces van neurodegeneratie. Deze genen werden gesequeneerd in een aantal FTDP-17 families van het consortium. We waren echter niet in staat om in deze genen mutaties te vinden die segregeerden met de aandoening. We breidden daarom de sequenering van het *tau* gen, het meest voor de hand liggende kandidaatgen, uit tot een groot aantal families in het consortium en tot niet-coderende gedeelten van het gen. We vonden drie missense mutaties: G272V, P301L en R406W, in respectievelijk exon 9, 10 and 13. Daarnaast vonden we drie mutaties in intronen, in een cluster van vier nucleotiden 13-16 bp 3' van de 5' splice site van exon 10. Al deze splice site mutaties destabiliseren een potentiële stem-loop structuur die betrokken is bij de regulatie van alternatieve splicing van exon 10. Dit veroorzaakt een meer frequent gebruik van de 5' splice site en een verhoogd gehalte aan tau transcripten die exon 10 bevatten. In een aantal FTDP-17 families van het consortium, waaronder FTDP-III, vonden we geen mutaties. De frequentie van *tau* mutaties werd onderzocht in de algemene FTD populatie in Nederland zoals vastgesteld in een genetisch-epidemiologische studie die liep van januari 1994 tot juni 1998. Een mutatie in het *tau* gen werd gevonden in 17.8% van de FTD patiënten en in 40.5% van de patiënten met een positieve familie anamnese voor dementie. Mutaties werden niet gevonden in sporadische gevallen. De door ons en anderen gevonden missense en splice mutaties voor FTDP-17 in het gen voor het tau eiwit, koppelen uiteindelijk tau disfunctie aan neurodegeneratie. Voor het eerst was er direct bewijs dat tau een centrale rol speelt in neurodegeneratie. Het mechanisme waardoor tau mutaties tot neurodegeneratie leiden wordt nog niet volledig begrepen. Het is nog onduidelijk of de aggregaten in FTDP-17, zoals voor veel andere neurodegeneratieve aandoeningen, zelf pathogeen zijn of meer een teken van het afsterven van de cel. Daarom hebben we de samenstelling van de tau aggregaten verder onderzocht door specifieke polyklonale antilichamen in konijn op te wekken tegen de P301L puntmutatie, en tegen het normale allel. In biochemische experimenten onderzochten we de relatieve proportie van mutant versus normaal tau eiwit in de sarkosyl-oplosbare en –onoplosbare eiwitfracties van verschillende hersengebieden. We toonden aan dat, hoewel zowel gemuteerd als normaal tau eiwit aanwezig is in de onoplosbare neerslag van de cerebrale cortex, de belangrijkste component van deze neerslag uit gemuteerd tau bestaat. In de oplosbare fractie vonden we een sterke verlaging van het niveau van gemuteerd P301L eiwit, vooral in het aangedane gebied. Deze bevindingen suggereren dat de lage waarde van gemuteerd eiwit in de oplosbare fractie veroorzaakt wordt door een selectieve vermindering die het resultaat is van de selectieve aggregatie van mutant eiwit in de sarkosyl-onoplosbare neerslag. Verder vonden we een verhoging van tau-immunoreactieve splitsingsproducten ten opzichte van normaal tau. Het is daarom mogelijk dat een verandering van de proteolyse van gemuteerd P301L eiwit ook een rol kan spelen in het ziekteverloop.

ABBREVIATIONS

Aβ	Amyloid β-peptide
ABC	Anterior bulbar cells
AD	Alzheimer's disease
APP	Amyloid precursor protein
BAC	Bacterial artificial chromosome
CAMPK II	Calmadulin-dependent kinase II
CBD	Corticobasal degeneration
CDK52/5	Cyclin-dependent kinases
cM	Centi-Morgan
DDPAC	Disinhibition-dementia-parkinsonism-amyotrophic complex
DLDH	Dementia lacking distinctive histopathology
DTT	Dithiothreitol
ESE	Enhance sequence element
EST	Expressed sequence tagged
FISH	Fluorescence in situ hybridization
FPSG	Familial progressive subcortical gliosis
FTD	Frontotemporal dementia
FTDGC	Frontotemporal dementia candidate genes
FTDP-17	Frontotemporal dementia and parkinsonism linked to 17
GFAP	Glial fibrillary acidic protein
GSK-3β	Glycogen synthase kinase 3β
HDDD2	Hereditary disphasic disinhibition dementia
HFTD	Hereditary frontotemporal dementia
kD	Kilo-Dalton
LOD	Logarithm of the odds
MAP	Microtubule associated protein
MAPK	Mitogen-activated protein kinases
MARK	Microtubule-affinity regulating kinase
NFT	Neurofibrillary tangle
NI	Neuronal inclusion
NT	Neuropil thread
PAC	Phage artificial chromosome
PCR	Polymerase chain reaction
PDPK	Proline-directed protein kinases
PHF	Paired helical filament
PNS	Peripheral nervous system
PP-1/2A/2B	Protein phosphatases
PPND	Pallido-ponto-nigral degeneration
PSP	Progressive supranuclear palsy
RAPIP8	Rap2 interacting protein 8

SAP kinases	Stress activated kinases
SCA	Spinal cerebellar ataxia
SDS	Sodium dodecyl sulphate
SF	Straight filament
SP	Senile plaque
SSCP	Single strand conformational polymorphism
STS	Sequence tagged sites
U1 snRNP	Small nuclear RNA-protein particle 1
Ube3A	E3 ubiquitin-protein ligase
VHR	VH-1 related dual-specific phosphatase
YAC	Yeast artificial chromosome
3R	Tau protein with 3 repeats
4R	Tau protein with 4 repeats

Curriculum vitae of Patrizia Rizzu

Date of birth	23 July 1964 in Sassari, Italy
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1988	Degree in Biological Sciences, summa cum laude, from the University of Sassari, Italy. Title of the thesis: "The switch of ß-globins in normal and ß-thalassemic newborns'. Advisor Prof. B. Masala
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