INDEX

SUMMARY 4

INTRODUCTION **7**

Frontotemporal Lobar Degeneration 11

TAR DNA binding protein-43 (TDP-43) 13

Frontotemporal Dementia 15

Molecular Genetics of *Frontotemporal* Lobar Degeneration 18

MAPT Gene 19

VCP Gene 20

CHMP2B Gene 21

PGRN Gene 22

Additional genes and genetic risk factors of Frontotemporal Lobar Degeneration 23

FTLD-Tauopathies 25

Microtubule Associated Protein Tau 26

Gene organization 27

Protein structure and function 30

Tau projection domain 31

Tau microtubule assembly domain 34

Post-translational modifications of tau 35

Molecular classification of Tauopathies 40

MAPT gene mutations 40

Mutation altering the tau-MT interaction 41

Mutation altering the tau mRNA splicing 42

Mutation altering Tau-MT interaction and tau RNA splicing 42

Regulation of exon 10 alternative splicing: cis-acting factors 43

Alternative splicing regulation at the Exon 10 5′ splice site: Stem loop theory 45

Alternative splicing regulation at the 5′ splice site–Linear sequence theory 47

Regulation of exon 10 alternative splicing:Trans-acting factors

Phosphorylation of SR proteins 48
Disruption of *tau* exon 10 splicing in tauopathies 49
AIM OF THE STUDY 51
MATERIALS AND METHODS 52
Clinical description 52
Genetic screening and Pedigrees 52
Neuropathology 55
Minigene constructs 56
Cells preparation and Transformation of *E.coli* Top10 58
Site-directed mutagenesis 58
Analyses on Thermodynamic Stability of the RNA Stem Loop Structure 59
Bioinformatic Analysis 59
Ultraviolet Melting 59
Cell cultures and transfection 60
RNA extraction and RT-PCR analysis on *MAPT* minigenes 61
RNA extraction and RT-PCR analyses on brain tissue 61
Statistical analysis 62
Tau Analysis 63
Immunohistochemistry 63
Biochemistry 63
Sarkosyl-insoluble tau extraction 63
Soluble tau extraction 64
Tau dephosphorilation 64
Immunoblotting 64
RESULTS 66
Minigene Design 66
The two novel mutations affect E10 alternative splicing 68
Effect of the patient's compound heterozygous condition
on the E10 alternative splicing 69

Minigene expression of the 5'splice site E10+3 mutation 70
Brain Tissue Study 72
Detection of increased levels of Tau3R transcripts 72
Tau Analysis 73
Immunohistochemical detection of increased levels of Tau 3R inclusions 73
Biochemical characterization of pathological tau 75
Sarkosyl-insoluble tau: *detection of increased levels of Tau 3R isoforms* 75
Soluble tau: *detection of increased levels of fetal Tau 3R isoform* 77
Thermodynamic stability of the stem loop regulatory structure increased by the E10+4 mutation 79
DISCUSSION 82
REFERENCES 89

SUMMARY

Frontotemporal lobar degeneration (FTLD) is a heterogeneous syndrome encompassing different nosological entities characterized by behavioural and personality change, accompanied by deterioration of executive function, language and movement. Clinically FTLD results in at least three distinct syndromes: Frontotemporal dementia (FTD), Semantic dementia (SD) and Primary progressive aphasia (PPA), while the pathological classification is based on histopathological presence or absence of neuronal inclusions of tau and/or ubiquitin proteins accumulating in the neuronal/glial inclusions, being forms of FTLD differentiated in tau-positive, ubiquitin-positive and tau-negative.

The most common clinical manifestation of FTLD is FTD, characterized by atrophy of the frontal and temporal lobes, with neuronal loss, gliosis and spongiosis of the superficial layers. FTD is mostly a presenile disorder showing changes in personality, impaired social conduct, emotional blunting, loss of insight, disinhibition, perseverative behaviour and hyperorality; cognitive deterioration, especially in language and in executive functions, appear later. Despite most cases of FTD are sporadic, approximately 10%-50% of FTD patients have a positive family history for dementia. Familial FTDL was associated to mutations in four genes: Microtubules associated protein tau (*MAPT*) and Progranulin (*PGRN*) genes that are responsible for the most genetic forms of FTD; instead, Valosin containing protein (*VCP*) gene is involved in rare forms of FTD with inclusion body myopathy and Paget's disease of the bone and Charged multivescicolar body protein 2B (*CHMP2B*) is mutated in some families with a combination of FTD and Amyotrophic lateral sclerosis (ALS).

Mutations in *MAPT* gene are responsible for 10%-20% of familial FTD. Alternative splicing of exons 2, 3 and 10 in *MAPT* pre m-RNA results in the expression of six isoforms. Exclusion or inclusion of Exon 10 gives rise to tau isoforms with three (tau3R, $E10^-$) o four (tau4R, $E10^+$) microtubule-binding repeats. In normal adult human brain the overall ratio of 3R to 4R tau is generally 1, whereas in fetal brain only the shortest tau isoform with 3R is expressed, indicating that tau expression is developmentally regulated.

To date, 44 different potential pathogenic *MAPT* mutations have been reported, divided into two groups depending on the primary molecular mechanism involved: missense or deletion mutations that commonly modify tau interaction with microtubules and splicing mutations that affect the alternative splicing of exon 10, leading to changes of the ratio of 3R-tau/4R-tau. However, a third group of mutations exists that might have effects at protein and RNA levels.

In the present study we report the molecular effect of two novel heterozygous *MAPT* gene mutations, a T to C transition at position -15 of intron 9 [T(-15)C] and an A to C transversion at position +4 of intron 10 (E10+4), identified in a patient with sporadic FTD, clinically and neuropathologically ascertained.

Considering that both mutations are located in the splicing regulatory regions surrounding Exon 10, we analyzed their molecular effect on the alternative splicing of *MAPT* pre-mRNA in a minigene model system and in *brain tissue*.

Semi-quantitative RT-PCR analyses, in minigene costructs and *in brain tissue*, have shown that the two novel mutations cause a novel Exon 10 splicing effect giving rise to a higher increase of mRNAs transcripts lacking Exon 10 (E10- or Tau3R) when compared with FTD-Ub+ control.

Immunohistochemical and biochemical analyses on *brain tissue* evidenced neuronal and oligodendroglial tau deposits mostly made of Tau3R isoforms and an increased increased availability of shorter Tau3R isoform respectively.

Data obtained with minigenes derived by the phenotipically healthy patient's parents demonstrate that when the mutations are inherited in a non compound heterozygous condition the ratio of E10 including/E10 excluding transcripts is quite normal.

Although the molecular mechanism underlying exon 10 splicing regulation remain to be completely elucidated, the exon 10 splice donor site is predicted to give rise to a RNA stem loop structure considered crucial for the quantitative regulation of exon 10 alternative splicing. Most of previously characterized mutations identified in the upper part of the stem loop strongly alter mRNA splicing by destabilizing the secondary structure, with a corresponding increase of E10 inclusion and 4RTau expression. Considering that the E10+4 mutation is located into the exon 10 splice donor site, we

also investigated the effect of the E10+4 mutation on the thermodynamic stability of the RNA stem loop structure. Our data, based on bioinformatic prediction of the stem loop sequence thermostability and Ultraviolet Melting experiments demonstrated a strong increasing of stability in the stem-loop structure carrying the E10+4 mutation. This higher stability could be important for the skipping of exon 10, even though the E10+4 mutation alone is not able to give rise to a pathologic phenotype.

We cannot exclude that the T to C transition, localized in a regulatory region upstream of exon 10, could also alter the binding of specific trans-splicing factors increasing the effect of the E10+4 mutation, giving rise when both mutation are present in the compound heterozygous condition (namely the FTD patient) to the E10 exclusion and the altered 4R/3R tau ratio observed.

Thus, we can hypothesize a trans-acting regulatory effect of both mutations with known, or unknown splicing factors, which might have contributed to the very atypical clinical and pathological FTD phenotype of the patient.

INTRODUCTION

Neurodegenerative diseases represent a large group of sporadic and familial progressive neurological disorders affecting 5-10% of the population over the age of 65 and their prevalence rises steeply to 20-30% of the population over 80 years. Although they arise for unknown reasons and progress in a relentless manner particularly, but not exclusively, in the elderly the most consistent risk factor for developing a neurodegenerative disorder is increasing age.

Future demographic projections are predicting the number of people aged 60 years or greater to reach nearly 1.2 billion by 2025 (http://www.who.int/ageing/en/). Thus, it can be anticipated that, over the next generations, the proportion of elderly people will double, and, with this, possibly the proportion of persons suffering from some kind of neurodegenerative disorder (**Serge Przedborski et al, 2003; Tanner C.M et al, 1992**). It is estimated that more than 20 million people currently have dementia worldwide and that this number will double by 2020 (**Ferri et al., 2005**).

The number of neurodegenerative diseases is currently estimated to be a few hundred, and, among these, many appear to overlap with one another clinically and pathologically, rendering their practical classification quite challenging. Furthermore, the same neurodegenerative process, especially at the beginning, can affect different areas of the brain, making a given disease appear very different from a symptomatic standpoint.

The most common classification of neurodegenerative disorders is still based on the predominant clinical feature or on the topography of the predominant lesion, or often on a combination of both. Accordingly, neurodegenerative disorders of the Central Nervous System may be first grouped, for example, into diseases of the cerebral cortex, the basal ganglia, the brainstem and cerebellum, or the spinal cord. Then, within each group, a given disease may be further classified based on its main clinical features (**Bertram L. et al, 2005**).

Although traditionally divided into sporadic and familial forms, there are almost certainly important influences in sporadic neurodegenerative disorders mediated through environmental and/or genetic and/or epigenetic element that contribute to the

development of neurodegeneration. Potential interaction between these elements may influence disease phenotype, onset and severity.

Among neurodegenerative disorders, dementia are most common diseases and are characterised by progressive loss of specific populations of central nervous system neurons in specific functional anatomic systems (**Fig 1**).

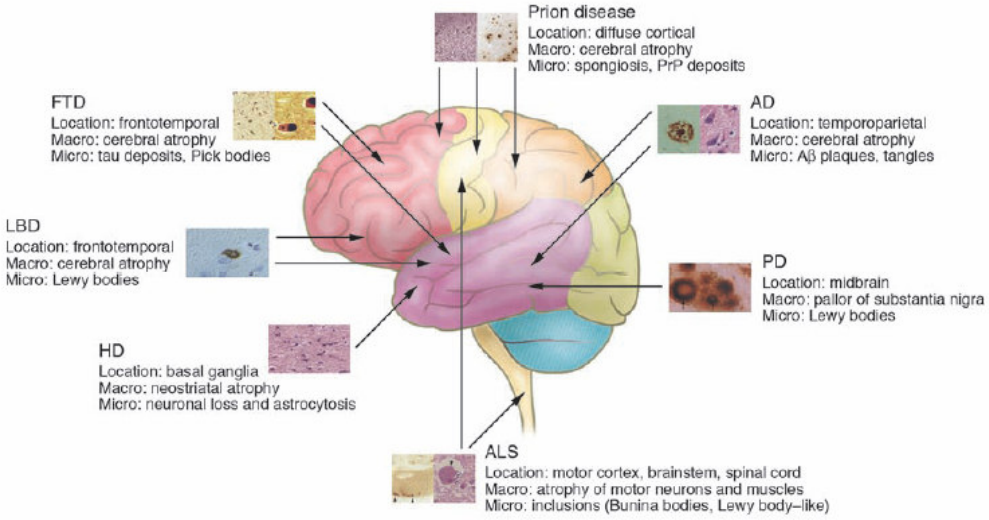

Figure 1 Anatomical location of the Macroscopic and Microscopic changes characteristic of the neurodegenerative diseases

Neuronal degeneration results in severe cognitive decline usually presenting with disturbances in mood, behaviour and personality. The onset of the disease depends on how fast the neurodegenerative process evolves, can range from a few months to several years, although the neurodegenerative process may suddenly accelerate, especially under the influence of intercurrent deleterious factors such as infections.

Over the past two decades, significant advances in neurohistological techniques, such as immunohistochemistry, have replaced or supplemented many of the classical histological approaches and have shown that abnormal protein aggregation in the affected brain areas represents a unifying biochemical and histomorphological hallmark of a wide variety of neurodegenerative diseases.

The protein aggregates form microscopically-visible cellular deposits called “inclusion bodies” localized, in the majority of cases, in the cytoplasmic compartment (**C.A. Ross et al, 2004; John Woulfe, 2008**) and appear to be associated with the neurodegenerative process. The biochemical study of these neuropathological lesions led to the identification of their molecular components and provided an insight into our understanding of these neurodegenerative disorders. Furthermore, great progress was also achieved from studies of familial forms that have identified specific gene mutations which cause the inherited forms of the different disorders. In most cases, the mutated genes were found to encode altered proteins that compose the neuropathological lesions. Thus, the classification of neurodegenerative diseases is increasingly becoming based on the identity of the protein which accumulates (**Tab.1**).

Tauopathies
Alzheimer's disease
Amyotrophic lateral sclerosis/Parkinson-dementia complex
Argyrophilic grain disease
Corticobasal degeneration
Dementia pugilistica
Diffuse neurofibrillary tangles with calcification
Down's syndrome
Frontotemporal dementia/parkinsonism linked to chromosome 17
Gerstmann–Straussler–Scheinker disease
Guadeloupian parkinsonism
Myotonic dystrophy
Niemann-Pick disease type C
Pick's disease
Postencephalitic parkinsonism
Progressive subcortical gliosis
Progressive supranuclear palsy
Subacute sclerosing panencephalitis
Tangle only dementia

Synucleinopathies
Parkinson's disease
Dementia with Lewy bodies
Multiple system atrophy
Neurodegeneration with brain iron accumulation
Pure autonomic failure
Meige's syndrome
Prion Proteinipathies
Creutzfeld–Jakob disease (familial, sporadic, and transmitted)
Fatal insomnia (familial and sporadic)
Gerstmann–Straussler–Scheinker disease
Kuru

Polyglutaminopathies
Huntington's disease
Dentatorubropallidoluysian atrophy
Spinal and bulbar muscular atrophy (Kennedy's disease)
Spinocerebellar ataxia 1, 2, 3, 6, 7, and 17

TDP-43 proteinopathies
Frontotemporal lobar degeneration with ubiquitin-only inclusions
Amyotrophic lateral sclerosis
RNA-mediated diseases
Fragile X-associated tremor ataxia syndrome
Myotonic dystrophy
Spinocerebellar ataxias 8, 10, 12
Huntington's disease-like type 2

Others
Neuronal intermediate filament inclusion body disease
Neuronal intranuclear inclusion disease
Neuroferritinopathy
TDP-43-negative FTLD-U

Table 1 Protein-based classification of neurodegenerative diseases

Frontotemporal Lobar Degeneration

Frontotemporal lobar degeneration (FTLD) is the third most common cause of neurodegenerative dementia, after Alzheimer disease (AD) and Dementia with Lewy bodies (DLB) (**Cairns N.J. et al, 2007**).

FTLD is a heterogeneous syndrome encompassing different nosological entities characterized by behavioural and personality change accompanied by deterioration of executive function, language and movement (**Brun et al., 1998; Neary et al., 1998**).

Clinically it results in at least three distinct syndromes: Frontotemporal dementia (FTD), Semantic dementia (SD) and Primary progressive aphasia (PPA). Over the past decade, many case reports, as well as large clinicopathological studies, have recognized an overlap among these three syndromes and some extrapyramidal syndromes like cortical basal degeneration (CBD), progressive supranuclear palsy (PSP) and motor neuron disease (MND) (**Josephs KA et al, 2006; Hodges JR et al 2004; Kertesz A et al 2005; Forman M.S. et al, 2006**). With the advent of immunohistochemistry it is now well established that more than 15 different pathologies can underlie FTLD and related disorders (**McKhann GM et al, 2001; Cairns N.J. et al 2007**). The nomenclature of FTLD has evolved as knowledge has advanced. Much of what is FTLD descends from the findings of Arnold Pick, in the early 1890s, who described a patient presenting with childish behaviour, memory deficits and aggressiveness. At autopsy, the patient presented lobar atrophy (**Pick A, 1892**) 'ballooned' neurons, argirophilic and eosin-positive neuronal inclusions. Over time, the disease was referred to as Pick's disease (PiD) and the intraneuronal inclusions became known as Pick bodies. In the recent years a more refined molecular characterization has been done. In 1994, The Lund-Manchester group published the first clinical and neuropathological criteria for FTD and adopted the clinical term 'Frontotemporal Dementia' (FTD) (**Lund and Manchester Groups, 1994**), successively updated in 1998 (**Neary D. et al, 1998**).

FTLD is pathologically heterogeneous and can be classified in two major subtypes as Tau-Positive (FTLD-τ) and Tau-Negative groups, on the basis of immunohistochemical and biochemical characterization of proteins accumulating in the neuronal and/or glial inclusions.

Tau positive group known as tauopathies, approximately one-third of FTLD cases, show inclusions made of insoluble aggregates of the microtubule-associated protein tau in the cytoplasm of neurons and glial cells (**Fig 2**).

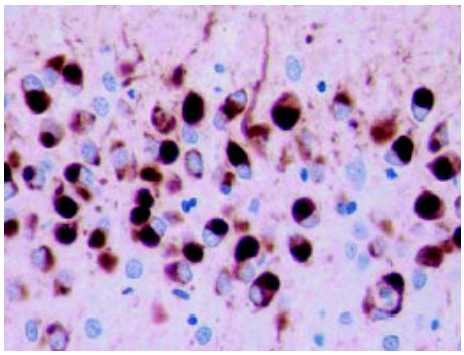

Figure 2 Tau positive inclusions

Several cases of Pick's disease have been included in this group showing that the round argyrophilic inclusions (Pick bodies) were Tau-positive (**Morris H.R. et al, 2001; Spillantini M.G. et al, 1997**).

Tau negative group, known as non-tauopathies, represent about 50-66% of all FTLD cases and with the advent of ubiquitin immunohistochemistry, were observed to have ubiquitin positive neuronal cytoplasmic inclusions (**Fig 3**) and renamed "Frontotemporal Lobar Degeneration with Ubiquitin-only–immunoreactive" changes (FTLD-U) (**Kertesz A et al, 2000; K.A. Josephs et al, 2004; Shi J, Shaw CL et al, 2005**).

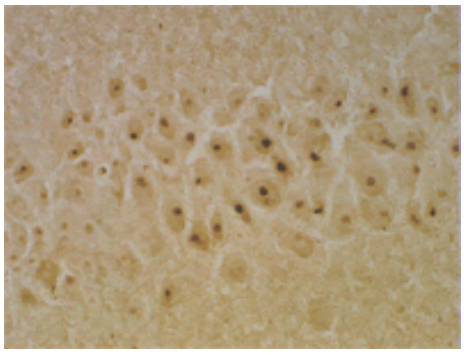

Figure 3 Ubiquitin positive neuronal inclusions.

One-third, or more, of these FTLD-U cases also have rare to occasional neuronal intranuclear inclusions (FTLD-U NII) (**Mackenzie IR et al, 2006**).

Dementia Lacking Distinctive Histopathology (DLDH), in which none of these kinds of inclusion are present, was defined as a third pathologic FTLD subtype.

TAR DNA binding protein-43 (TDP-43)

Recent discoveries of *TARDBP* gene, localized on chromosome 1q, have transformed our understanding of the FTLD-U and DLDH disorders.

TARDBP encodes TAR DNA binding protein-43 (TDP-43), a ubiquitously expressed and highly conserved nuclear protein involved in the regulation of transcription and alternative splicing (**Buratti E. et al, 2001; Buratti E. et al, 2005; Ayala Y. et al 2005**). Its pathology is characterised by increased phosphorylation, nucleus-to-cytoplasm translocation and co-localisation with ubiquitin in neurons and glial cells **(Neumann M. et al, 2006)**.

TAR DNA binding protein-43 (TDP-43), on the basis of data coming from immunohistochemistry and biochemistry, is the major pathologic protein of the inclusions in majority of sporadic and familial FTLD-U cases (**Cairns NJ et al, 2007; Kwong K et al, 2007;Neumann M et al, 2007**) (**Fig 4**), even though there are FTLD-U cases without TDP-43 inclusions (**Roeber S. et al, 2008; Josephs K.A. et al, 2008; Mackenzie I.R. et al, 2008**).

Furthermore, a proportion of cases associated with DLDH pathology have shown TDP-43 inclusion (**Fig 4**). These case, reclassified as FTLD-U, are now recognized as the major pathology underlying FTLD (**Samir Kumar-Singh et al 2007; Joseph K.A. et al, 2004**).

Recent studies have demonstrated that abnormal TDP-43 immunoreactivity also occurs in FTLD-τ as Alzheimer's Disease, Pick's Disease (PiD) (**Fig 4**), Pure Hippocampal Sclerosis (HS), Lewy Body Disease (LBD) and in the Amyotrophic Lateral Sclerosis-Parkinsonism Dementia Complex (ALS-PDC) (**Amador-Ortiz C. et al, 2007; Freeman S.H. et al, 2008; Nakashima-Yasuda H. et al, 2007; Geser F. et al, 2008**).

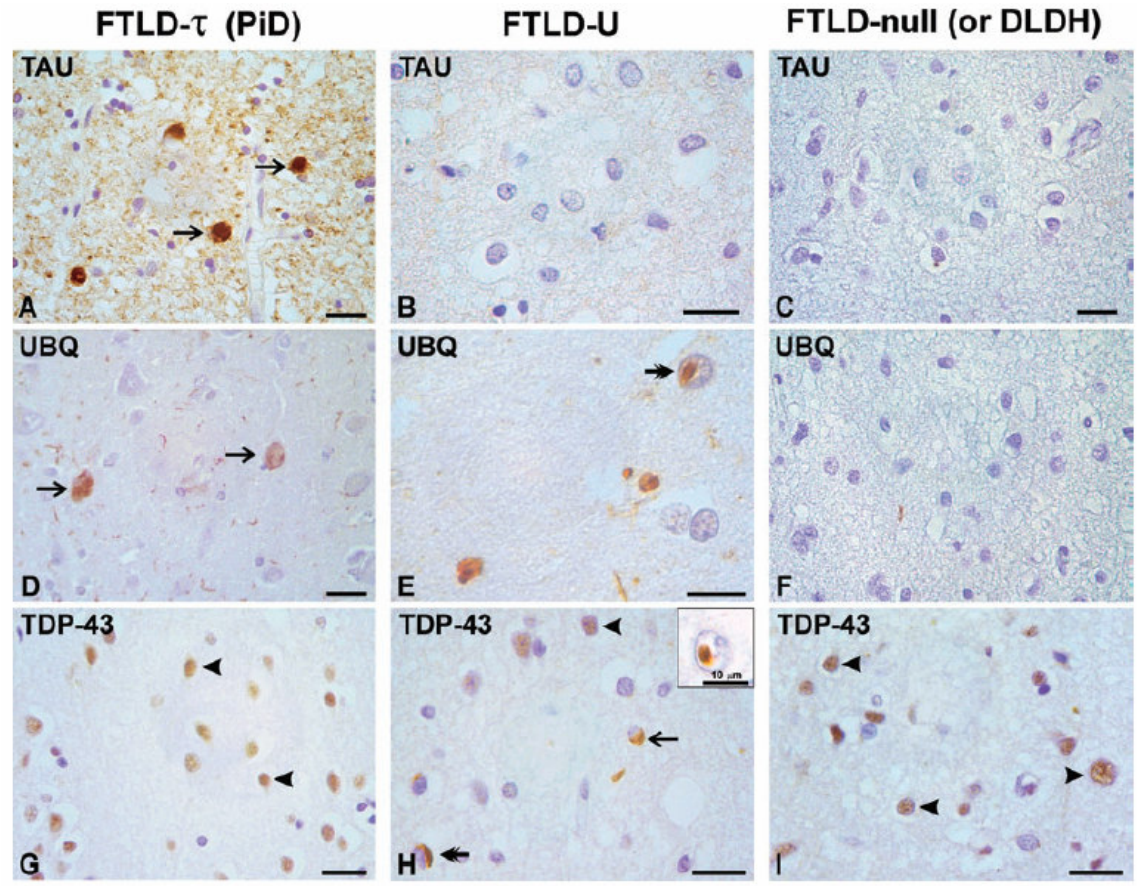

Figure 4 Pathological features of FTLD- τ, FTLD-U and DLDH. Tau (**A–C**), ubiquitin (**D–F**) and TDP-43 (**G,H,I**) immunostaining is shown on serial sections from superior frontal cortex of Pick's disease (left panel), FTLD-U with a *PGRN* IVS0 + 5G > C mutation (middle panel), and DLDH (right panel). Tau-reactive cytoplasmic inclusions (arrows) are present in Pick's disease (**A**), but absent in FTLD-U (**B**) and DLDH (**C**). Ubiquitin-reactive inclusions are present in Pick's disease (**D**) and FTLD-U (**E**), but not in DLDH (**F**). TDP-43 commonly stains neuronal nuclei (arrowheads in **G,H,I**) and also both cytoplasmic and intranuclear inclusions in FTLD-U (arrow and double-headed arrows in **H**).

It remains to be seen whether mutations in the *TDP-43* gene can also cause FTLD-U; however, the recent discovery of pathogenic missense mutations in a highly conserved glycine-rich domain of the *TARDBP* gene, in autosomal dominant ALS and SALS families and sporadic cases, confirms the importance of TDP-43 in the pathogenesis and demonstrates that defects in *TARDBP* are sufficient to cause TDP-43 proteinopathy (**Fig 5**) (**Gitcho MA et al, 2008; Sreedharan J et al, 2008; Kabashi E et al, 2008; Van Deerlin VM et al, 2008**).

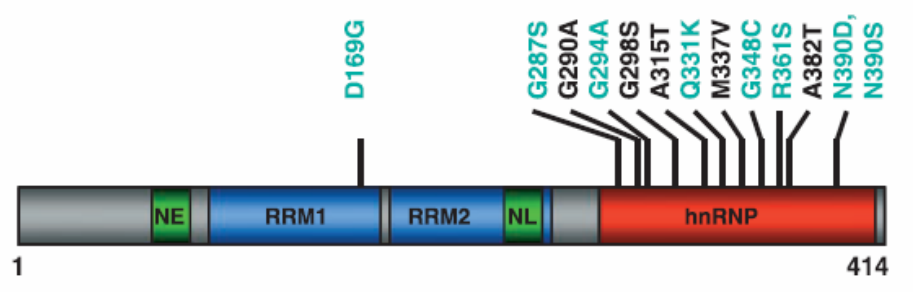

Figure 5 Mutations in the TAR DNA-binding protein 43 (TDP-43). Schematic diagram of functional domains and mutations in the coding region are indicated using the amino acid numbering of the 414 amino acid protein. RRM1, RNA recognition motif 1; RRM2, RNA recognition motif 2; NL, nuclear localization signal; NE, nuclear export signal; hnRNP, heterogeneous nuclear ribonucleoprotein interaction domain. TARDBP mutations: autosomal-dominant FALS (black), SALS (green).

Frontotemporal Dementia

Frontotemporal dementia (FTD) is the most frequent clinical phenotype under the spectrum of Frontotemporal Lobar Degenerative Diseases (FTLD), accounting for 5% to 10% of all dementia patients and 10% to 20% of patients under 65 years of age. Mean age of onset is approximately 52 to 58 years and the mean duration of the disease is 5-15 years. Nevertheless, recent instances reported onset of FTD symptoms before 30 and later than 80 years of age (**S.Spina et al, 2006; A.C. Bruni et al, 2007**). The incidence of FTD is almost even between the sexes. (**Graff-Radford NR and Woodruff BK, 2007**).

Up to 50% of FTD have a "familial component" and a part is inherited in an autosomal dominant pattern of inheritance (**Rosso SM et al, 2003; Chow TW et al, 1999; Poorkaj P et al, 2001**). To date, it is unknown whether these familial cases are due to shared genetic factors and/or environmental factors, or a combination of both genes and environment.

It is also described a high prevalence of FTD sporadic cases; it is thought that there are certainly important genetic influences mediated through a number of different genes (polygenic influences) each of which can promote the development of FTD phenotype.

In Table **2** are listed the FTD clinical symptoms (**Neary et al, 1998**). The feature of the disease that allows recognition is the profound alteration in personality and social conduct, which herald the onset of the disease. In its early course the disease is often mistaken for a psychiatric disorder, because abnormalities in memory are not a feature of the illness until later.

Core diagnostic features
Insidious onset and gradual progression
Early decline in social interpersonal conduct
Early impairment in regulation of personal conduct
Early emotional blunting
Early loss of insight
Supportive diagnostic features
Behavioral disorder
Decline in personal hygiene and grooming
Mental rigidity and inflexibility
Distractibility and impersistence
Hyperorality and dietary changes
Perseverative and stereotyped behavior
Utilization behavior
Speech and language
Altered speech output
Aspontaneity and economy of speech
Press of speech
Stereotype of speech
Echolalia
Perseveration
Mutism
Physical signs
Primitive reflexes
Incontinence
Akinesia, rigidity, and tremor
Low and labile blood pressure
Investigations
Neuropsychology: significant impairment on frontal lobe tests in the absence of severe amnesia, aphasia, or perceptuospatial disorder
Electroencephalogram normal
Brain imaging: predominant frontal and/or anterior temporal lobe atrophy

Table 2. The Clinical Diagnostic Features of FTD

Depending on the distribution of the pathology within the frontal and temporal lobes, there are three major FTD behavioural subtypes: the disinhibited type, the apathetic type and the dysexecutive type. Anatomically, the disinhibited type is connected with neurodegeneration of the orbitofrontal and anterior temporal neocortex; the apathetic

type is associated with atrophy in the medial frontal-anterior cingulate region and the dysexecutive type is correlated with degeneration of the dorsolateral prefrontal.

The disinhibited type is characterized by personality change, impulsivity, irritability, lability and socially inappropriate behaviour. The apathetic type lacks initiative and is emotionally indifferent, while the dysexecutive type has problems with planning, self-direction and ability to shift and maintain behavioural sets (**Mega MS et al, 1994**). Admixtures between these types are common due to variations in the regional localisation of atrophy. Furthermore, patients often have word finding difficulties where speech is nonspontaneous, economical and often stereotyped. Progressive reduction in speech usually leads to mutism after several years of disease. Orientation in time and space, visuospatial skills and memory initially remain intact, but deteriorate with progression of the disease (**Neary D et al, 2000; Lindau M et al, 2001**). Due to the degeneration of extrapyramidal neurons, parkinsonian signs may also be present, sometimes already from the onset of the disease. Parkinsonism involves bradykinesia, postural instability and rigidity without resting tremor.

The combination of clinical observations, neurological examination, neuropsychological tests, information gained from laboratory investigations and morphological and functional brain imaging (e.g. CT, MRI, SPECT) are necessary to achieve accuracy of clinical diagnosis generally made according to international criteria that allow to differentiate among different types of dementia and is based on clinico-pathological features, namely, peculiar clinical profiles in combination with characteristic brain lesions (**Graeber et al., 1997; Forstl, 1999; Duckett and Stern, 1999; McKhann et al., 2001**).

Finally, the diagnosis of FTD should be based on neuropathological examination, that only definitively differentiates from other types of dementia.

Neuropathological features of FTD include neuronal degeneration in the frontal and temporal cortices, sometimes with subcortical changes, basal ganglia atrophy and depigmentation of the substantia nigra. (**Josephs KA et al, 2006; Hodges JR et al 2004; Kertesz A et al 2005; Forman MS et al, 2006**).

However the pattern of atrophy can vary involving both hemispheres in equal or differing way and sometimes also involving the parietal cortex.

Neurodegeneration usually encompasses both sides of the frontal and anterior temporal lobes symmetrically, but can also appear asymmetrically in the hemispheric or predominantly temporal lobe.

At microscopic level neuronal loss, gliosis, spongiosis and cellular inclusions are present. For instance, affected neurons frequently display intracellular inclusions, named neurofibrillary tangles (NFTs), primarily composed of hyperphosphorylated tau protein (**Spillantini et al., 2000**).

However, based on immunohistochemical staining and on the pattern of intracellular inclusions, FTD can be further classified into three major subtypes (**Neary D et al, 1996**). The most common type (Frontal Lobar Degeneration type) includes microvacuolation of the superficial neurons and spongiform changes. The second type (Pick type) is mainly characterized by widespread and abundant gliosis, in addition to swollen neurons and inclusion bodies in regions such as dentate gyrus. In the third form (MND type), patients suffer from MND in combination with pathology noticed in other subtypes. Classification into one of these subtypes is sometimes difficult when typical differentiating features are not always present.

Molecular Genetics of Frontotemporal Lobar Degeneration

The great clinical and neuropathological heterogeneity of FTLD suggest the existence of several genetic factors underlying or modifying the pathogenesis of this prevalent and untreatable disorder. Genetic studies of FTLD autosomal dominant families led to the discovery of four major genes. Micotubule Associate Protein Tau (*MAPT*) and Progranulin (*PGRN* also known as *GRN*) genes that when mutated cause FTLD-Tau Positive and FTLD-Tau negative pathology respectively [26,27], Valosin-Containing Protein (*VCP*) [28] and Charged Multivesicular Body Protein 2B (*CHMP2B*) genes associated to the major cases of FTLD-U [29] (**Tab. 3**) (**O. Bugiani, 2007; Jill S et al, 2007**).

Gene name	*MAPT*	*PGRN*	*VCP*	*CHMP2B*
Gene location	17q21.1	17q21.32	9p13.3	3p11.2
Phenotype	FTDL	FTDL	IBMPFD	FTDL
Mutations	**44**	**63**	**12**	**4**

Table 3 Frontotemporal Lobar Degeneration (FTLD) Causative Genes

MAPT Gene

The unraveling of FTLD genetics began in 1994 with the identification of a locus linked to chromosome 17q21.2 in 13 families with a variety of clinical and pathologic phenotypes. At a consensus conference in 1996, the clinical, pathologic, and genetic findings of these families were compared and the term "frontotemporal dementia with parkinsonism linked to chromosome 17" (FTDP-17) was introduced to best describe the predominant symptoms in these families (**Foster NL et al, 1997**). In 1998, as the microtubule-associated protein tau (*MAPT*) gene had been localized in the FTDP-17 families to the 17q21 candidate region, it becames the obvious positional and functional candidate gene (**Fig 6**) **(Poorkaj P et al, 1998).**

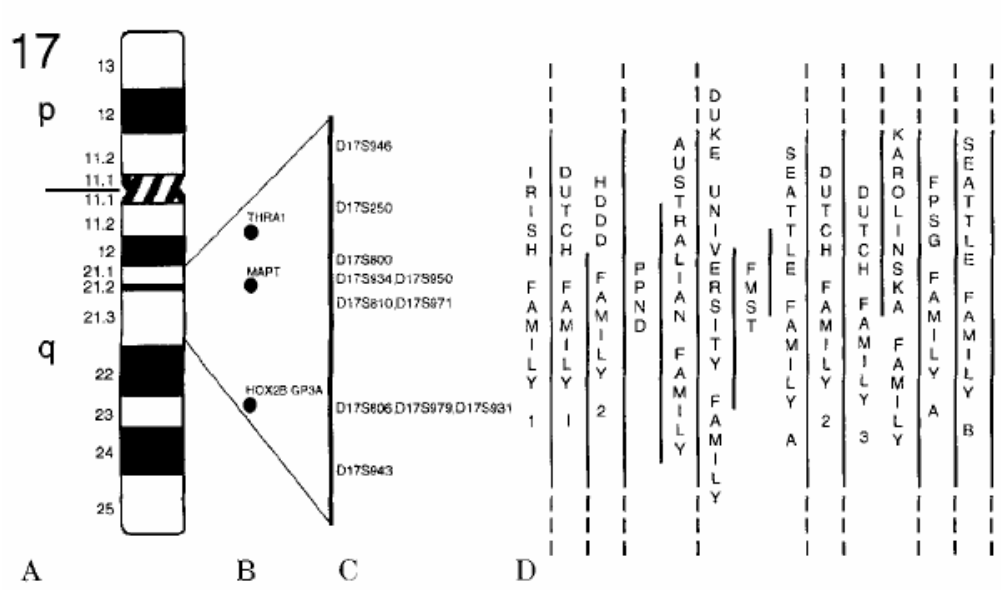

Figure 6 Linkage analysis for fiontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) families.

Subsequently several mutations within the *MAPT* gene were identified (**Hutton M et al, 1998; Spillantini MG et al, 1998**).

This gene encodes the microtubule-associated protein tau whose transcript undergoes complex, regulated alternative splicing, giving rise to several mRNA transcripts that are differentially expressed in the nervous system, depending on stage of neuronal maturation and neuron type. The tau protein promotes microtubule assembly and stability, and might be involved in the establishment and maintenance of neuronal polarity (For further details about gene and protein see below: paragraph…)

To date, 44 different potential pathogenic *MAPT* mutations have been reported which result in abnormal tau aggregation and are thought to cause disease through a toxic gain of dysfunction (AD and FTD Mutation Database http://www.molgen.ua.ac.be/FTDMutations).

MAPT mutation frequencies in FTLD populations have been shown to vary from 0 to 50%, depending on the family history of dementia and the use of clinical or pathologic inclusion criteria (**Fabre et al., 2001; Binetti et al., 2003; Rosso et al., 2003; Stanford et al., 2004**).

VCP Gene

In 2004, mutations in *VCP* on chromosome 9p13 were associated in FTD with inclusion body myopathy and Paget disease (IBMPFD), a rare multisystem disorder with autosomal dominant inheritance (**Watts, G.D. et al. 2004**). The locus for IBMPFD was mapped to chromosome 9p21-p12 in 2001 by genome-wide linkage analyses in a large Illinois family (**Kovach MJ et al, 2001**).

VCP is a member of the AAA-ATPase gene super family (ATPase associated with diverse cellular activities) involved in multiple cellular functions including acting as a molecular chaperone in endoplasmic reticulum–associated protein degradation (ERAD), stress response, programmed cell death, and interactions with the ubiquitin-proteasome system. Most of the *VCP* mutations are situated within the domain involved in the ubiquitin binding. They gives rise to significant phenotypic variation and are likely to cause a loss or alteration of the normal function of VCP, leading to impaired ubiquitin-

proteasome system activity and the accumulation of ubiquitinated proteins within cells (**Watts GD et al, 2004; Forman MS et al, 2006**). To date, 12 missense mutations in *VCP* gene have been reported. (AD and FTD Mutation Database http://www.molgen.ua.ac.be/FTDMutations) (**Guyant-Marechal L et al, 2006; Haubenberger D et al, 2005; Schroder R et al, 2005**).

One year later VCP gene identification, a large Danish family originating from the Jutland with "non specific dementia" was linked to the *CHMP2B* gene on chromosome 3p13 and a complex mutation was subsequently identified in this gene (**Brown J et al, 1995; Skibinski G et al, 2005**).

CHMP2B Gene

Human *CHMP2B* codes for a protein of 213 amino acids with a predicted coil-coil domain. It is a component of the endosomal secretory complex required in transport III (ESCRITIII) which is involved in the degradation of surface receptor proteins and formation of endocytic multivesicular bodies (MVB) (**Babst M, 2005**). Dysfunction of the ESCRT complexes results in the inability of MVBs to internalize membrane bound cargoes, leading to dysmorphic endosomes and poor protein turnover.

The *CHMP2B* mutation was located in the acceptor splice site of exon 6 and was shown to produce two aberrant transcripts that could give rise to a gain- or loss-of-function disease mechanism.

Overexpression of the two Danish CHMP2B mutant proteins in cell culture disrupted the CHMP2B localization and indeed resulted in the formation of dysmorphic organelles of the late endosomal pathway (**Skibinski G et al, 2005**). These data suggest that endosomal trafficking dysfunction might be an important factor leading to the neurodegeneration in this family.

To date 4 mutation in *CHMP2B* gene were reported and no other families have been found with a segregating *CHMP2B* mutation (AD and FTD Mutation Database http://www.molgen.ua.ac.be/FTDMutations) (**Cannon A et al, 2006; Momeni P et al 2006**).

PGRN Gene

Recently, was reported the linkage of FTDL-U families with chromosome 17 and systematic mutation analyses of positional candidate genes led, in July 2006, two groups of researchers to the identification of mutations in the Progranulin (*PGRN*) gene. (**Cruts M et al, 2006; Baker M et al, 2006**). *PGRN* encodes a precursor glycoprotein of 68 kD, composed of 7.5 tandem repeats of a highly conserved motif with 12 cysteines. Proteolytic cleavage by an elastase-like activity results in the generation of a family of 6-kD peptides called granulins. PGRN is generally thought to be a secreted mitogenic factor that is expressed in a variety of tissues **(Bateman A et al 1998)**. In non brain tissues, progranulin is thought to function as a growth factor that regulates cell cycle progression and cell motility in multiple processes including development, inflammation, and wound repair (**He Z. and Bateman A., 2003**; **He Z et al, 2003; Daniel R. et al, 2003**). However, the function of progranulin in the brain, observed in a subset of pyramidal neurons and in activated microglial cells, has received relatively little attention prior to it being linked with FTD (**Daniel R et al, 2000**). The fact that pathologic mutations in *PGRN* lead to a depletion of the functional protein suggests that progranulin is likely critical for neuronal survival (**Baker M et al, 2006**).

To date, 63 different pathogenetic *PGRN* mutations have been reported (AD and FTD Mutation Database http://www.molgen.ua.ac.be/FTDMutations) (**Fig 7**) (**Rademakers R et al, 2007**).

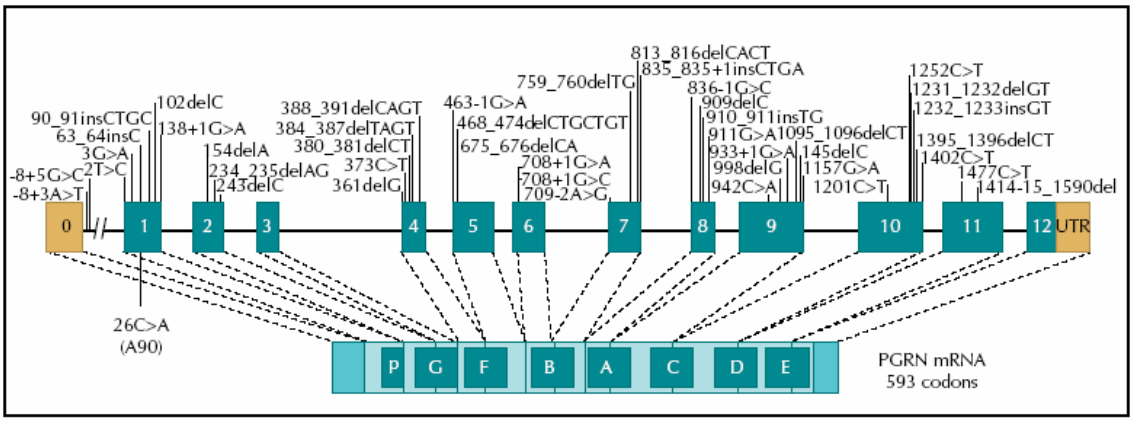

Figure 7 Null mutations in the progranulin gene (*PGRN*) and mRNA encoding the PGRN protein, showing all mutations identified to date. Letters in boxes refer to individual granulin peptides. Mutations are numbered relative to the largest *PGRN* transcript

The majority of mutations are nonsense, frameshift, and splice-site mutations that create null alleles; additionally, several missense mutations have been reported that are predicted to result in a nonfunctional protein (haploinsufficiency) although the pathogenicity of these mutations remains to be confirmed (**Cruts, M. et al. 2006**; **Le Ber I et al, 2007**).

Progranulin mutation frequencies in FTLD populations have ranged from 5 to 10%, although a frequency of 13 to 25% has been reported in the subset of FTLD population in which a positive family history is identified. *PGRN* mutations have also been found in individuals without any family history.

A comparison of *PGRN* mutations carriers showed a mean diseases age of onset is approximately 59 years with a range of 48 to 83 years and the mean age of death is 65 years with a range of 53 to 87 years. Penetrance is estimated to be 90% at age 70 (**Gass J et al, 2006; Le Ber I et al, 2007**). No phenotype/genotype correlation has been found but it is supposed that different genetic variants or environmental factors can influence the disease phenotype contributing to the massive range in onset age.

Additional genes and genetic risk factors of Frontotemporal Lobar Degeneration

Together, all FTLD genes, associated with significant interfamilial and intrafamilial phenotypic variation, explain the disease in less than half of the familial patients and in a minority of apparently sporadic patients, suggesting that additional genes and genetic risk factors are involved. It has now been linked to chromosome 9 an additional FTLD locus. Although candidate gene sequencing at this locus revealed the presence of a disease segregating nonsense mutation in the Intraflagellar Transport 74 *(IFT74)* gene in one independent family, the pathogenicity of this mutation is controversial and no causal *IFT74* mutations were identified in other FTD (**Morita M et al, 2006; Vance C et al, 2006; Valdmanis PN et al, 2007**). IFT74 is a 600 amino acid coiled-coil domain-containing protein that localizes to the intracellular vesicle compartment and is a component of the intraflagellar transport system responsible for vesicular transport of material synthesized within the cell body into and along dendrites and axon (**Momeni P et al, 2006**). Presenilin 1 (*PS1*) gene is another candidate gene. *PS1* mutations were been

associated either with familial FTDL-τ either with FTLD-U. (**Raux et al., 2000; Tang-Wai et al., 2002; Dermaut et al., 2004 Zekanowski et al., 2006; Livia Bernardi et al, 2008**). Furthermore emerging evidence indicates that the tau locus itself could be a susceptibility factor playing a key role in sporadic FTLD-Tau positive. Tau gene sequencing (**Andreadis A et al, 1992**) has revealed several polymorphisms which are in complete linkage disequilibrium, mostly inherited as two distinct haplotypes, H1 and H2, with relatively few recombination events (**Baker M et al, 1999**).

Haplotype-based association studies have shown a significant association between the H1/H1 genotype and Progressive Supranuclear Palsy (PSP) [**Baker M et al, 1999**; **Conrad C et al, 1997 Di Maria E et al, 2000**]. A similar association has also been found in corticobasal degeneration (CBD) [**Di Maria E et a, 2000; Houlden H et al, 1999**], while the results are conflicting in both FTD [**Hughes A et al, 2003; Sobrido MJ et al, 2003; Verpillat P et al, 2002**; **Zekanowski C et al, 2003**] and Pick's disease (PiD) (**Morris HR et al, 2002**). It was shown that H1 haplotype expresses significantly more 4Rtau *MAPT* transcripts than H2. These data suggest a mechanism for the increased susceptibility of H1 carriers to neurodegeneration. Additionally, carriage of the H2 haplotype was reported to be associated with a lower age at onset of FTD and functionally show a more severe decline of glucose utilization in frontal brain areas although no increased risk of the disease was attributed to this haplotype (**Borroni et al., 2005; Simon M. et al, 2007**).

In addition, few years ago Conrad and colleagues characterized a previously undescribed gene (**Conrad et al., 2002**), named Saitohin which is located in the intron between exons 9 and 10 of the human *MAPT* gene, in a critical region for the alternative splicing of exon 10 (**Hutton et al., 1998**). A single nucleotide polymorphism (Q7R; CAA to CGA), correlated with a higher incidence of Alzheimer disease patients **Combarros et al., 2003; Conrad et al., 2002**, was discovered in this gene; STH Q7R polymorphism is in complete linkage disequilibrium with the MAPT haplotypes: STH Q allele is linked to H1 MAPT haplotype and the STH R allele is linked to H2 MAPT haplotype (**de Silva et al., 2003**; **Peplonska et al., 2003; Verpillat et al., 2002b; Zekanowski et al., 2003**).

The possible role of Apolipoprotein E (APOE), established as the major genetic risk factor in AD, as well as in other neurodegenerative diseases, in FTD is still debated (**Verpillat P et al, 2002**). There are reports of positive associations of ε2/ε 4 APOE alleles with H1 haplotype as a risk factor of developing FTD [**Ingelson M et al, 2001; Verpillat P et al, 2002**] but other authors have not found negative results as underlined by the results of L.Bernardi et al. in which H1 haplotype enhances the effect of the ε2 allele decreasing FTD risk (**Livia Bernardi et al, 2006**). This opposite effects probably accord to the population specific genetic background. In any case the genetic mechanism underlying the association of specific gene or genetic risk factor in familial and sporadic FTDL remain still unresolved.

FTLD-Tauopathies

FTLD-τ cover a significant number of FTLD and related disorders characterized by the accumulation of filamentous deposits, consisting of abnormally insoluble hyperphosphorilated tau protein, also referred as Neurofibrillary Tangles (NFTs), in neurons and glia (**Fig 8**) (**Spillantini, M.G. and Goedert, M.,1998; Spillantini, M.G. *et al;*1998**). This group of diseases became known as "Tauopathies", term first used to describe a family with frontotemporal dementia and abundant tau deposits (**Spillantini, M.G. et al. 1997**).

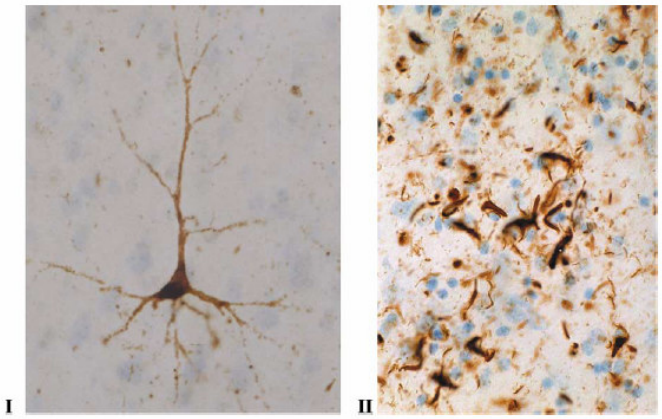

Figure 8 Examples of microscopic neuropathology in tauopathies. Photos are illustrating immuno-histochemical staining of brain tissue, using the AT-8 antibody against hyperphosphorylated tau. (I) A pyramidal cell in pretangle state from frontal cortex from a patient with frontotemporal dementia (FTD), and (II) the characteristic oligodendroglial inclusions (coiled bodies).

Tauopathies are now referred to a group of familial and sporadic disorders with, as shown in table **4**, widespread clinical symptoms and tau pathology in which tau accumulation is believed to be directly associated with neuronal death and disease development.

Disease	Clinical symptoms	Neuropathological features
Alzheimer's disease (AD)	Progressive dementia	Temporal and parietal lobar atrophy, neuronal NFTs and amyloid plaques
Amyotrophic lateral sclerosis/ parkinsonism–dementia complex of Guam	Bradykinesia, rigidity, dysarthria, progressive dementia and motor deficiency	Diffuse frontotemporal lobar atrophy, severe cerebral white matter atrophy, neuronal and glial NFTs
Corticobasal degeneration	Progressive asymmetrical rigidity and apraxia, progressive aphasia, impaired ocular movements, and dementia	Focal asymmetric cortical atrophy with nigral degeneration, and tau-positive neuronal and glial lesions in both the gray and white matter
Down's syndrome (DS)	Mental retardation, dementia	Hippocampal atrophy, NFTs and amyloid plaques
Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17)	Disinhibition, progressive aphasia	Frontotemporal lobar atrophy, gliosis, neuronal and/or glial NFTs
Gertmann–Sträussler–Scheinker disease with tangles	Ataxia, spastic paraparesis and dementia	Spongiform changes, gliosis, PrP plaques, NFTs
Niemann–Pick disease type C (NPC)	Severe childhood lipidosis, cerebellar ataxia, dysarthria, dysphagia, and progressive dementia	Neuroaxonal swelling, neurodegeneration, NFTs
Pick's disease	Disinhibition, progressive aphasia	Frontotemporal lobar atrophy, NFTs
Progressive supranuclear palsy (PSP)	Symmetrical akinetic–rigid parkinsonian syndrome, supranuclear gaze palsy, and cognitive changes	Asymmetric cortical atrophy with ballooned neurons, tau-positive neuronal and glial lesions

Tabella 4 Overview of selected tauopathies, presenting clinical and neuropathological characteristics

Microtubule Associated Protein Tau

Tau is a member of the microtubule-associated proteins (MAPs) family found in many animal species such as *Caenorhabditis elegans*, *Drosophila*, goldfish, bullfrog, rodents, bovines, goat, monkeys and human. Human cDNA for tau protein was isolated and cloned in 1989 by Goedert et al. (**Goedert M, Spillantini MG, Potier MC et al, 1989**; **Goedert M, Spillantini MG, Jakes R et al, 1989; Goedert M, Spillantini MG, Cairns NJ et al, 1992**). MAPs are abundant in the central nervous system where are normally expressed in axons of neurons. The main function of these proteins is to promote microtubule assembly, to reduce microtubule instability and to play a role in maintaining neuronal integrity and axonal transport. Furthermore, in some species the microtubule-

associated proteins can be found at low levels in astrocytes and oligodendrocytes, and can be expressed in glial cells, mainly in pathological conditions. It is possible to detect tau mRNA and proteins in several peripheral tissues such as heart, kidney, lung, muscle, pancreas, testis, as well as in fibroblasts.

Gene organization and Splicing

The human *MAPT* gene, coding for the tau protein is located over 150 kb on the long arm of chromosome 17 at position 17q21.3. It is a single copy gene composed by 16 exons (**Fig 9**).

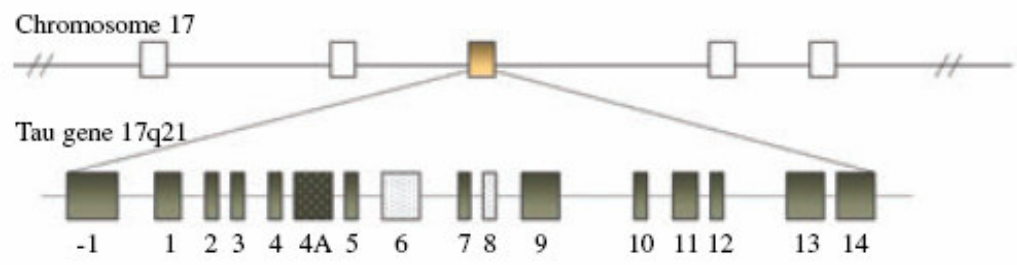

Figure 9 Schematic representation of *MAPT* gene location and genomic structure

Exons 1, 4, 5, 7, 9, 11, 12 and 13 are constitutive exons whereas exons 2, 3 and 10 are alternatively spliced and adult brain specific (**A. Andreadis et al, 1992**). Alternative splicing of these three exons gives rise to six mRNAs transcripts [**M. Goedert et al, 1989 Neron.; M. Goedert et al, 1989 EMBO; A. Himmler et al, 1989; K.S. Kosik et a, 1989**]. In particular, alternative splicing of exon 10 gives rise to tau isoforms with either three (exon 10^-) o four (exon 10^+) microtubule-binding repeats known as 3Rtau and 4Rtau respectively. In addition, alternative splicing of exons 2 and 3 results in 3Rtau and 4Rtau isoform with zero (0N), one (1N) or two (2N) N-terminal inserts. In the adult human brain the ratio of 3R to 4R tau is generally 1 (**Fig 10**).

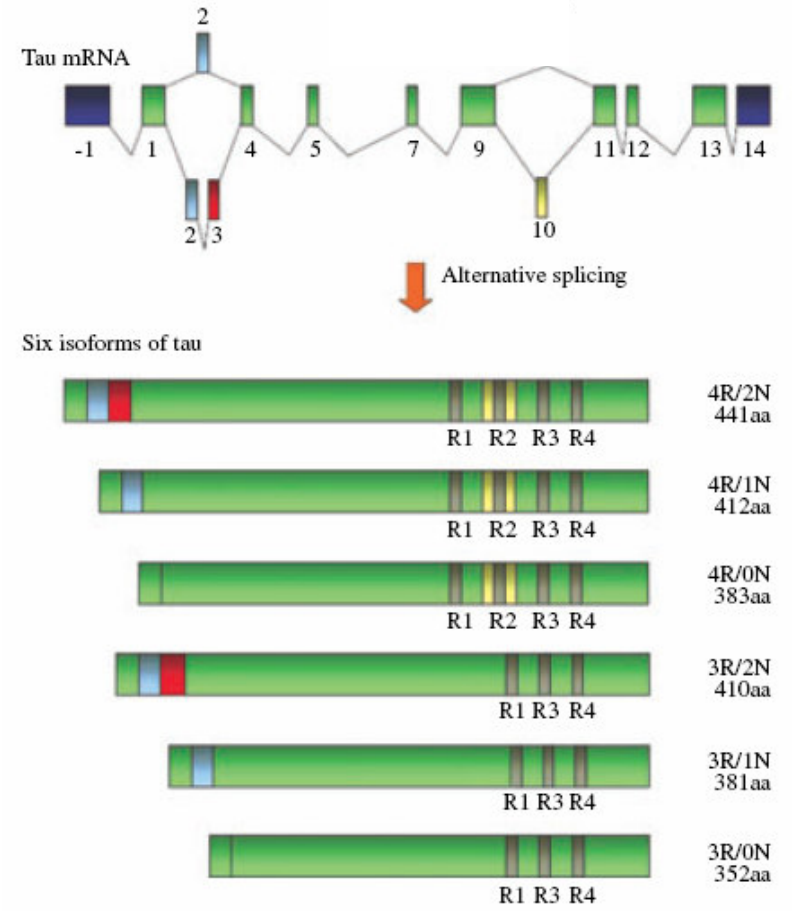

Figure 10 Schematic representation of the primary transcript and the six human tau isoforms expressed in human brain

Exons 4A, never present in the mRNA of human brain, is specific to peripheral tau proteins. Tau mRNA with either exons 6 or 8 have not been described in human. Some transcripts with exon 8 are found in bovine and rhesus monkey brains. Exon -1 is part of the promoter, and is transcribed but not translated. Exon 14 is found in messenger RNA, but it is not translated into protein. [**A. Andreadis et al, 1992; M. Goedert et al, 1989 Neron.; M. Goedert et al, 1989 EMBO; A. Sawa et al, 1994**].

Restriction and sequencing analyses show that the gene contains two CpG islands, one associated with the promoter region, the other with exon 9 (**A. Andreadis et al, 1992; A. Andreadis et al, 1995**). Two regions homologous to the mouse Alu-like sequence are present. The sequence of the promoter region also reveals a TATA-less sequence that is

likely to be related to the presence of multiple initiation sites, typical of housekeeping genes. Three SP1-binding sites, important in directing transcription initiation in other TATA-less promoters, are also found in the proximity of the first transcription initiation site (**A. Andreadis et al 1996; E. Sadot et al 1996**).

In *MAPT* intron 9 is present a region, that behaves like a gene giving rise to the expression of a novel protein known as saithoin. Saithoin has not yet been isolated and characterized; however, by analysis of its sequence it does appear to contain some similarities to certain nucleic acid binding proteins. Saithoin contains the regions SSYEESSR and SLAWEV similar to those present in some transcription factors, and thus it may play a role in transcription or nucleic acid metabolism. It is still not clear whether saithoin is a protein that is only expressed in human cells and not in other organisms such as the mouse (**Conrad C et al, 2002**).

Sequence analysis of the coding region and flanking intronic sequences in the *MAPT* gene has identified over 200 non coding polymorphisms in exons 1, 2, 3, 9, 11 and 13 in both controls and FTDP-17 patients (MGS pag234). Analysis of the occurrence of these polymorphisms revealed that they are in complete disequilibrium (LD) with each other and constitute two extended haplotypes, H1 and H2, which cover a region of ~1.8 Mb. It is the longest region of LD identified to date in the human genome, (43) including in the centre the *MAPT* gene and other genes, as corticotrophin-releasing hormone receptor 1 (*CRHR1*), N-ethylmaleimide sensitive factor (*NSF*), intramembrane protease 5 (*IMP5*) (44) (**Fig 11**).

The H1 haplotype is the predominant haplotype in all ethnic groups, whereas the H2 haplotype seems to be primarily of European origin (**Evans W et al, 2004**). Functional differences in transcriptional activity have been attributed to the different haplotypes, with the H1 haplotype reported to have more activity than the H2 haplotype (**Kwok JB et al, 2004**).

H1 and H2 differ in nucleotide sequence and intron size, but are identical at the amino acid level. Few years ago, it has been found that a ~900 kb segment from H2 chromosomes is inverted with respect to the H1 haplotype (**Fig 11**).

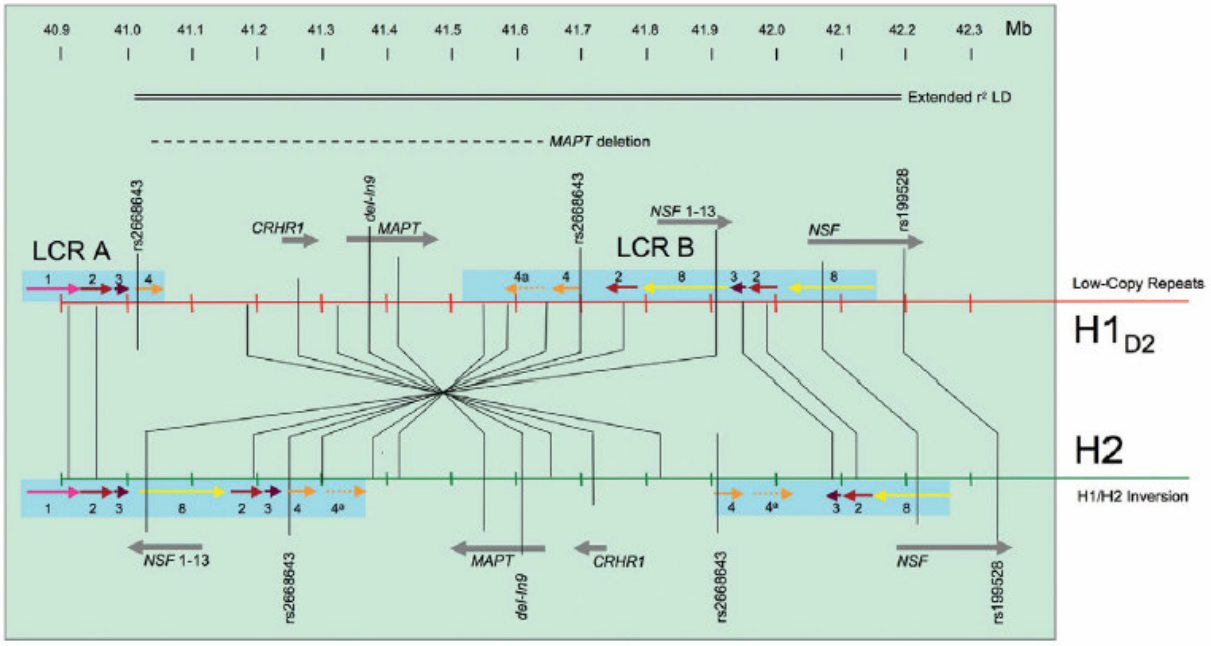

Figure 11 Inverted *MAPT* H1 and H2 Haplotypes

This inversion is thought to have occurred between 3 and 3.6 million years ago (**Stefansson et al., 2005**) and is almost entirely absent outside of European populations (**Evans et al., 2004**).

Further investigations into the genomic architecture of this region have identified a number of low-copy repeat (LCR) sequences prone to Chromosomal rearrangements. There is evidence to suggest that the non-allelic homologous recombination that generated the inversion at H2 occurred between two low-copy repeat sequences, LCR A and LCR B, located 250 kb centromeric and 180 kb telomeric to MAPT respectively (**Cruts et al., 2005**).

Protein structure and function

In the brain, tau proteins constitute a family of six isoforms which range from 352 to 441 amino acids. Their molecular weight is ranging from 45 to 65 kDa. Tau proteins differ from each other by the presence of either three (3R) or four repeat-regions (4R) in the carboxy-terminal (C-terminal) part of the molecule and the absence or presence of one or two inserts (29 or 58 amino acids) in the amino-terminal part (N-terminal) (**M.**

Goedert, M.G. Spillantini, R. Jakes et al, 1989; M. Goedert, M.G. Spillantini, M.C. Potier et al, 1989; A. Himmler et al, 1989). In adult human brain the protein levels of 3R and 4R tau isoforms are similar, whereas the fetal brain only contains the shortest 3R tau isoform, characterized by the absence of N-terminal inserts and the presence of three C-terminal repeats (3R) (**K.S. Kosik et al, 1989; M. Goedert and Jakes et al, 1990**) . It has been demonstrated most recently that there is a transient expression of 0N3R tau isoform during adult neurogenesis (**Bullmann et al, 2007**). Moreover, tau isoforms may be differentially distributed in neuronal subpopulations. For istance, tau mRNAs containing exon 10 are not found in granular cells of the dentate gyrus (**Goedert, M.G. Spillantini, R. Jakes et al, 1989**). Differently from humans, only 4R tau isoforms are expressed in adult rodent brain.

A number of functions of tau have been characterised. Its main physiological function is the promotion of assembly and stabilization of the microtubular network. As a result of this tau protein is essential for establishing neuronal polarity and axonal outgrowth during development and for maintaining axonal morphology and axonal transport in mature neurons. Tau performs these function through the its two large domains: the "projection domain" containing the amino-terminal two-thirds of the molecule and the "microtubule binding domain" containing the carboxy-terminal one-third of the molecule.

Tau projection domain

The projection domain can be divided into two regions: the amino-terminal region, encoded by exons 2 and 3, with a high proportion of acidic residues followed by a basic proline-rich region.

The N-terminal part is referred to as the projection domain because it projects from the microtubule (MT) surface giving rise to a crucial functions such as mediating interactions between tau and the neural plasma membrane and determining the spacing between MTs in axon (**R. Brandt et al, 1995; N. Hirokawa et al, 1988; J. Chen et al 1992**). It should be noted that projection domain may increase axonal diameter, for istance in peripheral neurons which often project a very long axon with large diameter, an additional N-terminal tau sequence encoded by exon 4A is present, generating a

specific tau isoform called 'big tau' with an approximate size of 100 kDa **(Couchie D et al, 1992; Goedert M et al, 1992; I.S. Georgieff et al, 1993)**.

Tau proteins bind to spectrin and actin filaments (**M.F. Carlier et al, 1984; I. Correas et al, 1990; J.P. Henriquez et al 1995**) and through these interactions may allow microtubules to interconnect with other cytoskeletal components such as neurofilaments (**Y. Miyata et al, 1986**) and may restrict the flexibility of the microtubules (**A. Matus et al, 1990**). There is also evidence that tau proteins interact with cytoplasmic organelles. Such interactions may allow for binding between microtubules and mitochondria (**D. Jung et L, 1993**).

Recent findings have shown an extensive colocalizzation of tau and dynactin complex suggesting an interection between these proteins and a direct involvement of tau in axonal transport (**Fig.12**) (**E. Magnani et al 2007**).

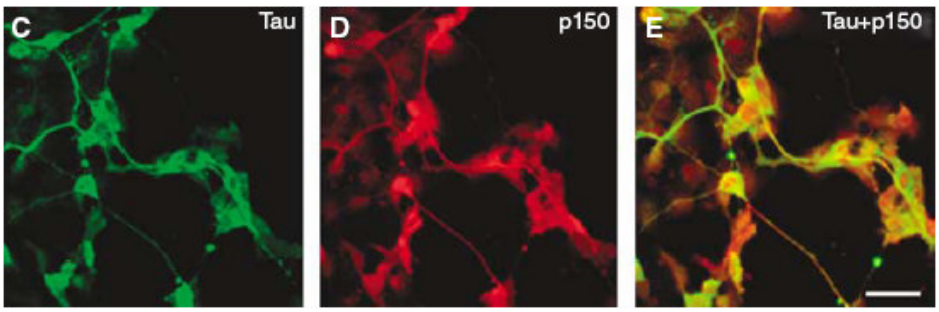

Figure 12 Colocalization of tau and dynactin p150

Dynactin complex, essential for cytoplasmic dynein-dependent organelle transport, consists of a short filament of an actin-like protein, ARP1, and around 10 other polypeptides, including a dimer of the heavy chain, known as p150 .

Axonal MTs are organized with their fast-growing (plus) ends pointing towards the axonal tip. MTs are assembled in the cell body and then transported along the axon. Tau is also found initially in the cell body, but after the development of one of the neurites into an axon, becomes concentrated in the distal axon. Tau's localization to the distal axon requires intact MTs and microfilaments and there is a model in which MT-bound tau is linked to the plasma membrane by a component that requires actin filaments for its

subcellular localization. Recently results suggest that the dynactin complex could be that component and support the idea that tau-stabilized MTs could be transported in the anterograde direction along the axon by dynein/dynactin motors linked to the axonal membrane.

It has been proposed a molecular model that shows a direct interaction between tau's N-terminal projection domain and the C-terminal region of p150 (**Fig 13**) (**E. Magnani et al 2007**).

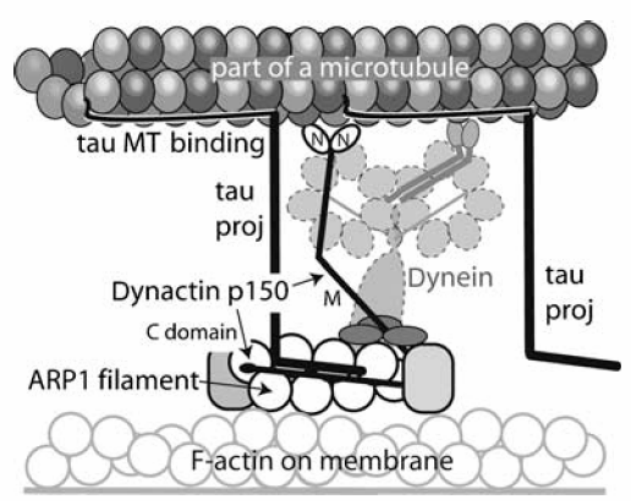

Figure 13 Proposed model of the role of tau in stabilizing the interaction between axonal MTs and the dynactin/dynein complex

In this model tau appears to facilitate and stabilize the binding of the dynactin complex with MT tracks by binding to the C-terminus of p150. Although other components of the dynactin complex bind to p150 in this extensive C-terminal region, a recent structural study has shown the presence of additional binding sites.

Recently, it was proposed another model in which tau protein may control the balance of microtubule-dependent axonal transport in the neuron, by locally modulating cytoplasmatic molecular motor proteins, dynein and kynesin, which transport cargo toward the minus-end (toward the cell center) and plus-end of microtubules (toward the cell periphery), respectively (**Ram Dixit et al 2008**). In the model is shown that in a healthy neuron, tau can be distributed in a proximal-to-distal gradient (**M. Kempf et al,**

1996; M. M. Black et al, 1996). Lower tau concentration at the cell body would allow kinesin to efficiently bind to microtubules and initiate anterograde transport of cargo, whereas higher tau concentration at the synapse would facilitate cargo release (**Fig.14**).

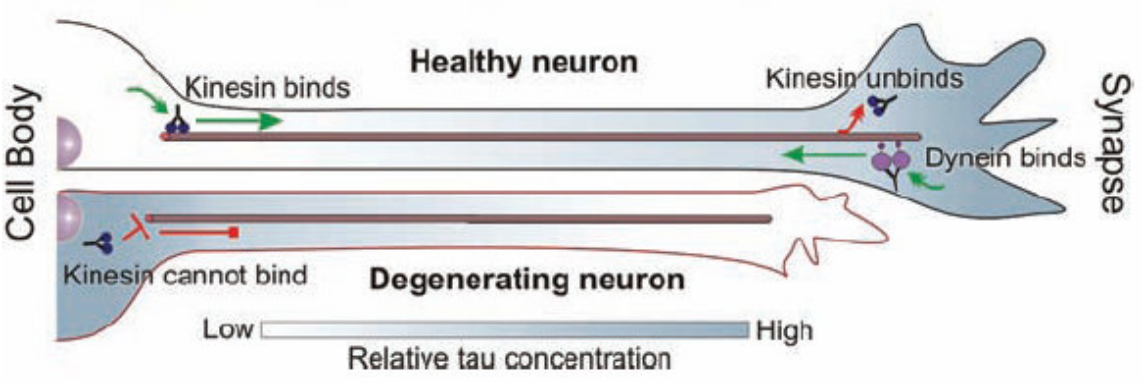

Figure 14 Model of the role of tau in the regulation of axonal transport

At the same time, dynein-driven retrograde transport from the distal axon would not be impeded due to dynein's lower sensitivity to tau (**Fig.14**) (**C. Ballatore et al, 2007; Ram Dixit et al, 2008**).

- ***Tau microtubule assembly domain***

Tau proteins bind microtubules through repetitive regions in their C-terminal part. These repetitive regions are the repeat domains (R1–R4) encoded by exons 9–12 (**G. Lee et al, 1989**). The three (3R) or four copies (4R) are made of a highly conserved 18-amino acid repeats separated from each other by less conserved 13- or 14-amino acid inter-repeat domains (**M. Goedert, M.G. Spillantini, M.C. Potier et al, 1989; A. Himmler et al, 1989**). Tau proteins, known to act as promoter of tubulin polymerization *in vitro*, are involved in axonal transport (**R. Brandt et al, Neurochem 1993; R. Brandt et al, J. Biol. Chem 1993**).

It has been demonstrated that adult tau isoforms with 4R (R1–R4) are more efficient to promote microtubule assembly than the fetal isoform with 3R (R1,R3,R4) (**M. Goedert, R. Jakes, 1990; K.A. Butner et al, 1991; N. Gustke et al, 1994**). Interestingly, the most potent part to induce microtubule polymerization is the inter-region between repeats 1 and 2 (R1–R2 inter-region) and more specifically peptide KVQIINKK within this sequence. This R1–R2 inter-region is unique to 4R tau, adult-specific and

responsible for a 40-fold difference in the binding affinities between 3R and 4R tau (**B.L. Goode et al, 1994**).

The heat schok proteins HSP70 and HSP90 can also bind to tau, and as a consequence of this interaction, the association of tau protein with microtubules increases, decreasing its self-association and hence the formation of tangles.

The nuclear tau isoforms are similar to cytoplasmic tau, but they show lower solubility, suggesting that they undergo specific modifications, either post-translational (phosphorylation) or through interactions less with other proteins. Prior to their addressing in the nucleus, tau proteins are phosphorylated in the cytoplasm. The function of nuclear tau and how it may be regulated by phosphorylation is still unknown (**J.A. Greenwood et al, 1995**).

Post-translational modifications of tau

Several modifications have been described for tau protein including phosphorylation, glycosylation, ubiquitinylation, deamination, oxidation, nitration, cross-linking, or glycation. The most studied of these has been phosphorylation. Tau plays a key role in regulating microtubule dynamics, axonal transport and neurite outgrowth, and all these functions are modulated by phosphorylation of numerous serine and threonine sites that are clustered in regions flanking the MT binding repeats.

The different states of tau phosphorylation result from the dynamic activity of specific kinases and phosphatases towards these sites.

Several tau kinases have been described and they are grouped into two different types: proline (PDPK) and non-proline (NPDPK) directed protein kinases (**Fig 15**) (**G. Drewes et al 1992; M. Goedert et al 1997; C.H. Reynolds et al 1997; R. Vulliet et al 1992; T.J. Singh et al 1996**).

PDPK modified Ser-Pro or Thr-Pro Pro tau motifs. Three main PDPK have been describe to phosphorylate tau, GSK3, also known as tau kinase I, cdk5 (or tau kinase II) and stress kinases like JNK and p38. Recently it was shown that GSK-3 phosphorylation of tau modulates its axonal transport by regulating binding to kinesin-1 (**Jian-Zhi Wang et al 2008; Cuchillo-Ibanez I et al, 2008**).

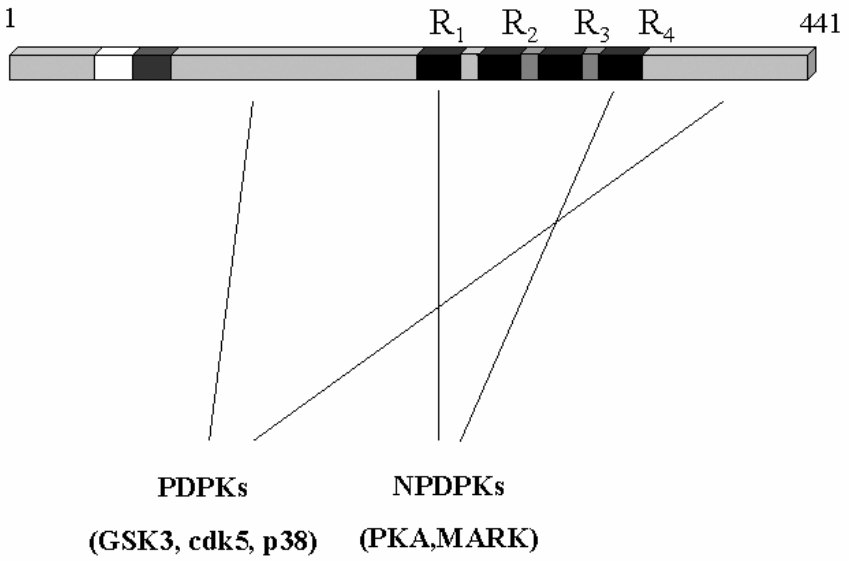

Figure 15 Scheme of tau molecule, showing the localization of some of the sites that can be phosphorylated by proline directed protein kinases (PDPKs) and non-proline dependent protein kinases (NPDPKs).

NPDPK modify Ser or Thr residues where not followed by prolines. Among them are: cyclic-AMP dependent kinase; Ca2+/calmodulin dependent kinase (CaMPKII), protein kinase C (PKC) or microtubule affinity regulating kinase (MARK).

There are seventy nine putative Ser or Thr phosphorylation sites on the longest brain tau isoform (441 amino-acids). Using phosphorylation-dependent monoclonal antibodies against tau, mass spectrometry and sequencing, at least thirty phosphorylation sites have been described. All of these sites are localized outside the microtubule-binding domains with the exception of Ser 262 (R1), Ser285 (R1–R2 inter-repeat), Ser305 (R2–R3 inter-repeat), Ser324 (R3), Ser352 (R4) and Ser356 (R4). Most of these phosphorylation sites are on Ser–Pro and Thr–Pro motives. A number of consequence sites on non Ser /Thr–Pro sites have also been identified. The phosphorylation status of tau is also regulated by phosphatases such as phosphatase 1A (PP1), phosphatase 2A (PP2A) and phosphatase 2B (PP2B) (**M. Goedert et al 1995; S. Goto et al 1985; H. Yamamoto et al 1988; H. Yamamoto 1990; Jian-Zhi Wang et al 2008;**).

The phosphorylation of tau is developmentally regulated; it is higher in fetal neurons and decreases with age during the development possibly reflecting the high degree of plasticity in the developing CNS **(Goedert et al 2001)**. In developing neurons, the phosphorylation of tau also seems to influence its distribution and its functions. Tau that is phosphorylated in its proline-rich region is mainly present in the somatodendritic compartment, whereas when this region becomes dephosphorylated, it can be found principally in the distal region of the axon. Additionally, tau phosphorylated in its carboxy-terminal domain is also found mainly in the distal axonal region.

The abnormal tau phosphorylation that occurs in neurodegenerative conditions not only results in a toxic loss of function (e.g. decreased microtubule binding) but probably also a toxic gain of function (e.g. increased tau-tau interactions) that perhaps even increase cell death (**Jian-Zhi Wang et al 2008)** (**Fig.16**).

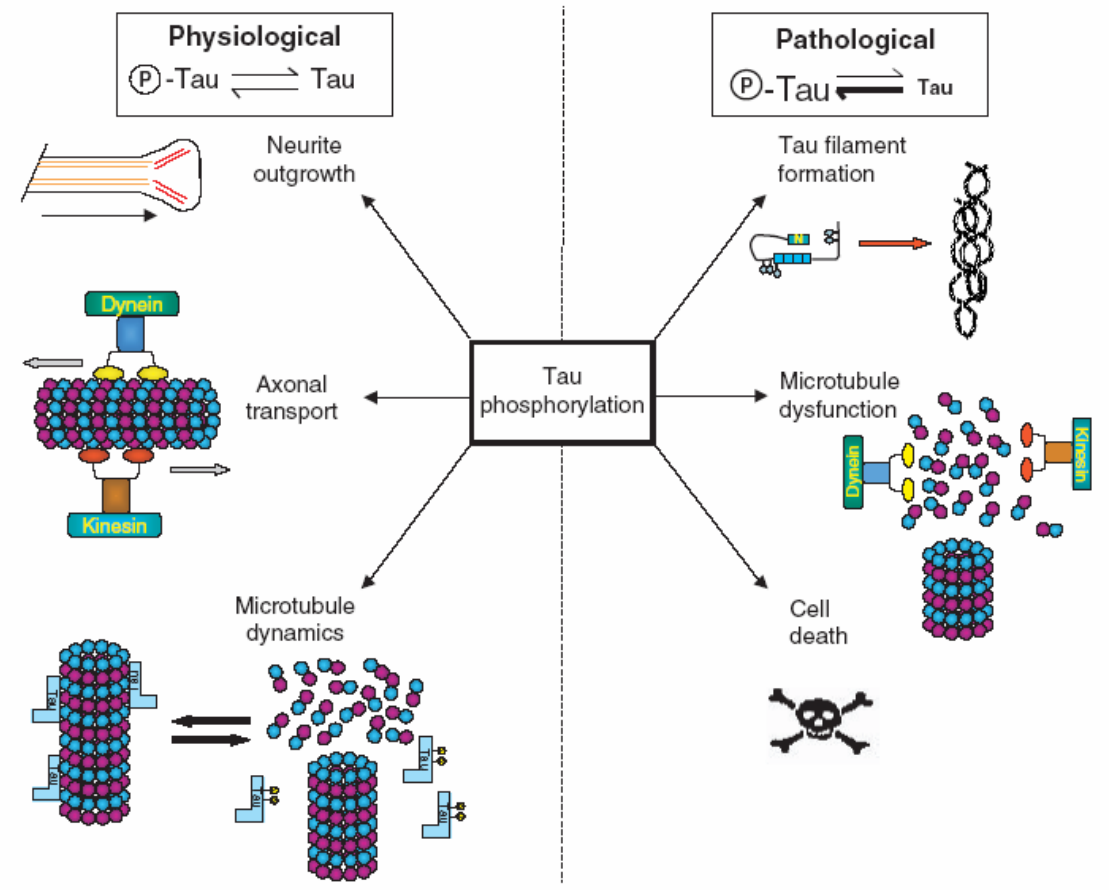

Figure 16 Physiological and pathological roles of tau protein in the neuronal cells.

The modification of tau by phosphorylation affects its interaction with microtubules, and indeed, hyperphosphorylated tau is the major component of paired helical filaments (PHF) or straight filaments that form neurofibrillary tangles (NFT's).

Formation of NFTs is an early event in the dementia cascade, and the number of NFTs correlates with disease severity. The accumulation of abnormal tau filaments into tangles is a hallmark of the tauopathies. In a subset of these disorders, similar tau aggregates also accumulate in glial cells in structures called glial fibrillary tangles (GFPs). The most typical example of a tauopathy is Alzheimer's disease, in which two main pathological structures form in the brains of patients: senile plaques (composed of the β-amyloid peptide), and NFT. The formation of PHF from tau molecules may follow different steps and could involve tau phosphorylation, a conformational change in the protein, and finally polymerization. The cytotoxicity mediated by tau in AD could be due to its hyperphosphorylation associated with the induction of apoptotic cell death. Aggregated tau also accumulates in progressive supranuclear palsy (PSP), corticobasal degeneration, frontotemporal dementia with parkinsonism-chromosome 17 type (FTDP-17), Pick's disease (PiD), Down syndrome, postencephalitic Parkinsonism, Niemann Pick's disease, and numerous other neurodegenerative diseases. NFTs are also prominent in normal aging, but to a diminished extent compared to disease states. The aggregated tau found in these different neurodegenerative diseases is hyperphosphorylated relative to tau in non-disease adult brains, and the disease phosphorylation pattern resembles that found in fetal brain. Normal tau proteins, infact, are also phosphorylated in fetal and adult brain, but they do not aggregate to form filamentous inclusions. Moreover, non-phosphorylated recombinant tau form filamentous structures under physiological conditions *in vitro*, when sulfated glycosaminoglycans are or other polyanions present. These data suggest that, in addition to phosphorylation, other mechanisms may be involved in the formation of pathological tau filaments (**Gail V et al 2004; Jian-Zhi Wang et al 2008**).

Molecular classification of Tauopathies

Comparative biochemistry of tau aggregates in brain tissue from patients affected by different tauopathies showed a differentiation in both phosphroylation and content of tau isoforms, wich enable a molecular classification of tauopathies. Five classes and four different electrophoretic patterns of tauopathies have been defined depending on the type of tau aggregates that constitute the "Bar Code" (**Fig17**).

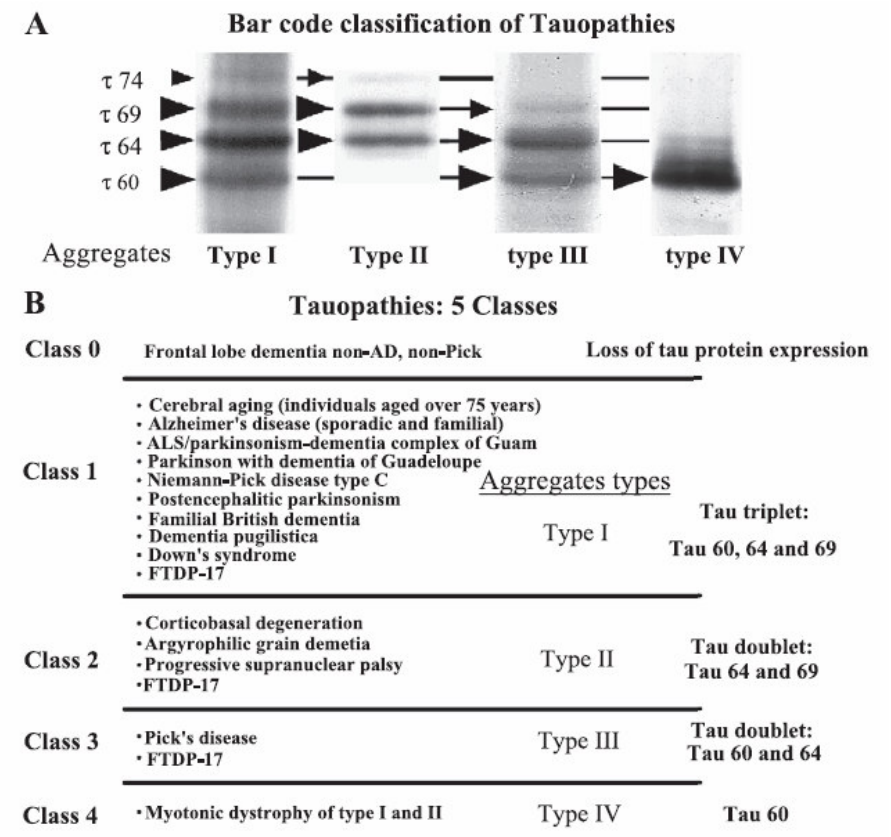

Figure 17 The bar code of tauopathies and their classification

These are composed of pathological tau bands at 60, 64, 69 and 74 kDa which correspond to pathological tau found in aggregates (**N. Sergeant et al, 1997; M. Goedert, M.G. Spillantini, N.J. Cairns et al, 1992**). The τ60 is composed of shortest tau isoforms. T64 and τ69 are a mix of tau isoforms with exon 10 or exon 2 alone, and

exon 2 +10 or exon 2+3 respectively. However, a τ68 to τ72 KDa component, according to their degree of phosphorilation, is present in only very low amounts and correspond to the longest tau isoform (2+3+10+). No tau aggregates are observed in Class 0. Type I or class I is characterized by the presence of the four pathological tau components (τ60, τ64, τ69, τ74), whereas Type II and III include two major pathological tau components at 64 and 69 kDa or 60 and 64 kDa, respectively. Finally, type IV is characterized by a strong pathological tau component at 60, 64 and 69 KDa components, is observed depending on the severity of the affected region analyzed (**N. Sergeant et al, 2005**)

Recent developments have shed light on the significance of tau phosphorylation and aggregation in pathogenesis. Furthermore, emerging evidence reveals the central role played by dominant mutations in the *MAPT* gene and tau pre-mRNA processing in tauopathies.

MAPT gene mutations

For most tauopathies, the role of tau aggregation in the initiation and progression of neurodegenerative disease is unknown. However, the discovery of missense and splice site mutations established that abnormal regulation of *MAPT* gene or abnormal tau protein could trigger neurodegeneration.

To date, 44 different potential pathogenic *MAPT* mutations have been reported in a total of 117 tauopathy families (**AD and FTD Mutation Database http://www.molgen.ua.ac.be/FTDMutations**). With the exception of two mutations in exon 1 (**Hayashi S et al, 2002; Poorkaj P et al 2002**), all coding mutations are clustered in exons 9 to 13, which encode the microtubule binding domains and its flanking regions (**Fig 18**). The intronic mutations are all located in the introns flanking the alternatively spliced exon 10.

MAPT mutations can be divided into two groups depending on the primary disease mechanism involved: (1) missense or deletion mutations that commonly modify tau interaction with microtubules, and (2) splicing mutations that affect the alternative splicing of exon 10, leading to changes of the ratio of 3R-tau/4R-tau. However, a third group mutations exists that might have effects at protein and RNA levels.

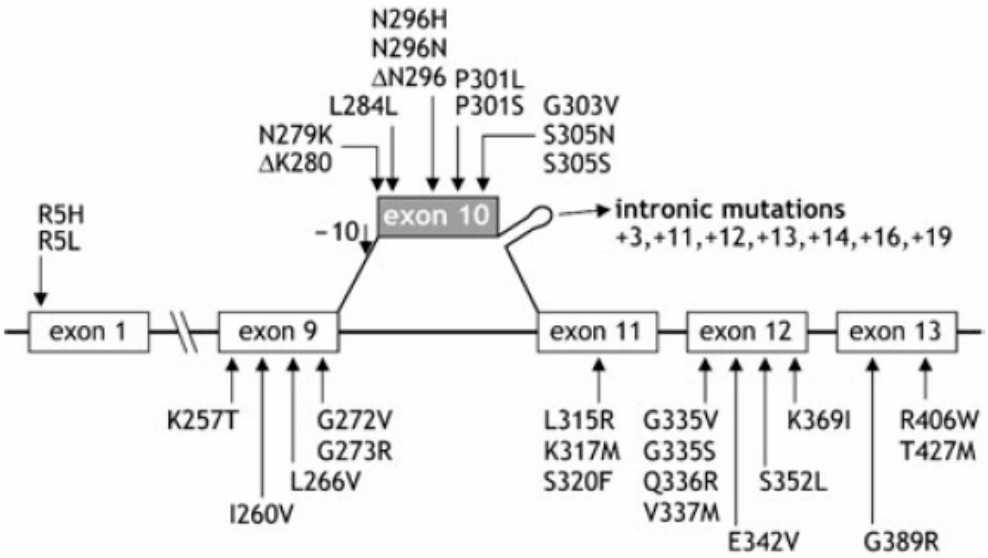

Figure 18 Overview of *MAPT* gene mutations

Mutation altering the tau-MT interaction

In vitro studies have demonstrated that coding-region mutations in exons 9, 11, 12 and 13 of MAPT gene, disrupt tau-MT interactions reducing the ability of tau to promote MT assembly. This effect is present in all six tau isoforms (**Rizzini, C. *et al.* 2000; Grover A et al, 2003; Hogg et al, 2003; van Herpen E et al, 2003; Zarranz et al, 2005; Rosso et al, 2002; Neumann M et al, 2005; Giaccone G et al, 2005; Hong M et al, 1998; Pickering-Brown S et al, 2004; Hasegawa M et al, 1998; Lippa et al, 2000; Nicholl et al, 2003; Neumann M et al, 2001; Murrell, J.R. et al. 1999**). A reduction in MT assembly has also been reported for mutations located outside the MT binding domain of tau, that is exon 1 mutations (**Hayashi S et al, 2002; Poorkaj P et al 2002**). The effect of exon 1 mutations on MT could possibly be mediated by a conformational change in the tau amino-terminal projection domain, leading to alteration of tau trafficking and/or compartimentalisation, affecting tau interaction with MT and possibly altering the regulation of MT dynamics.

The Q336R mutation in exon 12 represents an exception, due to its slightly increased ability to promote MT assembly (**Pickering-Brown SM et al, 2004**).

E10 mutations share the same mechanism but affect the biochemical properties of only 4R isoforms (**D'Souza et al 1999; Grover A et al, 2002; Spillantini et al, 2000; Hasegawa M et al, 1998; Ros et al, 2005; Stanford et al, 2000)**

The reduction in MT binding increases the pool of unbound tau in the neuron, which is subsequently available for pathologic aggregation.

Mutation altering the tau mRNA splicing

Exon trapping experiments has shown that mutations in intron 9 as wells as in intron and exon 10, except those at codon 301 (P301L and P301S), are considered splicing regulation mutations that disturb the normal splicing balance of exon 10, usually resulting in more inclusion of exon 10 with an increased 4R/3R tau ratio as the final outcome **(Rizzu, P. *et al.* 1999; Spillantini MG, Murrell JR et al 1998; D'Souza et al, 1999; Varani et al, 1999; Neumann et al. 2005; Miyamoto et al, 2001; Hutton et al, 1998; Hong et al, 1998; Malkani et al, 2006)**

Only the +19 and +29 mutation have been reported to reduce exon 10 splicing leading to an increase of 3R tau (**Stanford PM et al, 2003**). The +29 change was reported as a rare polymorphism, before being considered a mutation (**D'Souza I et al, 1999; Stanford PM et al, 2003**). The family carrying the +29 mutation has now been found to have a *Progranulin* mutation (**Pickering-Brown SM et al, 2006**), establishing that it is a benign polymorphism.

The primary effect of the intronic mutations is exerted at the level of RNA splicing and leads to altered expression of tau isoforms (**Lee VM et al, 200; Stanford PM et al, 2003; Clark LN et al, 1998; Hutton M et al, 1998;; van Swieten JC et al, 2007**).

Mutation altering Tau-MT interaction and tau RNA splicing

Several missense mutations locate in exon 10 (ΔK280, ΔN296, N296H and S305N) potentially exert their effects at both protein and RNA levels. Mutations ΔK280, ΔN296 N296H reduce (**Grover A et al, 2002; Iseki E et al, 2001; Yoshida H et al 2002**), but S305N (**Hasegawa M et al 1999**) stimulates the ability of tau to promote MT assembly *in vitro*.

The N296H and S305N mutations increase the splicing-in of exon 10 and lead to an overproduction of 4R tau (**Grover A et al, 2002; Hasegawa M et al 1999)**

It has been reported that the ΔN296 mutation leads either to increased (**Yoshida H et al 2002**) or unaltered (**Grover A et al, 2002**) exon 10 splicing but ΔK280 give rises to an overproduction of 3R tau isoforms reducing the splicing-in of exon 10. A mutation in exon 12 (E342V) also affect tau at the RNA and protein levels. This mutation reduces the ability of tau to promote MT assembly *in vitro* and seems to affect the splicing-in of exon 10 **(Lippa et al, 2000)**.

Regulation of exon 10 alternative splicing: cis-acting factors

MAPT exon 10 has two weak splice sites, a weak 5' splice and a weak 3' splice site. The exon is flanked by unusually large intron 9 (13.6 kb) and intron 10 (3.8 kb). These features of *tau* exon 10 lead to much complicated regulation. Several short *cis*-elements in exon 10 and intron 10, which modulate the use of the weak 5' and 3' splice sites, have been identified and extensively characterized. The 5' end (first 45 nucleotides) of exon 10 contains three non redundant exon splicing enhancer (ESE): a SC35-like enhancer, a polypurine enhancer (PPE), and an A/C-rich enhancer (ACE) sequence. Following the ESEs region, there is an exon splicing silencer (ESS). In addition, two regulatory elements within exon 10 are also present, an AT rich silencer and a purine-rich enhancer directly downstream of the silencer that binds (**Wang et al., 2005; Jiang et al., 2003**).

The 3' end of exon 10 contains another ESE sequence between the ESS and the 5' splice site. Immediately downstream of the 5' splice site in intron 10 is present an intronic bipartite regulatory sequence composed of the intron splicing silencer (ISS) and the intronic splicing modulator (ISM) elements. Deletion assay revealed opposite effects of the ISS and ISM on E10 splicing (**Fig 19**).

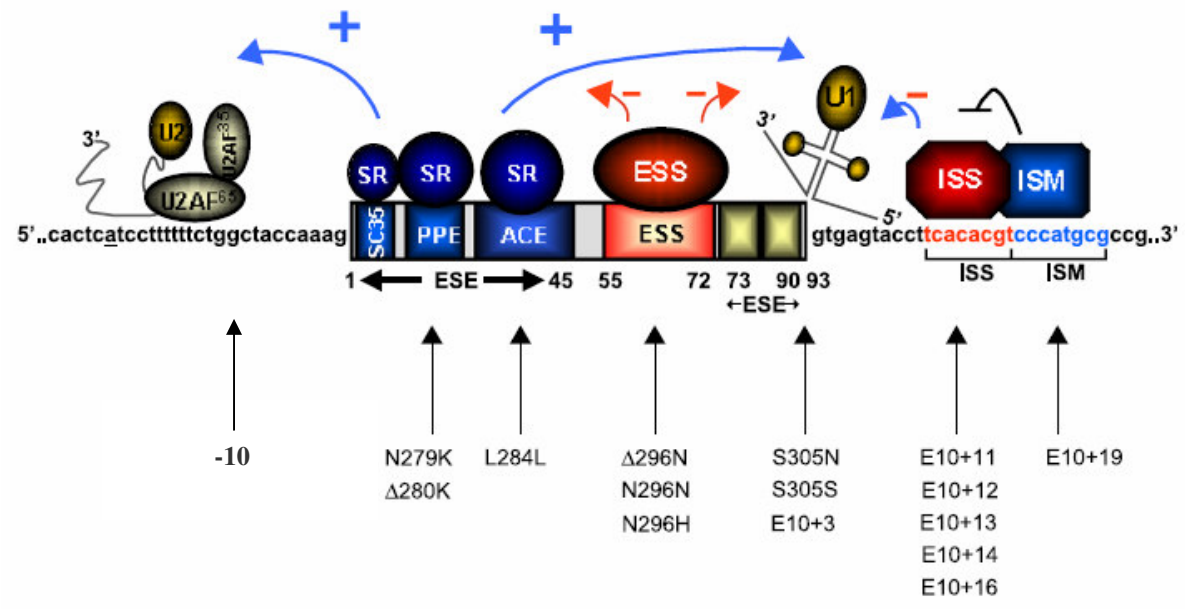

Figure 19 Model for *MAPT* Exon 10 splicing regulation showing a network of positive (blue arrows) and negative interactions (red arrows) Known disease mutations are shown at the bottom with vertical arrows pointing toward the specific regulatory element which they affect.

The ISM is not an enhancer by itself, but functions only in the presence of the ISS and counteracts ISS-mediated inhibition of the 5' splice site. Mutations in these elements may disrupt their function in alternative splicing of exon 10. A total of 15 mutations within the six elements (PPE, ACE, ESS, ESE, ISS and ISM) have now been identified in individuals with tauopathies. These mutations include N279K and Δ280 in PPE; L284L in ACE; N296H, N296N and Δ296N in ESS; P301S G303V in ESE; E10+11, E10+12, E10+13, E+10+14 and E10+16 in ISS; and E10+19 in ISM. They all alter the alternative splicing of exon 10 by either promoting or inhibiting exon 10 inclusion. In particular mutations N279K and L284L strengthen an exon splicingenhancer element located in the 5′ region of exon 10, resulting in increased levels of exon 10-containing mRNA and soluble 4R tau (**D'Souza I et al 2002; D'Souza I et al 1999**). The same mechanism has recently been found for a mutation located at position –10 of the intron that precedes exon 10 (**Malkani R et al 2006**). The silent mutation N296N leads to increased levels of exon 10- containing transcripts by disrupting an exon splicing-silencer (**D'Souza I et al 2002; Spillantini MG et al 2000**) or by creating an exon

splicing-enhancer sequence (**Grover A et al, 2002**). Conversely, it has been reported that the +19 mutation increases the splicing out of exon 10 by altering a splicing silencer sequence (**D'Souza I et al 2002; Stanford PM et al, 2003**).

In addition to the regulatory sequences (*cis*-elements) within exon 10 and intron 10, distal exonic sequences appear to affect exon 10 splicing of tau as well. Disease related mutations within exon 9 and exon 12 are reported to alter exon 10 splicing. However, how and by which mechanism these distal sequences regulate exon 10 splicing remain to be elucidated.

- **Alternative splicing regulation at the Exon 10 5′ splice site: Stem loop theory**

Exon-intron interface at the 3' end of exon 10 displays a high degree of self-complementarity, suggesting the presence of a RNA stem loop structure. Theoretical modelling of the *MAPT* pre-mRNA sequence by two different groups predicted the presence of a stem loop RNA structure spanning this splice site **(M. Hutton et al, 1998; Spillantini MG and Murrel JR et al, 1998**). Both groups proposed the theory that *MAPT* exon 10 5′ splice site mutations destabilize the stem–loop, thereby facilitating access of the splicing machinery to the splice site and increasing the splicing in of exon 10. Mutations within the predicted stem increase inclusion of exon 10 in transcripts, while mutations generated in the putative loop have no effect on splicing.

The +3 intronic mutation was also predicted to increase the binding of U1 small nuclear ribonucleoprotein (snRNP) to the 5′ splice site leading to increased inclusion of exon 10 and excessive production of 4R tau **(Spillantini MG and Murrel JR et al, 1998)**. However, the stem–loop structures predicted by the two groups differ both in the number of nucleotides in the loop and in the length of the stem. A subsequent study by Varani and co-workers, using NMR spectroscopy of RNA oligonucleotides, confirmed the existence of a stem–loop, but suggested an alternative structure to the two that had been proposed previously (**L. Varani et al 1999**). This proposed structure consists of a single stable G–C base pairing separated from a double-helix stem of six base pairs by an unpaired adenine, and an apical loop of six base pairs (**Fig 20**). In the same NMR study, it was also confirmed that the stability of the stem–loop was reduced by known

intronic mutations, leading to an increase in the number of transcripts containing exon 10 or 4R tau.

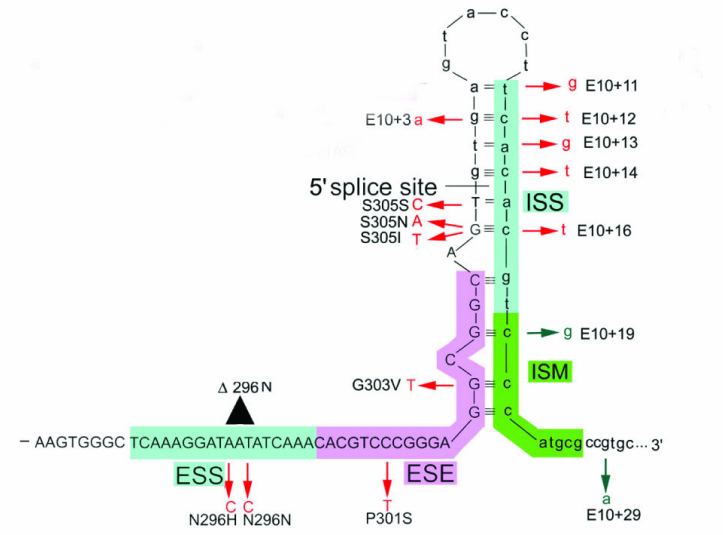

Figure 20 Interface between exon 10 and intron 10. Mutations that cause an increase (red), decrease (dark green) or not yet known change (black) in the ratio of 4R/3R-tau are indicated. Triangle indicates deletion mutation.

In rodents, this stem-loop structure is destabilized by the transition A to G at position E10+13, which is also seen in FTDP-17. This replacement might explain why adult mice and rats express 4R tau predominantly in their brains. Furthermore, recent studies have demonstrated that stem loop variants that increase the stability of the stem loop result in decreased inclusion of exon 10 and increased expression of 3R tau (**Christine P. Donahue et al, 2006**). Although the secondary structures can be observed in *in vitro* experiments, the existence of this stem loop under physiological conditions and its role in splice regulation are controversial because there are multiple splicing regulatory proteins that coat the pre-mRNA possibly preventing secondary structures from forming. Importantly, regulation through the stem–loop structure does not necessarily allow for regulation by trans-acting factors that may affect differential splicing seen throughout development.

- **Alternative splicing regulation at the 5′ splice site–Linear sequence theory**

An alternative hypothesis is that the inhibitory sequence in intron 10 is a linear cis-acting element that binds trans-acting splicing factors (**D'Souza I et al 1999; D'Souza I et al 2000; D'Souza I et al 2002**). Some of the mutations that destabilize the stem loop structure may also function to increase exon 10 inclusion by increasing affinity for the U1 and U6 snRNPs which have both been implicated in exon 10 splicing inclusion.

A proposed intronic silencer at position exon 10+11 to exon 10+18 retains its function even after it has been translocated to different positions, including a position within exon 10 as well as a heterologous setting, where the complementary bases are not present to form a stem–loop structure. An intronic splicing modulator located at position exon 10+19 to exon 10+26, acts in conjunction with the intronic splicing silencer to regulate the splicing of exon 10. This splicing modulator is disrupted by the exon 10+19 mutation and results in increased 3R tau isoforms. These two elements seem to act in opposition and it has been suggested that trans-acting factors that bind the modulator sterically hinder other trans-acting factors from attaching to the silencer, which in turn allows access to the 5′ splice site.

Regulation of exon 10 alternative splicing:Trans-acting factors

Alternative splicing is highly regulated by *trans*-acting factors in addition to *cis*-elements. These splicing factors are divided into two major groups, hnRNPs (heterogeneous nuclear ribonucleoproteins) and SR (serine/arginine rich)/SR-like proteins. Both of them are involved in alternative splicing (**Dreyfuss G et al, 2002; Graveley BR et al, 2000**). SR/SR-like proteins are components of spliceosome. In addition, hnRNPs are also involved in pre-mRNA transport, RNA stability and translational regulation. SR proteins are highly conserved in eukaryotes. They are characterized by containing one or two RNA-recognition motifs at the N-terminus, which determine RNA binding specificity, and an arginine-serine-rich (RS) domain at the C-terminus, which promotes protein-protein interactions within the splicing complex (**Caceres JF et al, 1997; Zahler AM et al, 1992**). They are essential for both constitutive splicing and alternative splicing. For constitutive splicing, SR proteins are

required for the formation of the early prespliceosomal complex to stabilized U1 snRNP article and U2AF (**Eperon IC et al, 1993; Krainer AR, Maniatis T et al, 1985**). In alternative splicing, SR proteins function in modulating 5' splice site in a concentration-dependent manner.

SF2/ASF (splicing factor 2/alternative splicing factor) is a well-studied SR protein. It binds to PPE enhancer of exon 10 and plays essential and regulatory role in tau exon 10 splicing (**D'Souza I et al, 2006**). FTDP-17 mutations N279K and Δ280 K alter the normal PPE sequence by adding or removing an AAG copy and lead to increase or decrease in the binding ability of SF2/ASF, resulting in exon 10 inclusion and exclusion, respectively. In addition recent study, based on minigene model system, showed the roles of many SR proteins in exon 10 splicing.

SRp30c and SRp55, which inhibit inclusion of exon 10, bind the AT rich silencer within exon 10 (**Wang et al., 2005**). htra2beta1 which activates inclusion of exon 10 purine-rich enhancer directly downstream of AT rich silencer (**Jiang et al., 2003**). 9G8 directly interacts whit the proximal downstream intron of exon 10 and inhibit inclusion of exon 10 (**Lei Gao et al, 2007**).

SR proteins that bind the same region give rise to different E10 regulation. For istance human Tra2β was demonstrated to interact with the PPE region at 5′ side of *tau* exon 10 and to stimulate its inclusion (**Shinichi Kondo et al, 2004**). In contrast SRp54 binds the same region but enhances the exon 10 exclusion. It is shown that SRp54 competes with Tra2β for binding to PPE exonic element and then antagonizes its activity (**Jane Y. Wu et al, 2006**). It should be pointed that all these studies were carried in cultured cells, and some observations are contradictory. Variations in the minigene size used, various types of cells with different compositions and levels of endogenous splicing factors as well as SR protein kinases and phosphatases, and different stages of cells may contribute to the inconsistent results among studies.

- **Phosphorylation of SR proteins**

The RS domain of SR proteins is extensively phosphorylated on serine residues, and phosphorylation plays an important role in regulating their nuclear activities. To date, multiple kinases, including SR protein kinase 1 (SRPK1) (**Gui JF et al, 1994**), SRPK2

(**Wang HY et al, 1998**), cdc like kinase (Clk/Sty) (**Colwill K et al, 1996**), DNA topoisomerase I (**Rossi F et al 1996**), cAMP-dependent protein kinase (PKA) and AKT (**Kvissel AK et al, 2007 and Patel NA et al, 2005**), have been shown to phosphorylate the RS domain. Phosphorylation of the RS domain of ASF/SF2 promotes its interaction with pre-mRNA and other splicing factors and regulates the shuttling crossing nuclear membrane (**Colwill K et al, 1996; Ngo JC et al, 2005; Xiao SH et al, 1998**) . It has been shown that phosphorylation of ASF by SRPK1 drives it from cytosol into the nucleus and by Clk/Sty causes its release from speckles, the storage compartment of inactive SR proteins (**Ngo JC et al, 2005; Koizumi J et al,1999**) . Thus, both SRPK1 and Clk/Sty help recruit ASF into nascent transcripts, resulting in enhancement of its role in regulation of alternative splicing. In the case of tau exon 10 splicing, activation of SRPK1 and Clk/Sty could increase the nuclear concentration of active ASF/SF2 that might result in an increase in exon 10 inclusion.

Recently, it has found that dual-specificity tyrosinephosphorylated and regulated kinase 1A (Dyrk1A), a critical kinase linked with DS, also phosphorylates ASF/SF2 at sites rather than those by SRPK and Clk/Sty and drives ASF/SF2 into speckles, resulting in suppression of its promotion in exon 10 inclusion.

The activity of SC35, which promotes tau exon inclusion, is regulated by phosphorylation with glycogen synthase kinase-3β (GSK-3β), a protein kinase that may be involved in the pathogenesis of AD (**Takashima A et al, 2006**). Inhibition of GSK-3β activity in cultured neurons caused an increase in tau exon 10 inclusion (**Hernandez F et al, 2004**). However, the splicing competency of GSK-3β-phosphorylated SC35 in general or on tau is unknown. This issue is especially relevant because GSK-3β might be up-regulated in AD brain (**Pei JJ et al, 1997**) and a SC35-like ESE at the 5' end of tau exon 10 appears essential for exon 10 splicing (**D'Souza I. et al, 2000**)

Disruption of *tau* exon 10 splicing in tauopathies

Most *MAPT* mutations that affect exon 10 alternative splicing give rise to an overproduction of 4R tau that may lead to an excess of free 4R tau. As 4R tau appears likely to aggregate more readily than 3R tau, this could drive tau aggregation, resulting

in the formation of tau filaments and associated cellular dysfunction as seen in neurodegenerative disorders known as tauopathies. In addition to FTDP-17, dysregulation of *MAPT* exon 10 splicing in both familiar and sporadic cases may also contribute to other human neurodegenerative disorders, such as PiD, PSP, and corticobasal degeneration. Some tau gene mutations can cause hereditary PiD and PSP (**Bronner IF et al, 2005; Neumann M et al, 2001; Pickering-Brown S et al, 2000; Ros R et al, 2005**). Only 3R-tau inclusions were previously found in the brains of both familial and sporadic cases of PiD. However, several groups recently observed 4R-tau inclusions as well, suggesting that a disruption of 3R-tau/4R-tau ratio at either directions may contribute to PiD. Changes of 3R-tau/4R-tau ratio are also seen in PSP and corticobasal degeneration, in which 4R-tau is up-regulated in majority of the cases (**Yoshida M et al, 2006**). In FTDP-17, the altered *MAPT* exon 10 splicing is the result of tau mutations at the cis-elements that regulate the splicing, though the detailed mechanisms might be different in different mutations. Much less is known about the mechanisms by which the 3R-tau/4R-tau ratio is altered in other tauopathies. Further investigation on the mechanisms will help identify new therapeutic targets for the treatment of those tauopathies caused or contributed by disruptions of 3R-tau/4R-tau balance. How the imbalance of 3R-tau/4R-tau causes or contributes to neurofibrillary degeneration and dementia is currently not understood. Since equal levels of 3R-tau and 4R-tau appear to be essential for normal function of the mature human brain, it is possible that the 1:1 ratio of 3Rtau/ 4R-tau bound to MTs is required for maintaining the normal dynamics of MTs in mature neurons. Because the MT-binding and MT assembly activity of 3R-tau is smaller than that of 4R-tau (**Goode BL et al, 1994; Goode BL et al, 2000; Alonso AD et al, 2001; Lu M et al, 2001**), any changes of the 3R-tau/4Rtau ratio could alter the MT dynamics and cause problems in the neuron. It is also possible that in the mature neuron, 3R-tau/4R-tau only at an 1:1 ratio bind to MTs. Access amounts of either 3R-tau or 4R-tau due to disrupted tau exon 10 splicing could resulted in increased concentration of free 3R-tau or 4R-tau in the cytoplasm. Compared to MT-bound tau, free tau is more vulnerable for hyperphosphorylation and aggregation into NFTs (**Sengupta A et al, 2006**).

AIM OF THE STUDY

In the present study we report the molecular effect of two novel heterozygous *MAPT* gene mutations identified in a patient with a sporadic Frontotemporal Dementia (FTD), clinically and neuropathologically ascertained.

Sequence analysis of the *MAPT* gene of the index patient showed a T to C transition at position -15 of intron 9 [T(-15)C] and an A to C transversion at position +4 of intron 10 (E10+4).

It is known that mutations in intron 9 as wells as in intron 10 and exon 10, except those at codon 301 (P301L and P301S), are considered splicing regulation mutations. These mutations modifying regulatory sequences disturb the normal splicing balance of exon 10, usually resulting in more inclusion of exon 10 with an increased 4R/3R tau ratio as the final outcome. Thus the aim of this study was to investigate the molecular effect of both mutations on exon 10 alternative splicing in a minigene model system and in index patient's brain tissue.

Although the molecular mechanism underlying MAPT exon 10 splicing regulation remain to be completely elucidated, the exon 10 splice donor site is predicted to give rise to a RNA stem loop structure considered crucial for the quantitative regulation of exon 10 alternative splicing. Considering that the E10+4 mutation is located into the exon 10 splice donor site, we also performed experiments to test if the E10+4 mutation affects the thermostability of the RNA stem loop structure giving rise to an altered alternative splicing regulation.

MATERIALS AND METHODS

Clinical description

The index patient, a 46 year-old woman, presented with progressive personality changes and behavioural disturbances (apathy, disinhibition, lack of critique and judgement and absence of insight to the Regional Neurogenetic Centre, AS6-Lamezia Terme (Calabria Southern Italy). Symptoms progressed with memory loss, strong reduction of verbal fluency evolving to complete mutism, behavioural stereotypies, episodic myoclonus, urinary incontinence, hyperorality. Neurological examination, normal in the early periods, successively evidenced amimia, akinesia, right deviation of the head and of the eyes, upper and lower limbs spasticity, hyperreflexia, supraspinal signs. No amyotrophy was present (normal EMG examination). FLAIR MRI brain scanning showed massive fronto-temporal cortical atrophy with enormous ventricular enlargement. Death occurred at 57.

Genetic screening and Pedigrees

Genetic analysis were performed at the Regional Neurogenetic Centre. Informed consent has been obtained from all subjects participating in the study and of the legal guardian of the patient.

Genomic DNA was extracted from peripheral blood buffy-coats using standard phenol-chloroform procedures. Coding exons of *MAPT* gene and intronic sequences flanking exons 9-12, coding exons and non coding exon 0 of *PGRN* gene, coding exons of *PSEN1* (3–12) and *PSEN2* (3–12) genes and exons 15–18 of the APP gene were amplified by polymerase chain reaction (PCR) using specific primers, as previously described (**Cruts et al., 2006, 1998; Hutton et al., 1996; Goate et al., 1991**). A total of 200 ng of genomic DNA was amplified in a 50µl reaction mixture containing 20 pmol of each primer, 0.2mMdNTPs, 1U Taq (Eppendorf AG, Germany), 1.5mM $Mg(OAc)_2$ and 1× buffer. PCR products were purified using a polymerase chain reaction purification kit (Nucleospin, Macherey-Nagel). For each exon, the purified product was sequenced in both directions using the Big Dye kit (PerkinElmer) and relevant PCR primers according

to the manufacturer's protocol. Sequencing was performed using an ABI310 automated sequencer.

The APOE genotyping was performed through PCR and *HhaI* (Invitrogen, Life Technologies) restriction enzyme digestion (**Hixson and Vernier, 1990**). Three polymorphisms in introns 2 (+18 C/T), 9 (insertion/deletion *Δ*238), and 13 (+34 T/C) of *MAPT* gene were used to reconstruct the extended Tau haplotypes H1 and H2 (www.alzforum.org/res/com/mut/tau/taupolytable.asp).

Sequence analysis of the *MAPT* gene of the index patient (II:1**;Fig.21**) revealed two novel heterozygous mutations: a T to C transition at position -15 of intron 9 [T(-15)C], and an A to C transversion at position +4 of intron 10 (E10+4). Sequencing of the *MAPT* gene in the genomic DNA of her healthy parents revealed that the father (I:1; **Fig.21)** was heterozygous for [T(-15)C], and the mother was heterozygous for (E10+4) (I:2**;Fig .21**).

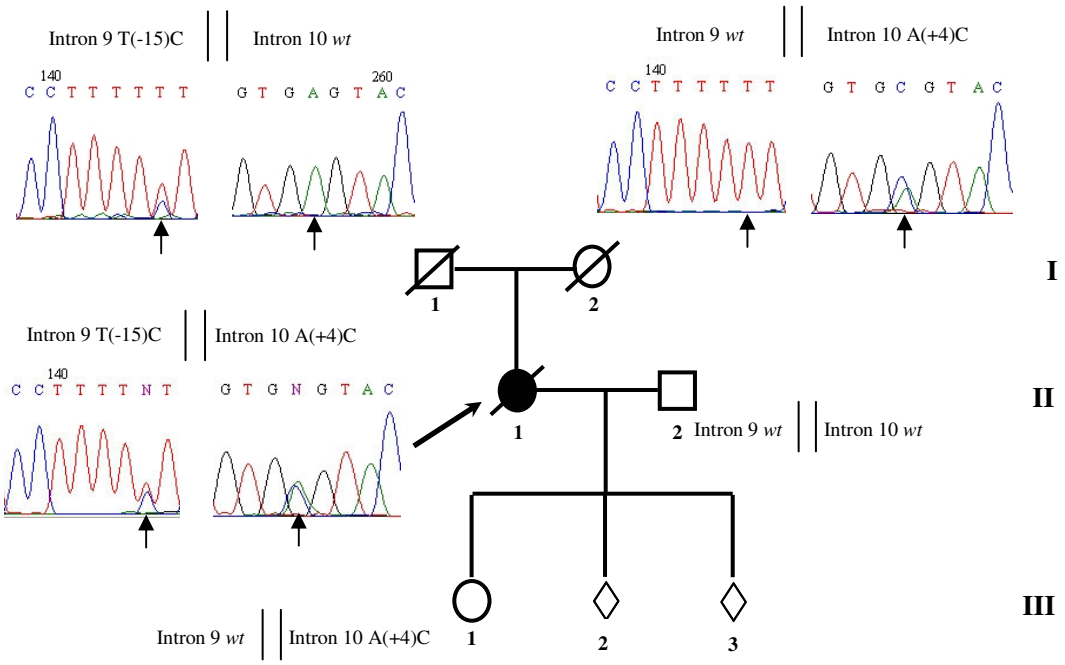

Figure 21 Family tree of the index patient. It is shown the segregation of T(-15)C and E10+4 mutations of *MAPT* gene in the family. The index patient (II-1) is compound heterozygous and showed FTD phenotype. The other family members are only heterozygous for either of the mutations and are cognitively healthy.

The mutations were not found in the genomic DNA of 138 patients with FTD used as control. *Tau* haplotype and ApoE genotype of the index patient were H1H2 and ε2/ ε3 respectively and no mutations were found in the *PSEN1*, *PSEN2*, *APP* and *PGRN* genes. Genetic analysis carried out on the other cognitively healthy family members (labelled with star in the pedigree in **Fig.22**) revealed that they were only heterozygous for either of the mutations. However only one sister (III:3;**Fig.22**) of the index patient is compound heterozygous for the two mutations but, to date, she has not shown neurological and psychiatric abnormalities.

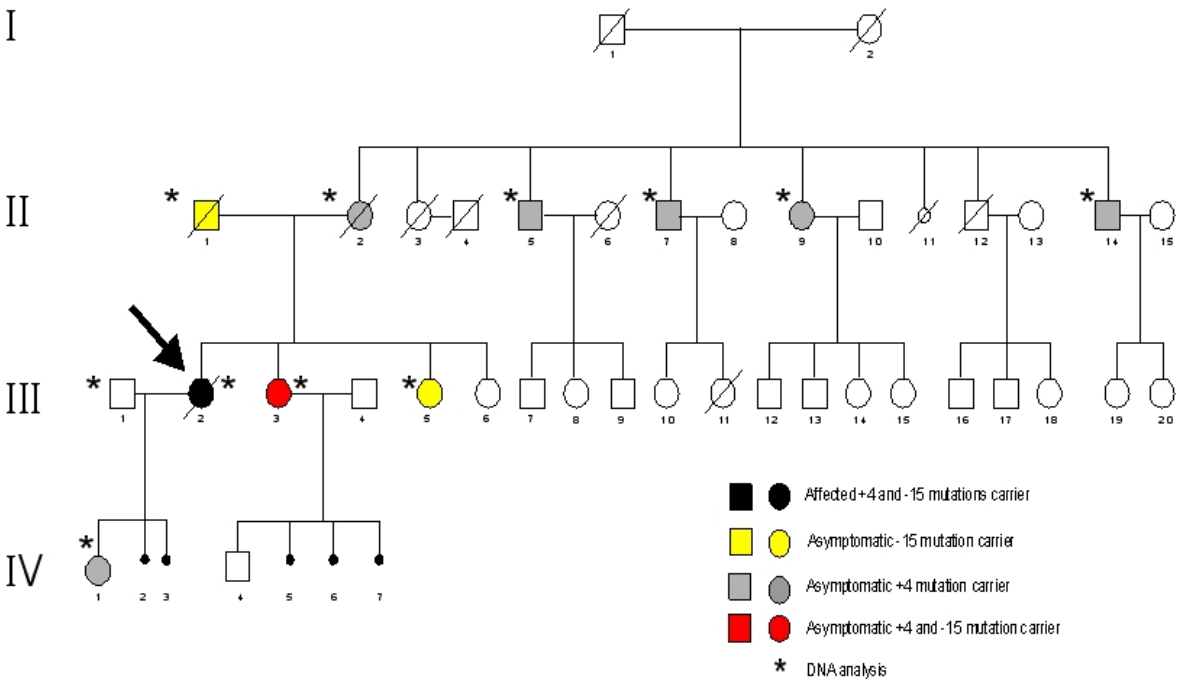

Figure 22 Pedigree of the index patient's family. Blackened symbols denote the compound heterozygousity of the index patient (-15,+4). Coloured symbols show: in yellow the asymptomatic -15 mutation carrier, in grey the asymptomatic +4 mutation carrier and in red asymptomatic -15 and +4 mutation carrier.

Neuropathology

Autopsy and standard neuropathological examinations were carried out in the Geriatric Unit AS3-Rossano (Calabria Southern Italy). Ethical approval was obtained for the use of all human tissues presented in this thesis.

Brain weighted 650 grams. Atrophy was sharply circumscribed and severe in the frontal, parietal and temporal lobes with relative sparing of Rolandic and calcarine regions (**Fig 23** A,B). The ventricles, especially the frontal and temporal horns of the lateral ventricles were severely dilated (**Fig 23** C). Hippocampus was destroyed (**Fig 23** D).

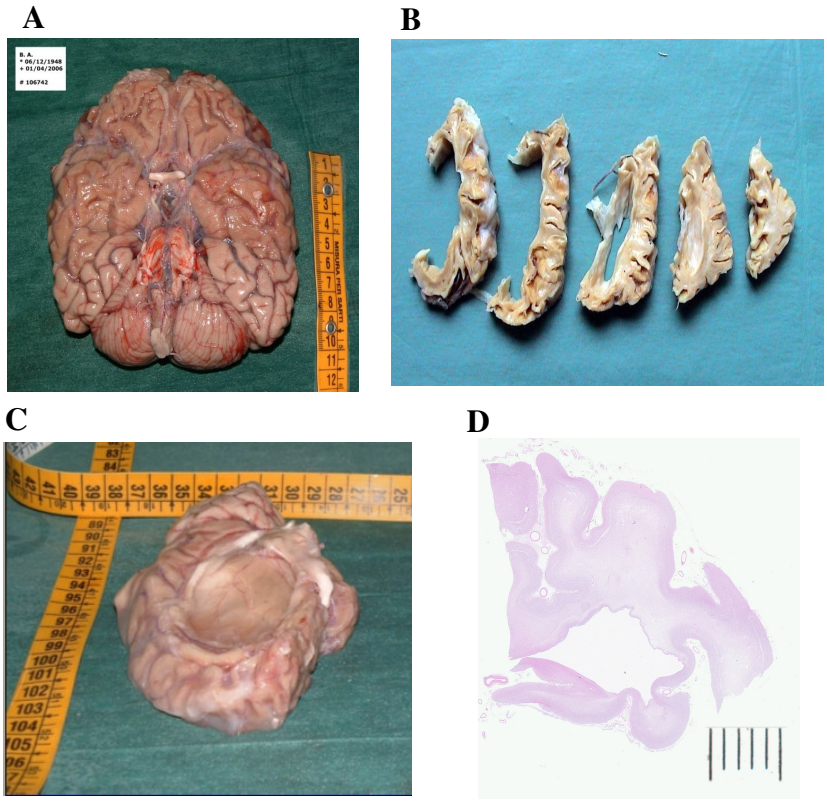

Figure 23 Neuropathological Findings in the index patient brain. (**A**) Severe frontotemporal cortical atrophy. (**B**) Sagittal sections of right frontal lobe.(**C**) Lateral Ventricle. (**D**) Destroyed. hippocampus

Histopathology evidenced nearly exceptional neuronal loss in all layers of frontotemporal and insular cortices with intense protoplasmatic gliosis, white matter

degeneration and relative sparing of primary motor and sensory cortices; area 17 was normal. The putamen was nearly normal, while the pallidus and thalamus were severely affected. The cerebellum and brain stem were globally spared, with the exception of substantia nigra and griseum central mesencephali, that showed severe neuronal loss.

Minigene Constructs

MAPT DNA's fragments, containing intact exons 9-11 as well as flanking Exon 10 intronic sequences of minimal length for a correct splicing, were amplified by PCR from the index patient and a FTD-ubiquitin positive (FTD-U+) genomic DNAs (**Qingming Y. et al, 2004**). Oligonucleotides were designed to intronic sequences flanking exons using the softwares Oligo Explorer and Oligo Analyser and containing different restriction enzyme sites at their 5' ends (**Tab 5**)**.** PCR primers were also designed with 1-2 extra nucleotides (red in Table) at their 5'-end adjacent to the recognition site for the restriction enzyme, considering that many restriction enzymes do not cut efficiently when their recognition sites are located close to the end of a PCR product.

Name	Sequence (5' to 3')
Int-9-For Int-9-Rev	EcoRI GGAATTCGGCTTCACTCCCTTCCTTCCTTCCCA KpnI GGGGTACCGCAGCCAGCCACTCTCACCTT
Int-10-For Int-10-Rev	KpnI GGGGTACCAATCCCAGCTTCGTAAAGCCCGCT BamHI CGGGATCCTCATCTGCCCTATTCTGTCCACACA
Int-11-For Int-11-Rev	BamHI CGGGATCCCTTCTCTCTCCTCCTCTCTCATCTCCA XbaI GCTCTAGATCCACAGGGCTACCTGGTTTAT

Table 5 Oligonucleotides (Invitrogen) used for minigene constructs

PCR reactions were performed using a high fidelity polymerases, either Pfu or Taq Plus Precise (Stratagene) according to the manufacturer's protocols in a Perkin Elmer GeneAmp PCR System 9700 thermocycler. PCR products were resolved with 0.5 µg/ml

ethidium bromide on 0.8-2% agarose gels in 1X TBE buffer [Tris 500.0mM, Boric acid 500.0mM, EDTA (pH 8.0) 10.0mM] and visualized under ultraviolet light (UV).

PCR products, purified with the QIAquick Purification kit (Qiagen), were sequenced on both directions using the BigDye Terminator Cycle Sequencing kit v3 (Applied Biosystems). Sequencing reactions were carried out in a total volume of 10µl containing 200ng of purified DNA, 3.2pmol of Forward or Reverse primers, 2µl of buffer5x, 4ul of sequenase kit [dNTPs, ddNTP, thermo sequenase Enzyme and Tris-HCl pH 9.0]. Amplification was performed for 25 cycles under the following conditions: denaturation at 94°C for 10 seconds, annealing at 55°C for 5 seconds, extension at 60°C for 4 minutes. The PCR products were purified using Centri-sep columns (Princeton Separation), dryed, resuspended in 18µl of Template Suppression Reagent (TSR) buffer and then runned on an ABI 310 DNA sequencer (Applied Biosystems). Electropherograms were analysed using the BioEdit Software.

1µg of each PCR fragments, purified and sequenced, were restricted at 37°C for 4h using the specific enzymes (10-12 U/µl) and 2µl of 10Xbuffer (50mM NaCl, 100mM Tris HCl, 10mM $MgCl_2$ 0.025% Triton X-100 pH 7,5) in a total volume of 20 µl. The fragments were then linked (at an equimolar ratio) through the restriction sites at the ends of PCR products at 5°C for 4h with 1U of T4 DNA ligase and 2µl of 5X buffer (250mM Tris-HCl/ pH 7.6/ 50mM $MgCl_2$/ 5mM ATP/ 5MM DTT 25% (v/v) glycole-polietilenico 8000). Exons 9-11 constructs (1075bp each) were then inserted (molar ratio 3:1) into a pcDNA3 expression vector, between the EcoRI and XbaI sites, under the control of the cytomegalovirus promoter and cloned into *E.coli* Top10 cells. Mutants and *wild-type* minigene constructs are detailed in *Results*. Constructs were validated by sequencing analysis.

pcDNA3 expression vector was a generous gifts from Prof Antonio Brunetti (Dipartimento di Medicina Sperimentale e Clinica, Università Magna Grecia, Catanzaro-Southern Italy).

Cells preparation and Transformation of *E.coli* Top10

20 ml of LB (Luria-Bertani) medium (pH 7.5, Bacto Triptone 1.0%, Yeast Extract 0,5%, NaCl 1.0%) were inoculated with a single colony of *E.coli* Top 10 (Invitrogen) and grown overnight at 37°C with vigorous shaking. 6 ml of the culture were transferred into 300 ml of L-Broth medium (Bacto Triptone 1.0%, Yeast Extract 0.5% e NaCl 0.5%) containing streptomicin (c.f.=50µg/ml) and grown to early logarithmic phase (OD_{600} =0.6). The culture was then centrifuged at 4000 rpm for 20 min at 4°C and the pellet washed three times at 5000 rpm for 25 min. The cells were then resuspended in 5ml volume of cold 10% glycerol, aliquoted and stored at -80°C.

Transformation was assayed using 20µl of electrocompetent E.*coli* Top 10 cells with 1ng of pcDNA3 vector or ligation reactions in a gene pulser Biorad set to 250Kv and 200Ω (**Dower W.J. et al, 1998**). In order to select trasformant Amp^r colonies, the cells were plated on LB medium supplemented with ampicillin and streptomicin (c.f.=50µg/ml) after an incubation at 37°C for 45'. Plasmid DNAs, isolated from the resulting clones by alkaline lysis (**Birnboim H.C. and Doly J., 1979**), were digested to check for the appropriate size bands and sequenced.

Site-directed mutagenesis

Site direct mutagenesis was performed in order to obtain minigene constructs carrying the E10+3 mutation. Mutagenic oligonucleotides (Invitrogen) b (Mut-3-Rev) and c (Mut-3-For) and flanking oligonucleotides a (Int-9-For) and d (Int-11-Rev) (Tab.5) were used to generate intermediate PCR products ab and cd that are overlapping fragments of the entire product ad (**Karin L Heckman and Larry R Pease 2007**). Final product AD, drived in PCR 2 by primers a and d was inserted into pcDNA3 expression vector to generate larger quantities of DNA and sequenced to ensure the presence of the desired mutation (**Fig.24**).

Mut-3-For (c) 5'-CGGCAGTAAGTACCTTCACACACGTCCCAT-3'

Mut-3-Rev (b) 5'-ATGGGACGTGTGAAGGTACTTACACTGCCG-3'

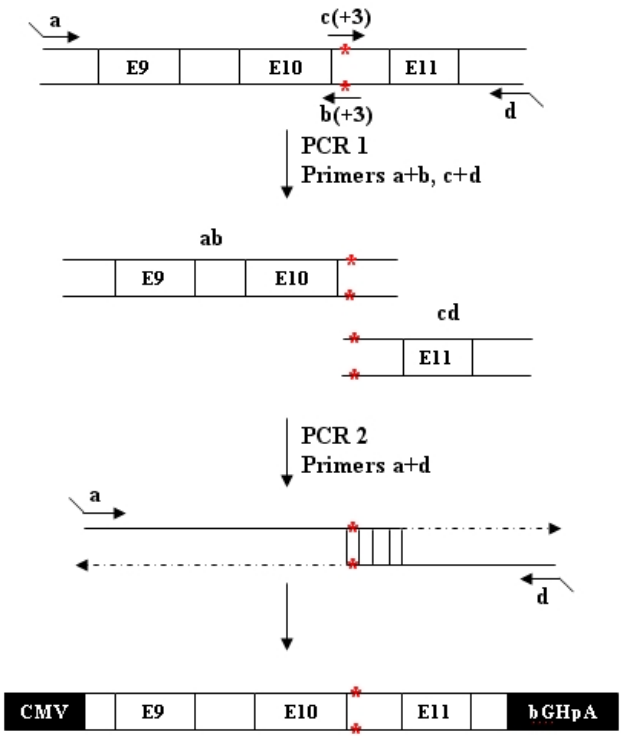

Figure 24 Site direct Mutagenesis using mutagenic oligonucleotides. Mutation E10+3 is indicated by the red asterik.

Analyses on the thermodynamic stability of the RNA stem loop structure

Bioinformatic Analysis

Thermodynamic analysis was performed on the first 25 nucleotide at the 5' end of Intron 10, that are known to give rise to a RNA stem loop structure.

The thermal stability of RNA secondary structure was calculated with the Poland server (www.biophys.uni-duesseldorf.de/local/POLAND/poland.html-Institut für Physikalische Biologie;Heinrich Heine-Universität Düsseldorf) which calculates the thermal denaturation profile of double-stranded RNA, DNA or RNA/DNA-hybrids molecules.

Ultraviolet Melting

Thermal denaturation experiments were performed on a CARY UV/VIS spectrophotometer (UV 2100) with a temperature controlled heating block. RNAs oligonucleotides (Invitrogen) including the stem loop sequence were used at 1µM in

0.1mM $NaPO_4$. The absorbance of RNA was measured at 260 nm, and temperatures were varied from 30 to 94 °C with 0.5°C/min temperature increments. Melting temperatures were calculated as previously described by Varani L. et al. (**Varani L, Hasegawa M, Spillantini MG, et al. 1999**).

RNAs Oligonucleodes (Invitrogen) used in the UV melting experiments:

WT 5'-GGCAGUGUGAGUACCUUCACACGUC-3'

+4 5'-GGCAGUGUGCGUACCUUCACACGUC-3'

+3 5'-GGCAGUGUAAGUACCUUCACACGUC-3'

Cell cultures and transfection

Human Epithelial Carcinoma (HeLa) and Human breast adenocarcinoma (MCF-7) cell lines were a generous gifts from Prof. Maria Luisa Panno (Department of Cell Biology, University of Calabria, Rende-Sothern Italy).

HeLa and MCF-7 cells were manteined in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine, 1% streptomycin/penicillin and phenol red, in a humified atmosphere of 5% CO_2 at 37°C. Transiently transfection were performed using *TransFast Transfection Reagent* (Promega). Different transfection conditions were tested such as effect of cell confluence, different amounts of Transfast/DNA ratio, different incubation times with the transfection mixture and medium variations (**Jennifer Oler, B.S., and Elaine Schenborn 1999**).

Exponentially growing cells, seeded at a density of about 2 x 10^5 cells for 60-mm dish, were incubated in DMEM medium supplemented with antibiotics for about 24h to reach 60-70% of confluence.

For a single transfection 2µg of each plasmid carrying *wild type* and mutants minigene were mixed with 6µl of TransFast Reagent with -S medium (DMEM serum and antibiotics free) in a final volume of 100µl. For cotrasfection experiments same amount (2µg) of *wild-type* or mutant constructs were mixed with 12µl of TransFast Reagent. Empty vector was also used as transfection's control.

The transfection mix was incubated at room temperature for 15 minutes in order to form a precipitate. Cells, on 1ml of –S medium, were exposed to transfection mix for 1h and then returned to 4ml of DMEM supplemented with antibiotics. To express the transfected plasmids, cells were grown for 48 hours in a humified atmosphere of 5% CO_2 at 37°C.

RNA extraction and RT-PCR analysis on *MAPT* minigenes

Total RNA was isolated using TRIzol Reagent 48 hours post transfection according to the manufacturer's protocol (Invitrogen). RNA samples were treated with DNase I (Amersham Pharmacia Biotech) and purified. RNA concentrations and purities were estimated by spectrophotometric measurement from the relative absorbance (230, 260 and 280nm). Reverse transcription was performed on 1µg of RNA in a volume of 20µl by using the ImProm-II™ Reverse Transcription System according to standard procedures (Promega). cDNAs were amplified in a total volume of 50µl with E9 and E11 oligonucleotides (**Tab.6**).

PCR were performed under the conditions: initial denaturation for 1 min at 94°C; 30 cycles of 1 min at 94°C, 2min at 60C° and 3 min at 72°C. At the end the reactions were held for 7 min at 72°C for the final extension. PCR products were separated by electrophoresis in 1% (w/v) agarose gel and stained with ethidium bromide. Gel bands corresponding to mRNA with and without E10 were excised from gel, purified using the JETquick Gel Extraction Kit (Genomed GmbH) and validated by sequences. For all quantitative experiments RT-PCR reactions were carried out in five independent experiments.

RNA extraction and RT-PCR analysis on brain tissue

Autopsy of the index patient has been obtained within five hours after death. Ethical approval was obtained for the use of all human tissues presented in this thesis.

The brain and the upper part of the spinal cord were analyzed. One hemisphere was sectioned on coronal slices and stored at -80. Small blocks from frontal, parietal, temporal, occipital and cerebellar cortices were cutted for RNA extraction (**Christine L.**

Miller , Robert H. Yolken 2003). The other hemisphere, cerebellum and brainstem were fixed in buffered formalin.

Total RNA was extracted from frontal, parietal, temporal, occipital and cerebellar cortices (200-300 mg) of the index patient as well as from frontal cortex of a FTD-Ub+ by using the RNeasy Lipid Tissue Midi kit according to the manufacturer's protocol (Qiagen). RNA concentrations and purities were estimated by spectrophotometric measurement from the relative absorbance (230, 260 and 280nm).

As previously described, Reverse Transcription was performed on 1µg of RNA extracted from the different brain areas and cDNAs were amplified with E9 and E12 specific primers (**Tab.6**). E1 oligonucleotide was also used in combination with E12 in order to analyze the transcriptional pattern of tau isoforms. For the quantification experiments 30 cycles were used.

RT-PCR products were separated by electrophoresis in 1% (w/v) agarose gel and stained with ethidium bromide. PCR bands corresponding to mRNA with and without E10 were excised from gel, purified and validated by sequences. For all quantitative experiments RT-PCR reactions were carried out in five independent experiments.

Name	Sequence (5' to 3')
Ex-9-For	CTCCAAAATCAGGGGATCGC
Ex-11-Rev	TATGATGGATGTTGCCTAATGAGCC
Ex-12-Rev	TTTTTATTTCCTCCGCCAG
Ex-1-For	AGTTCGAAGTGATGGAAGATCA

Table 6 Oligonucleotides (Invitrogen) used to detect minigene and tissue mRNA

Statistical analysis

Densitometry analysis of each band was measured by TotalLab-Software (Nonlinear Dynamics Ltd, Newcastle, UK) and the relative ratio between trascripts was calculated. Statistics were performed using the SPSS 11.0 Software (27 Statistical Package for Social Sciences, SPSS Inc, Chicago, IL, USA, 2001). Significance was estimated by the t-student test.

Tau Analysis

Immunohistochemistry

Paraffin embedded cryosections of different brain areas obtained from index patient and Alzheimer control brains were mounted on glass slides, deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol (100, 90 and 70%). The sections were incubated in boiling water (in a microwave) for 15 min and allowed to cool to room temperature. Endogenous peroxidase activity was quenched by incubating for 20 minutes the sections in PBS containing H_2O_2 (2%) and methanol (20%). Sections, microwaved in citrate buffer (10 mM, pH6) for 10 min., were then blocked in PBS/Tween20 (0.3%) containing normal horse serum (5%) to block non specific sites before exposure at 4°C overnight to primary monoclonal antibodies AT8 (1:1000) (Innogenetics), RD3 (1:3000) or RD4 (1;100) (Upstate Biotechnology). The sections, washed in PBS (3 x 10 minutes), were incubated in biotinylated horse anti-mouse IgG (Dako) for 2 hours at room temperature and then washed in PBS (3 x 10 minutes). The signal amplification step was performed by incubation with avidin-biotynilated-enzyme complex solution (Vector Laboratories) for 1 hour at room temperature. Antibody staining was visualized by treating the sections with 3,3'-diaminobenzidine (DAB)/H2O2 (0.3%) in 50 mM Tris buffer (pH7.4) for 3-5 minutes (Vector Laboratories). The sections were dehydrated in increasing concentrations of ethanol (70, 90 and 100%) and xylene for 2h and then covered.

Biochemistry

Sarkosyl-insoluble tau extraction

Sarkosyl-insoluble tau proteins were extracted from the frontal, parietal and occipital cortices of the index patient. Briefly, brain tissue (0.2-0.5g) was homogenised in extraction buffer (10mM Tris, 1mM EGTA, 0.8M NaCl, 10% sucrose) containing a cocktail of protease inhibitors (Complete, Roch, Penzburg, Germany). Homogenates were centrifuged at 20 000g for 20 minutes at 4°C and the supernatants transferred to new tubes. The pellets were re-extracted and homogenised as before. The combined supernatants were treated with sodium lauroylsarcosine (1%) at room temperature for 1

hour with gentle agitation. The mixture was then centrifuged at 540 000g for 1 hour at 4°C. The pellets were resuspended in 50 mM Tris (pH 7.4) at 0.1ml/g of initial tissue weight and stored at 4°C.

Soluble tau extraction

Soluble tau was prepared by homogenising in 2.5 % perchloric acid 200-500mg of the index patient's frontal cortex, as well as from Alzheimer control. The homogenates, left on ice for 15 minutes, were then centrifuged at 540 000g for 30 minutes at 4°C. The supernatants were collected and the perchloric acid was removed from the protein samples by dialysis against 100mM Tris (pH7.4)-5mM EDTA at 4°C overnight. Soluble tau protein was then dephosphorilated.

Tau dephosphorilation

Sarkosyl-insoluble and soluble tau proteins, resuspended in Tris buffer containing 6M urea/2mM DTT, were incubated at room temperature for 1hour with gentle mixing. After dialysis against 50mM Tris/2mM DTT, aliquots of proteins were treated with Lambda-phosphatase, 50U/µl, for 6 hours at 30°C in a final reaction volume of 20/µl (New England Biolabs). Incubations were terminated by the addition of 4X sample buffer.

Immunoblotting

Sarkosyl-insoluble and soluble Tau proteins were runned on 12% SDS-PAGE gels and blotted onto nitrocellulose membrane (Biorad). The Biorad semi-dry blotting system was used for protein transfer, which was carried out at 150mA for 3 hours in protein transfer buffer (25mM Tris pH 8.3, 192mM glycine, 20% methanol). The blots were incubated with 5% milk for 1h at room temperature to block non-specific antibody binding. The phosphorylation-independent anti-tau antibody BR134 (1:5000; Innogenetics) and phosphorylation-dependent anti-tau antibodies AT8, AT100 (1:1000; Innogenetics) and 12E8 (1:1000; a kind gift from Dr P Seubert - Elan Pharmaceuticals, San Francisco, CA, USA) were diluted in TBS containing 2% milk and primary antibody incubation was performed overnight at 4°C, on a shaking platform. The membranes were then washed

with TBS-Tween20 (4 x 10 minutes) and incubated with peroxidase-linked secondary antibodies at room temperature for 2h. The membrane were then washed in TBS-Tween20 (5x10 minutes) and visualized on photographic film using the ECL chemiluminescent substrate for peroxidase.

RESULTS

Two novel heterozygous mutations were identified on the MAPT gene of the FTD patient at the Regional Neurogenetic Centre, as described in Methods **(Fig.).** Both mutations, a T to C transition at position -15 of intron 9 [T(-15)C] and an A to C transversion at position +4 of intron 10 (E10+4), are located in the splicing regulatory regions surrounding Exon 10.

Minigene Design

To develop an expression cloning strategy that allows us to examine inclusion or exclusion of Exon 10 in cultured cells**,** we established to use a minigene approach, according to human tau minigene model that was previously established by Jiang et al.(**Jiang et al, 2000**)**.** In this system alternative inclusion of exclusion of the 93bp exon 10 leads to the formation of tau4R and tau3R, respectively, similar to the endogenous human MAPT gene. Minigene model system is the best alternative approach to study the molecular process of splicing and an important tool for the identification and analysis of the cis-acting regulatory elements and trans-acting factors that establish splicing efficiency and that regulate alternative splicing (**Thomas A. Cooper, 2005**). It should be pointed that most of the data on the molecular effect of MAPT gene mutations reported in literature are based on the exon trapping assay.

DNA fragments containing *MAPT* exons 9, 10, and 11 and flanking intronic sequences were obtained, as described in Methods, by PCR from the index patient or a FTD-ubiquitin positive (FTD-Ub+) control (*wt*) genomic DNAs (**Yu Q. et al, 2004**). Briefly, a 311bp fragment containing exon 9 (266bp) and the surrounding intron sequences (27bp upstream and 18 downstream respectively) was amplified with a forward primer and a reverse primer with non-priming EcoRI and KpnI sites respectively. A 641 fragment containing exon 10 (91bp) and the proximal portions of its flanking intron sequences (272bp upstream and 278 downstream respectively) was obtained using an upstream primer and a downstream primer with non-priming KpnI and BamHI sites respectively. A 82bp fragment containing exon 11 (82bp) and surrounding intron

sequences (28bp upstream and 13 downstream respectively) was amplified with primers containing non-priming BamHI and XbaI sites at the 5' and 3' ends respectively (**Fig 25**). The PCR fragments, purified and restricted, were finally linked through their unique restriction sites. The final constructs (1075bp) were cloned into the mammalian expression vector pcDNA3, between the EcoRI and XbaI sites, under the control of the cytomegalovirus promoter and transiently expressed in HeLa and MCF-7 cells.

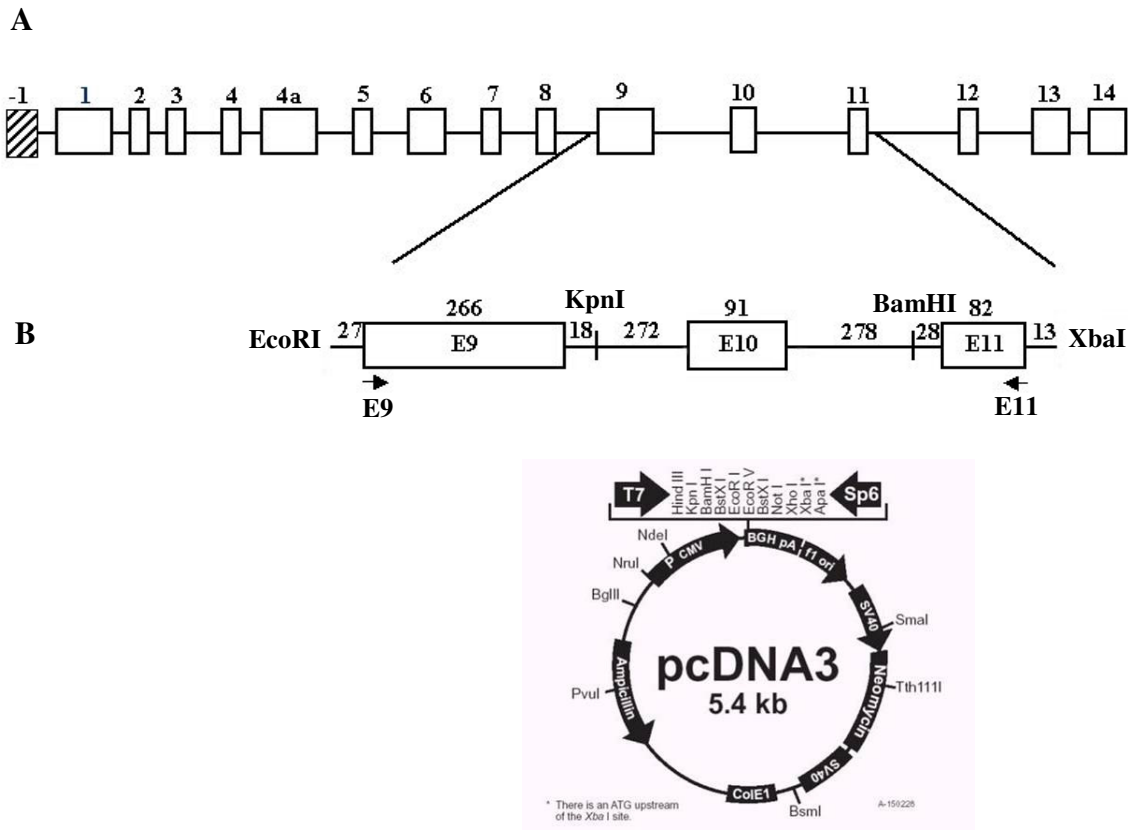

Figure 25 ***MAPT*** **gene and minigene constructs** (**A**) Schematic representation of human *tau* gene (drawn to scale). Open boxes represent exons and lines introns. (**B**) Schematic representation of minigene costructs. The minigenes composed by exons 9, 10 and 11 and flanking intronic sequences were inserted in a pcDNA3 vector. The lenghts of intron regions associated with each exon are shown with vertical lines indicating the fusion of two fragments. Positions of the RT-PCR primers to exons 9 (E9) and 11 (E11) are indicated.

The two novel mutations affect E10 alternative splicing

Alternative splicing patterns were examined by Reverse Transcription-Polymerase Chain Reaction (RT-PCR) on total RNA extracted from transiently transfected HeLa cells using E9 and E11 specific primers for internal sequences of exons. PCRs were first performed using a range of amplification cycles (15, 20, 25, 28, 30, 32, 35) to determine the optimum number of cycles for this analysis (data not shown). Quantification experiments were carried out after 30 cycles. As shown in figure **26** two products corresponding to transcripts including (439-bp) and excluding exon 10 (348-bp) were amplified.

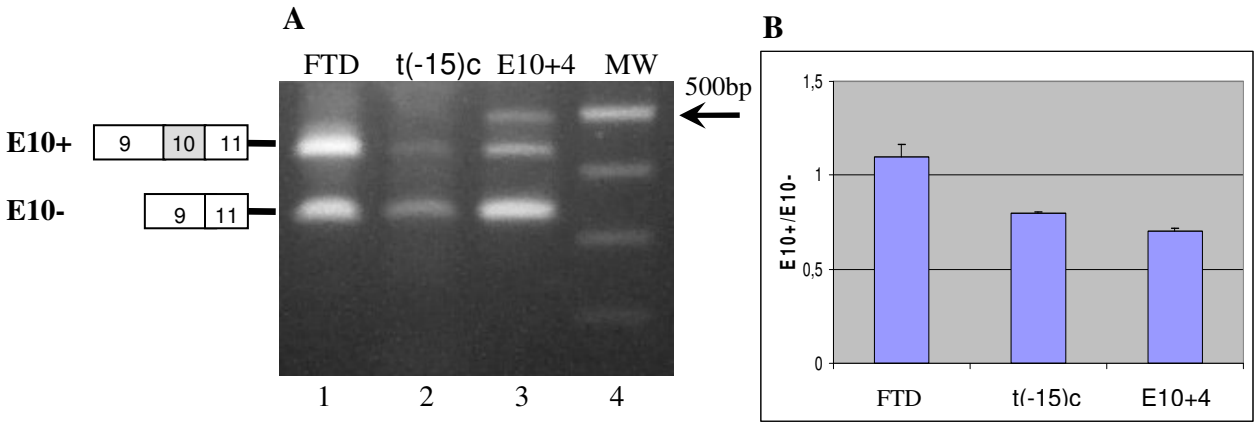

Figure 26 RT-PCR analysis on minigenes. Splicing patterns in HeLa cells of exon 10 in minigenes were examined by using RT-PCR. (**A**) Ethidium bromide-stained agarose gel of RT-PCR products. The 439bp band contains exon 10 (E10+), whereas the 348bp band lacks exon 10 (E10-). Lane 4 (MW) shows a 100bp DNA ladder. Lane 2 and Lane 3, [t(-15)c] and (E10+4) respectively, show a higher proportion of RNA excluding exon 10 when compared with the FTD-Ub+ control minigene (Lane 1). A schematic representation of the unskipped and skipped products is shown on the side of the gel. (**B**) The intensity of the bands shown in **A** was measured and the percentage of exon 10 exclusion was calculated. Each bar represents the mean +/- SD of five separate transfection experiments. Level of significance: $p<0.05$.

Splicing patterns derived from the expression of both mutant minigene constructs suggest a novel Exon 10 alternative splicing effect, that gives rise to a higher increase of mRNAs transcripts lacking Exon 10 (E10-).

The pre-mRNA transcribed from the minigene with the E10+4 mutation (Lane2) is spliced to give rise to a ~1,6-fold higher proportion of RNA excluding E10 when

compared with FTD-Ub+ *wt* minigene (FTD, Lane 1) where the ratio is close to 1. Similarly, the [T(-15)C] mutation (Lane 3) results in a lesser, but still significant increase in E10 splicing. Values are the average from at least 5 different transfections ($p<0.05$, 2-tailed *t*-test). Same results were obtained in MCF-7 cells (data not shown).

Electropherogram analysis of products corresponding to transcripts lacking E10, purified from gels, shows the exact junction between Exons 9 and 11 (**Fig 27**).

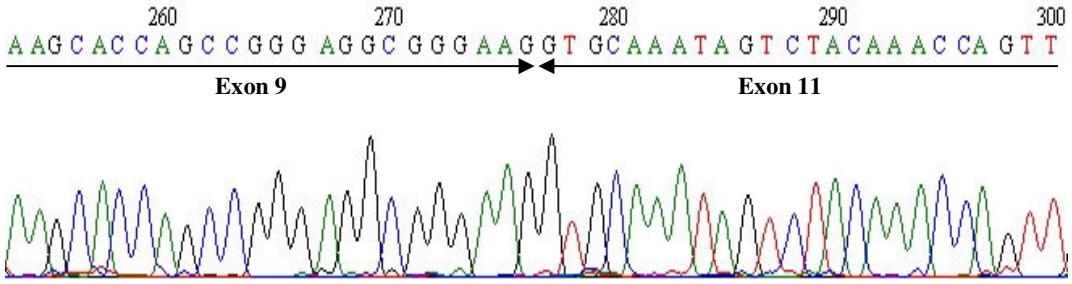

Figure 27 Electropherogram analysis of the products corresponding to transcripts lacking E10

Effect of the patient's compound heterozygous condition on the E10 alternative splicing

Considering that each single mutation dramatically affects E10 alternative splicing, we performed cotrasfection experiments to simulate *in vitro* the compound heterozygous condition of the index patient and the heterozygous condition for either of the mutations of her healthy parents.

We cotrasfected in HeLa cells, the minigene carrying the [T(-15)C] or E10+4 mutations with *wild type* minigene to simulate the heterozygous condition of index patient's father or mother described in pedigree (I-1 and I-2 respectively). It should be pointed that *wild type* minigene was obtained, as previously described, from a healthy control. The two mutant minigenes were also cotrasfected together to simulate the compound heterozygousity of the index patient (II-1 in pedigree).

RT-PCR analysis performed on total RNA, extracted from HeLa cells, using E9 and E11 specific primers are showed in figure **28**. While cotransfection of patient's parents (Lanes 2 and 3) shows, as the FTD-Ub+ control (FTD, Lane 1), a quite normal ratio of

E10 including/E10 excluding transcripts, in the affected patient (Lane 4) the co-expression of both mutations dramatically affects the ratio. Transcripts excluding E10 are spliced in a higher proportion. Values are the average from at least 5 different transfections ($p<0.05$, 2-tailed *t*-test).

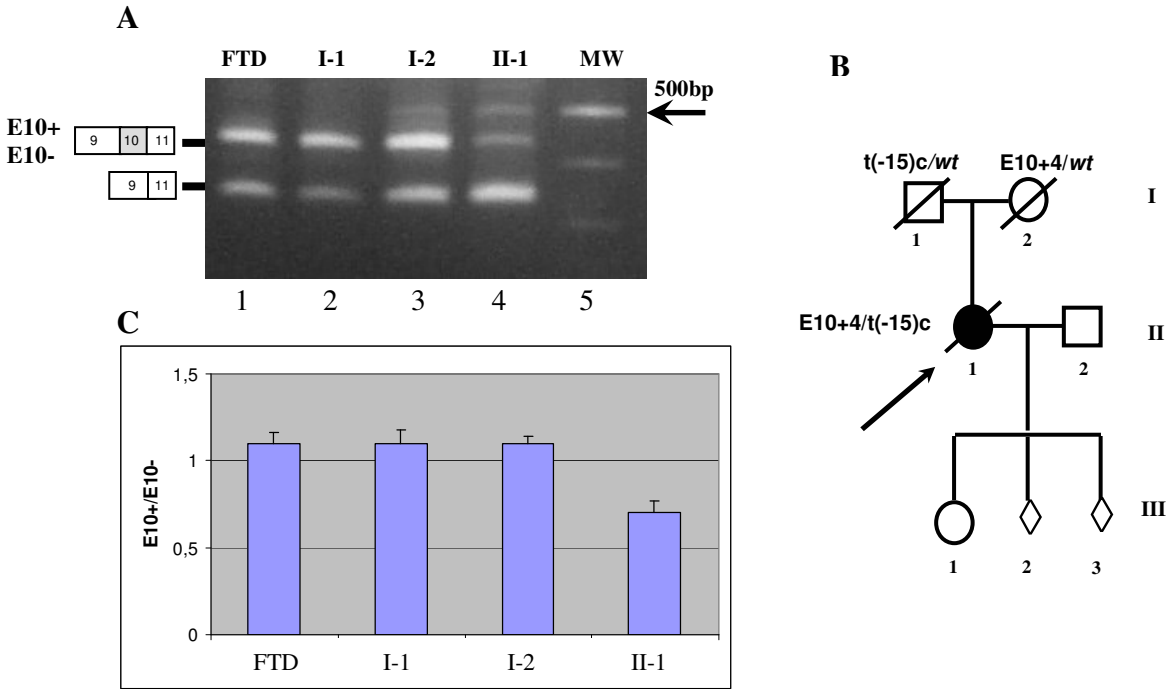

Figure 28 RT-PCR analysis on minigenes cotrasfection (A) Ethidium bromide-stained agarose gel of RT-PCR products The 439bp band contains exon 10 (E10+), whereas the 348bp band lacks exon 10 (E10-). Lane 5 (MW) shows a 100-base pair DNA ladder. In Lane 2 [t(-15)c/wt] and Lane 3 (E10+4/wt) both bands, as FTD-Ub+ control (Lane 1), show a quite similar intensities. Lane 4 [E10+4/t(-15)c] shows a higher proportion of RNA excluding exon 10. A schematic representation of the unskipped and skipped products is shown on the side of the gel. (**B**) Pedegree of the index patient's family. (**C**) The intensity of the bands shown in **A** was measured and the percentage of exon 10 exclusion was calculated. Each bar represents the mean +/- SD of five separate transfection experiments. Level of significance: $p<0.05$.

Minigene expression of the 5'splice site E10+3 mutation

As described in literature, *MAPT* gene mutations occurred in intron 10, immediately downstream the 5' splice site, increase splicing giving rise to transcript including Exon 10 (E10+). Exon trapping analysis on these mutation, as E10+3 the first mutation that

was been correlated to the FTD pathology, provided evidence for a quantitative correlation between thermodynamic stability of the RNA stem loop regulatory element and splice site utilization (VARANI). Considering the opposite molecular effect of the E10+4 mutation, we designed a minigene construct carrying the E10+3 mutation that in exon trapping analysis led to a 30-fold increase of E10 inclusion. Oligonucleotide directed mutagenesis, as described in materials and methods, was used to introduce the E10+3 mutation into the *wild type* minigene obtained from a healthy control. The construct was then inserted into the pcDNA3 vector and transiently expressed in HeLa cells. Cotrasfection experiment with *wild type* minigene was also performed to simulate the heterozygous condition of the FTD-17 patients carrying the E10+3 mutation, described in literature.

RT-PCR analysis performed on total RNA, extracted from HeLa cells, using E9 and E11 specific primers are showed in figure **29**. Either the single E10+3 mutation (Lane 2) or the heterozygous condition (Lane 3, E10+3/*wt*) give rise to a ~2 fold increase of E10 inclusion transcripts when compared with the FTD-Ub+ control transcripts (Lane 1). Values are the average from at least 3 different transfections ($p<0.05$, 2-tailed *t*-test).

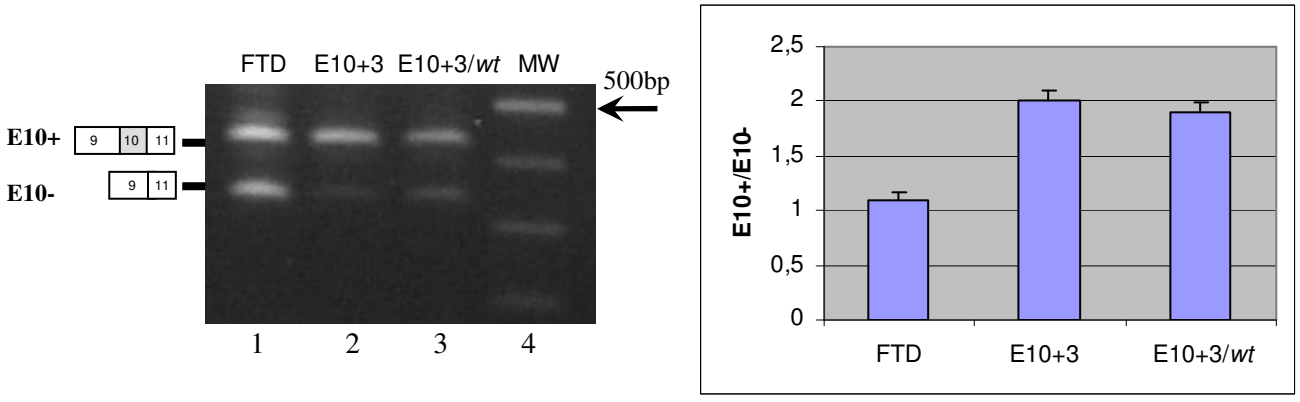

Figure 29 RT-PCR analysis on E10+3 minigenes. Ethidium bromide-stained agarose gel of RT-PCR products. The 439bp band contains exon 10 (E10+), whereas the 348bp band lacks exon 10 (E10-). Lane 4 (MW) shows a 100bp DNA ladder. In Lanes 2 (E10+3) and 3 (E10+3/*wt*) both bands show a higher proportion of transcripts including exon 10 when compared with the FTD-Ub+ control (Lane 1). Schematic representation of the unskipped and skipped products is shown on the side of the gel.

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Brain Tissue Study

Detection of increased levels of Tau3R transcripts.

Total RNA was extracted from different brain areas (Frontal, Temporal, Parietal, Occipital cortex and Cerebellum) of the index patient as well as from frontal cortex of the FTD-Ub+ control (FD) as described in Methods. E10 alternative splicing patterns were first analysed in the frontal cortex area of the index patient using E9 and E11 specific primers. Considering that the splicing pattern was comparable with that obtained with the minigene system (data not shown), we performed RT-PCR analysis in the different brain areas using E9 and E12 specific primers for sequence flanking microtubule binding domain, upstream and downstream respectively.

Two PCR products corresponding to Tau4R (552bp) and Tau3R (461bp) transcripts were amplified and validated by sequencing analyses (**Fig 30**).

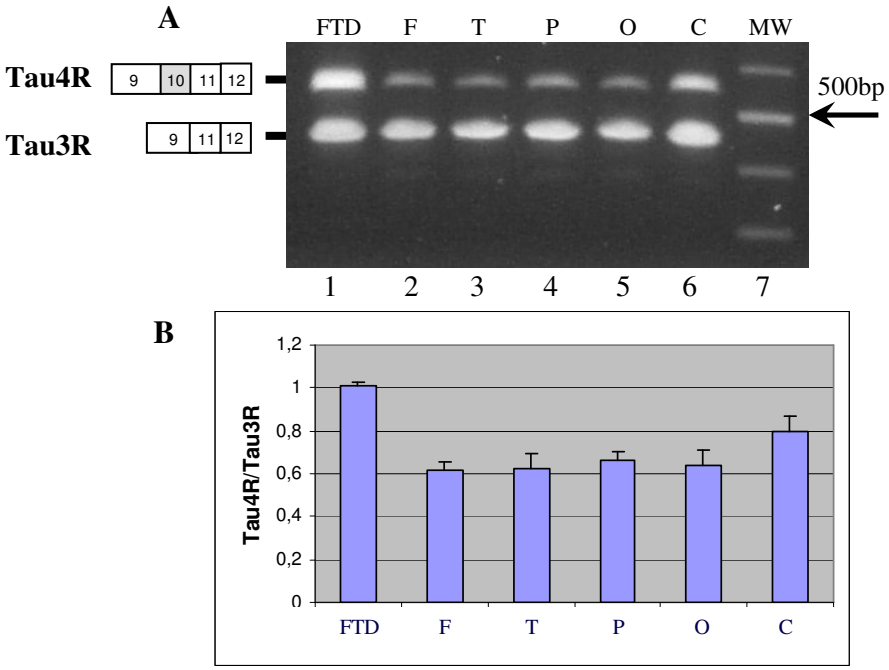

Figure 30 RT-PCR analysis in different brain sections of the index patient. (**A**) Agarose gel of RT-PCR products. The 552bp band corresponds to the Tau 4R transcripts, whereas the 461bp to the Tau3R transcript. Lane 7 (MW) shows a 100-base pair DNA ladder. In the FTD-Ub+ control (Lane 1) both bands show a quite similar intensities. Lanes 2-6 (**F**rontal, **T**emporal, **P**arietal, **O**ccipital and **C**erebellum sections) show a much stronger level of Tau3R transcripts (461bp fragment). (**B**) The intensity of the bands shown in **A** was measured and the ratio of Tau 4R:Tau 3R was calculated. Each bar represents the mean +/- SD of three separate transfection experiments. Level of significance: $p<0.05$

The transcripts corresponding to Tau3R isoforms (Lane 2-6), as shown in Figure **30**, are much stronger than Tau4R isoforms when compared with the FTD-Ub+ control transcripts (Lane 1), where the ratio was 1.01±0.02. In contrast the Tau 4R to Tau 3R ratio in the index patient is altered in all brain areas (Frontal = 0.61±0.04, Temporal= 0.63±0.07, Parietal= 0.66±0.04, Occipital=0.64±0.07) except for cerebellum where the ratio is higher (0.80±0.07). Values are the average from at least 3 different experiments ($p<0.05$, 2-tailed *t*-test).

Tau Analysis

We hypothesized that the increased expression of Tau3R transcripts could be responsible of an increase of Tau 3R isoforms *and that free tau isoforms might be aggregated into toxic inclusions in the brain tissue of the index patient*. To detect abnormal protein inclusions, biochemical and immunohystochemical analyses were performed in the laboratory of Prof M.G. Spillantini (Centre for Brain Repair, University of Cambridge-United Kingdom).

Immunohistochemical detection of increased levels of Tau 3R inclusions

In order to investigate the presence of tau inclusions in the brain of the index patient, immunohistochemistry experiments were performed using tau phosphorilation-dependent AT-8 antibody. Abundant, rounded, star-like and coiled pathological tau inclusions were observed in neurones, astrocytes (tufted astrocytes) and oligodendrocytes (**Fig 31** B-D) when compared with an Alzheimer (AD) brain control (**Fig 31**, A). AT-8 labelled Pick bodies were also found (**Fig 31** B-D).

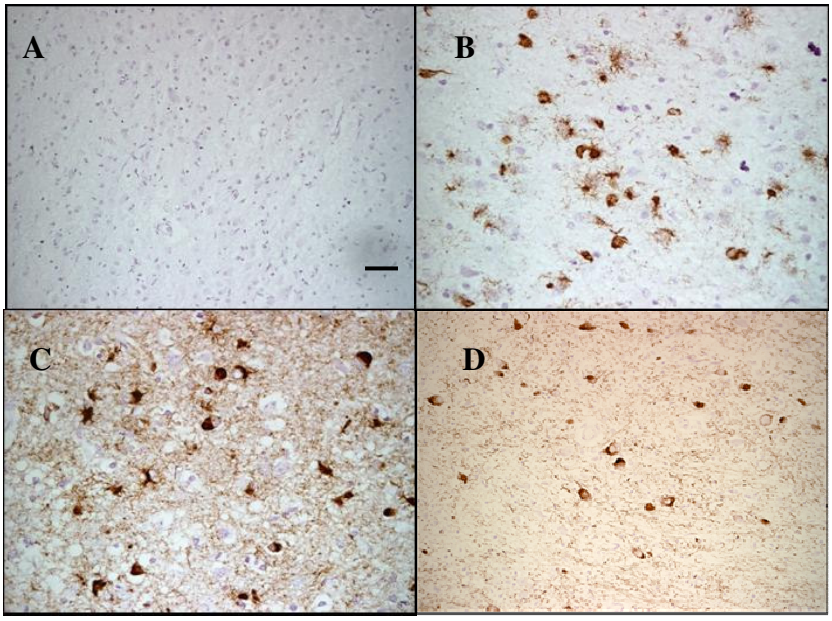

Figure 31 Immunostaining for phosphorilated tau. (A) AD control staining (Frontal cortex). (**B**) Frontal, (**C**) Parietal and (**D**) Occipital cortex of the index patient. Anti-tau antibody=AT8. Scale bar = 100 µm

Immunohistochemistry with monoclonal antibodies RD3 and RD4 specific for 3R and 4R tau isoforms respectively, performed on the index patient's *brain tissue,* revealed that the most of the AT8-positive inclusions were only composed of 3-repeat tau (**Fig 32**, B). No immunoreactivity was observed with the antibody RD4 (**Fig 32**, A) and no amyloid was found.

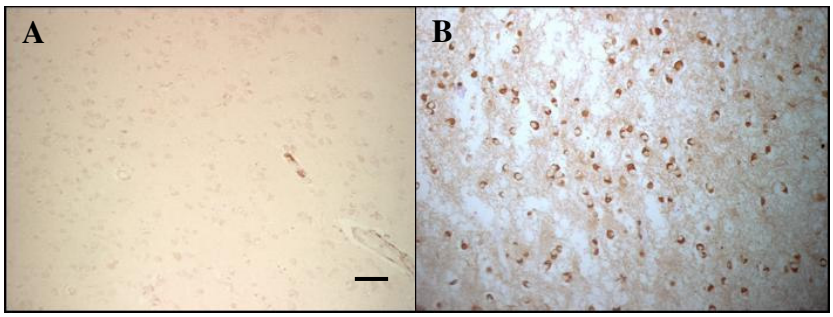

Figure 32 Immunostaining Immunostaining for phosphorilated tau. (**A**) Tau4R; (**B**) Tau3R Anti-tau antibody=RD3 and RD4. Scale bar = 100 µm

Biochemical characterization of pathological tau

Biochemical analyses on Sarkosyl-insoluble and soluble tau were performed in order to investigate the *expression pattern of tau isoforms* and to characterise the content of tau deposits.

- ***Sarkosyl-insoluble tau*: *detection of increased levels of Tau 3R isoforms***

Sarkosyl-insoluble tau extracted from the frontal cortex of the index patient as well as from an Alzheimer (AD) brain, used as a disease control for the phosphospecific antibodies, was resolved by SDS-PAGE. Western blot analysis with the phosphorylation-independent anti-tau antibody BR134 and phosphorylation-dependent anti-tau antibodies AT8, AT100 showed in the index patient's brain tissue (**Fig.33,** F) anomalous levels of Tau3R isoforms, when compared with the AD control (**Fig.33**, AD).

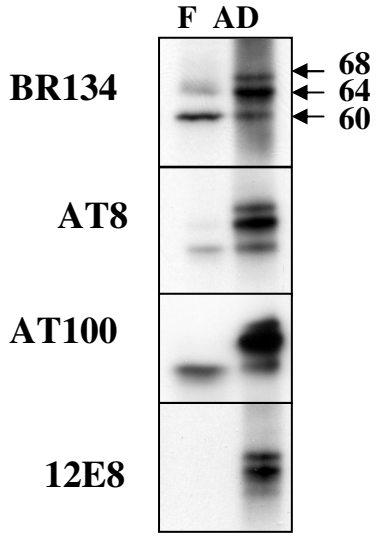

Figure 33 Immunoblotting of sarkosyl-insoluble tau. Three bands of 60, 64 and 68 KDa molecular mass were detectable in AD control (Lane 2) with anti-tau antibody BR134, AT8, AT100, and 12E8. In contrast only two bands of 60 and 64 KDa were detectable and No 12E8 immunoreactivity was visible in the frontal cortex of the index patient (Lane 1).

Immunoblotting with BR134 revealed 3 protein bands with molecular weights 68, 64 and 60 kDa in extracts obtained from the AD brain. In contrast, sarkosyl-insoluble tau extracted from the brain of the index patient was labelled with BR134 at 64 and much more at 60kDa, corresponding to shortest tau aggregates. The phosphorylation-dependent antibodies AT8 and AT100 predominantly labelled the 60kDa band of

insoluble tau, extracts from the brain of the index patient, indicating that sarkosyl-insoluble tau is highly phosphorylated. Sarkosyl-insoluble tau was also analysed using the phosphorylation-dependent anti-tau antibodies 12E8 that, as BR134, AT8 and AT100, labelled the neurofibrillary tangles, but not Pick bodies. 12E8 recognises tau phosphorilated at S262 and S356. The undetectable 12E8 immunoreactivity in the sarkosyl-insoluble tau prepared from the index patient's brain confirmed the presence of Pick Bodies (**A. Probst, M. Tolnay, D. Langui, M. Goedert and M.G. Spillantini 1996**).

The isoform composition of sarkosyl-insoluble tau was assessed by immunoblotting with the phosphorylation-independent anti-tau antibody BR134 following dephosphorylation by λ-phosphatase. Dephosphorylation of insoluble tau caused a shift in the electrophoretic mobility of the insoluble tau protein bands, with two major band aligning with recombinant tau isoforms, 3R0N and 3R1N, containing three repeats (**Fig34**). These data indicate that the pathological inclusions characterized by immunohistochemistry are composed mainly of Tau3R isoforms.

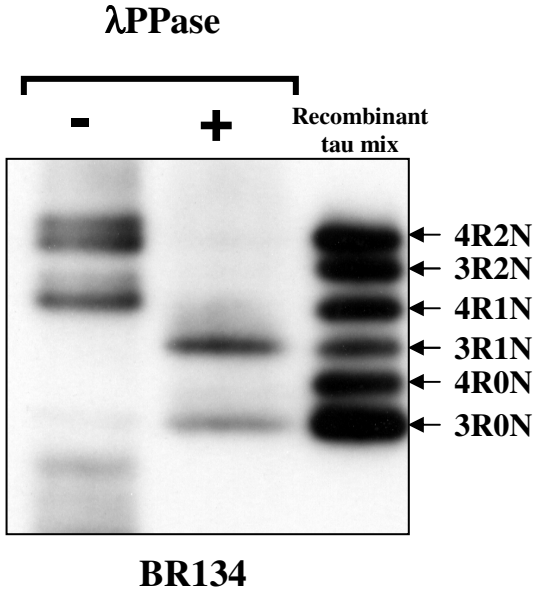

Figure 34 Immunoblotting of dephosphorilated sarkosyl-insoluble tau. Immunoblot of sarkosyl insoluble tau protein extracted from the frontal contex of the index patient before (-) and after (+) alkaline phosphatase treatment (Lane 2) compared with the pattern of recombinant human tau isoforms (Lane 3). Immunoblotting with BR134 detected two bands that co-migrated with the 3R0N and 3R1N isoforms.

- ***Soluble tau*: *detection of increased levels of fetal Tau 3R isoform***

Soluble tau was analysed to establish whether the specific incorporation of Tau3R isoforms in the inclusions is due to a selective aggregation of Tau3R isoforms (from a soluble pool containing 3- and 4-repeat isoforms in approximately 1:1 ratio) or whether it is due an increased availability of Tau3R isoforms in the soluble fraction of neuronal tau.

Soluble tau, extracted from the index patient's brain (frontal region) as well as from AD patient used as control, was subjected to dephosphorylation for a clear identification of the different tau protein isoforms and the products were resolved by SDS-PAGE. Immunoblotting with the phosphorylation-independent antibody BR134, as shown in figure **35**, revealed a major band that co-migrated with the 3R0N isoform of recombinant tau. Minor protein bands corresponding to 4R0N, 3R1N and 4R1N were also detectable. This pattern was distinct from the protein profile obtained after dephosphorylation of the soluble tau fraction derived from an AD brain, where the BR134-labelled bands of Tau3R and Tau4R isoforms were apparently in equal amounts.

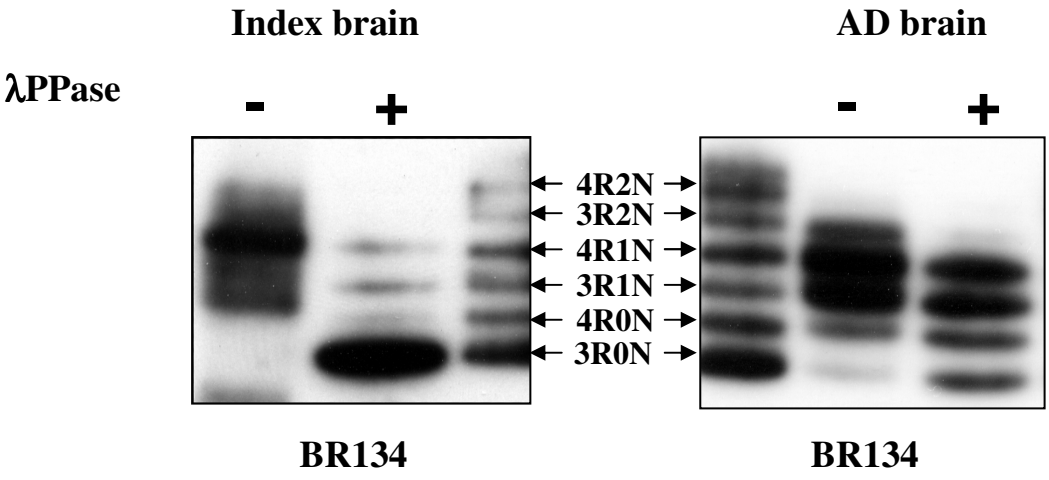

Figure 35 Immunoblotting of dephosphorilated Soluble tau. Immunoblots of soluble tau protein extracted from the frontal contex of the index patient and AD control brain before (-) and after (+) alkaline phosphatase treatment compared with the pattern of recombinant human tau isoform. Immunoblotting with BR134 detected a major band that co-migrated with the 3R0N isoform.

Considering the increased availability of shorter Tau3R isoforms in the index patient's brain, we analysed the transcriptional pattern of *MAPT* gene to investigate the amount of each tau mRNA transcripts. RT-PCR analysis were performed on same amount of total RNA extracted from index patient's Frontal brain section as well as from the FTD Ubiquitin positive control, using E1 and E12 specific primers respectively.

Preliminary data have shown that isoform transcriptional patterns in the brain tissue of the index patient is different when compared with the FTD-Ub+ control (**Fig 36**). The expression of the shorter 3R0N transcripts, like the corresponding 3R0N isoforms, seem to be enhanced than the others.

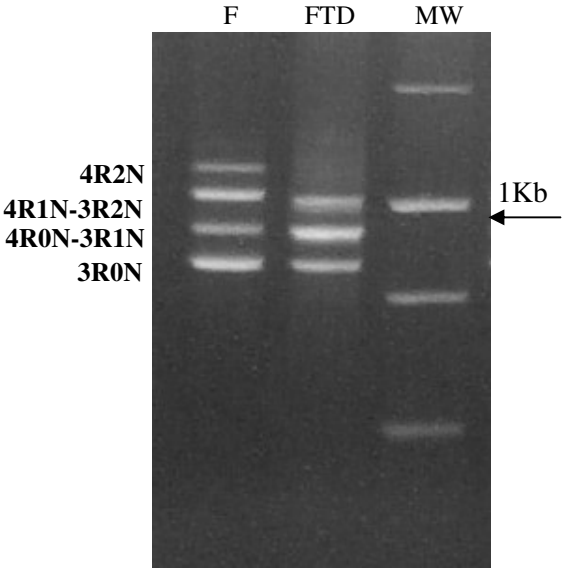

Figure 36 RT-PCR analisys of transcriptional pattern of *MAPT* gene. Ethidium bromide-stained agarose gel of RT-PCR products. Lane 3 (MW) shows a 1Kbp DNA ladder (MW). Lane 1 (index patient's frontal cortex) shows an increase of shorter 3R0N transcripts compared with a FTD-Ub+ control (Lane 2).

Thermodynamic stability of the stem loop regulatory structure increased by the E10+4 mutation.

Though the molecular mechanism of the Exon 10 splicing regulation remain to be completely elucidated, the RNA stem-loop structure located close to the splice donor site of the intron after exon 10 seems to be, as described, a regulatory region of Exon 10 alternative splicing. It's known that the thermodynamic stability of the structure is reduced by all reported *MAPT* mutations identified to date in this region (**Fig 37**), giving rise to an increased inclusion of exon 10 and increased expression of tau4R (**M. Hutton et al, 1998; Spillantini MG and Murrel JR et al, 1998; L. Varani et al, 1999**). *Recent studies have also demonstrated that stem loop variants that increase the stability of the stem loop result in decreased inclusion of exon 10 and increased expression of 3Rtau (**Donahue**).*

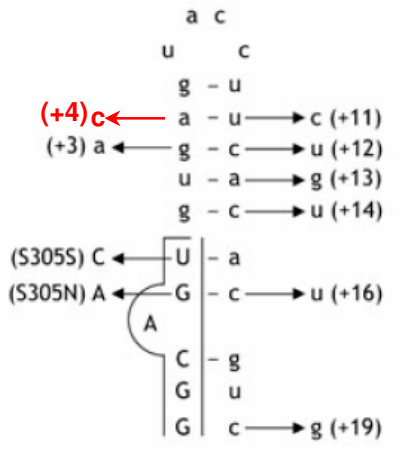

Figure 37 Predicted pre-mRNA stem loop structure

To investigate the effect of the E10+4 mutation on the thermodynamic stability of the RNA stem loop structure we performed bioinformatic analysis and UV melting experiments. We synthesized oligonucleotides representing the *wild-type* regulatory element, extends from nucleotides -5 to +19, and the two sequence variants with mutations at positions +4 and +3. NMR, UV melting and exon trapping experiments have demonstrated (VARANI) the E10+3 reduces stem loop's stability giving rise to an increase of transcripts with E10 and tau4R.

It should be underlined that the E10+4 mutation is located close to the exon 10 splice donor site predicted to give rise to a RNA stem loop structure considered crucial for the quantitative regulation of exon 10 alternative splicing. *In vitro* studies have shown that most intronic mutations downstream of exon 10 destabilize the stem loop and give rise to an increase of E10 inclusion and 4RTau.

Bioinformatic prediction of stem loop stability was carried out using Poland alghoritm, modified by Fixman and Freire and described by Steger (**Poland D., 1974**; **Fixman M. and Freire J. J**., **1977**; **Steger G., 1994**), using Gotoh standard parameters (**Gotoh O. 1983**). Bioinformatic analysis shown a largest rise in temperature due to the +4 mutation respect to *wt* and +3 mutation, that instead decrease stability.

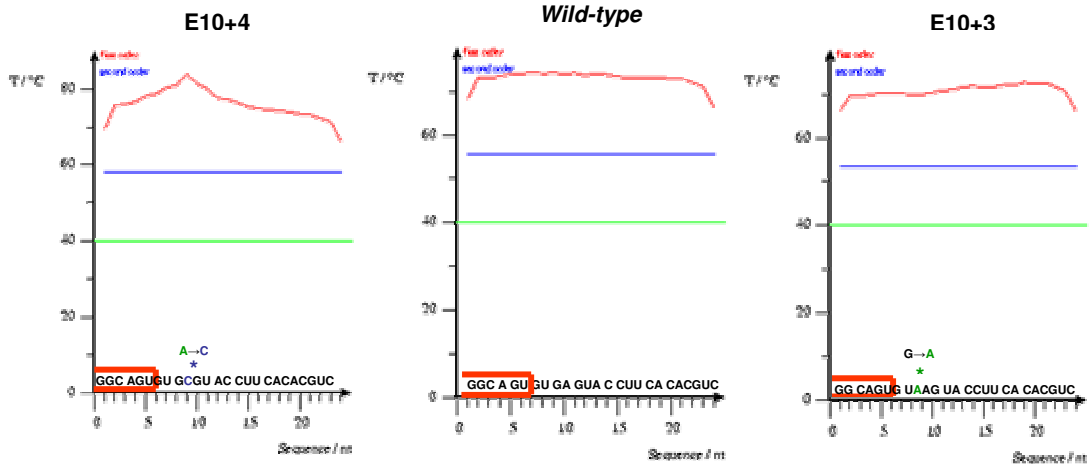

Figure 38 Bioinformatic prediction of stem loop stability.

Quantitative results were obtained by Ultraviolet (UV) spectroscopy. We recorded a set of UV melting curves (Optical Density as a function of temperature) for the stem loop RNAs E10+4, E10+3 and *wild type*, using a UV 2100 spectrophotometer with a temperature controlled heating block. The melting temperature, defined as the midpoint of the transition from fully structured to fully unstructured RNA, was estimated from the maximum in the derivative of the UV melting profile. The *wild-type* stem loop, as shown in figure **38**, had a melting temperature of 50°C. In contrast, E10+3 and E10+4 stem loop showed a decrease and an increase in melting temperature of 5°C respectively.

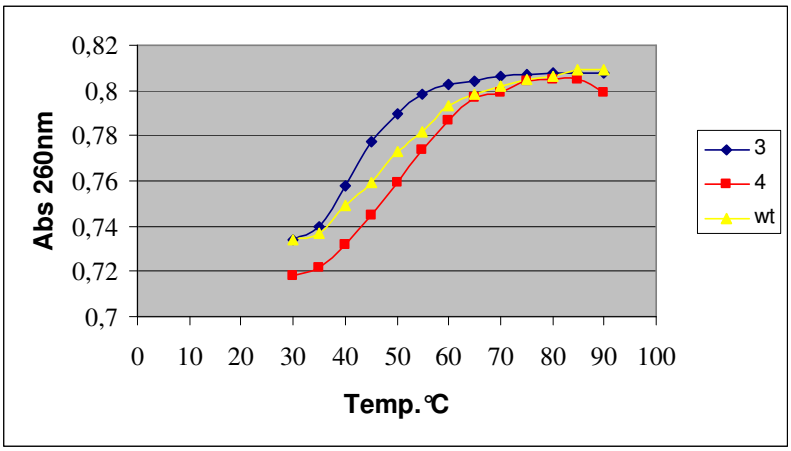

Figure 38 Ultraviolet (UV) melting experiments. UV melting curves were generated for three stem loop variants: *wild-type* (*wt*), E10+3 (3) and E10+4 (4). Normalized UV absorbance (Rel. Abs) is shown as a function of temperature. The values used to construct the melting curves are the average of at least three independent experiments.

DISCUSSION

The present study was aimed to analyze the molecular effect of two novel heterozygous Microtubule-associated tau (*MAPT*) gene mutations, a T to C transition at position -15 of intron 9 [T(-15)C] and an A to C transversion at position +4 of intron 10 (E10+4), identified in a patient with sporadic Frontotemporal Dementia (FTD). The patient started slowly and insidiously with behavioral disturbances and personality changes at age 46. Disease course evolved progressively into a complete FTD picture until death occurred at age 57. Genetic analysis carried out on her parents showed that the [T(-15)C] transition was transmitted by her father and the E10+4 transversion by her mother. All other family member analyzed were heterozygous for either of the mutations and cognitively healthy, except one patient's sister, compound heterozygous for the two mutations. The sister, to date, has not shown neurological and psychiatric abnormalities although a slight impairment of verbal memory (*Rey's words short term 28, nv 28.53)* was evidenced by the Mental Deterioration Battery together with slight deficit in attention and concentration. The index patient, similarly to her compound heterozygous sister, shows an higher number of abortions. Recent findings argue for a role of tau protein in chromosome stability by means of its interaction with both microtubules and chromatin. It was found that patients carrying the P301L mutation, in *MAPT* exon 10 and associated with FTD, consistently had several chromosome aberrations in their fibroblasts and lymphocytes: chromosome and chromatid breakages or gaps, aneuploidies, translocations, in addition to chromatin bridges and decondensed chromosomes (**Rossi G et al, 2008**).

Our *in vitro* and *brain tissue* experiments suggest that the two novel mutations, located in the splicing regulatory regions surrounding *MAPT* Exon 10, cause a novel Exon 10 splicing effect. It is known that *MAPT* gene undergoes extensive alternative splicing, leading to the expression of six tau isoforms in the adult human brain (**Andreadis A. 2005**). Exon 10 is one of the alternatively spliced exons and encodes the second of four imperfect microtubule-binding repeats in the C-terminus half of tau protein (*25*). Exclusion or inclusion of exon 10 gives rise to tau isoforms with three (tau3R, $E10^-$) or

four (tau4R, $E10^{+}$) microtubule-binding repeats. Alternative splicing pattern of human tau exon 10, specific for humans, undergoes developmental changes, switching from almost exclusively tau exon 10 skipping isoform during the fetal stage to approximatively a 1:1 ratio of exon 10+/exon 10- during the adult stage **(Andreadis A. 2005; D'Souza et al, 2005; Kar A. et al, 2005)**. Perturbation of this ratio, due to exonic or intronic mutations in *MAPT* gene, leads to the development of a neurodegeneration process. Functionally, *tau* mutations fall into two categories; those whose primary effect is at the protein level and those that influence the alternative splicing of tau pre-mRNA (**Andreadis A. 2005; D'Souza et al, 2005; Goedert, M et al 2005; Hutton, M et al 2001; Kar, A. et al 2005; Lee, V et al 2001; von Bergen, M. et al 2005).** Thus, *MAPT* exon 10 alternative splicing is clearly fundamental for neuronal function. More than 40% of genes important for neuronal function utilize alternative exon skipping/inclusion as a mechanism to generate functionally distinct protein product **(Gene Yeo et al, 2004, Qin Li et al 2007**). We analysed the molecular effect of the [T(-15)C] and the E10+4 mutations on the alternative splicing of *MAPT* pre-mRNA in a minigene model system and in *brain tissue*. We established to use a minigene model system, that is the best alternative approach to study the molecular process of splicing when tissue is not available (**Thomas A. Cooper, 2005**). According to human tau minigene model that was previously established by Jiang et al.(**Jiang Z. et al, 2000**), alternative inclusion or exclusion of the 93bp exon 10 leads to the formation of tau4R and tau3R, respectively, similar to the endogenous human *MAPT* gene. Splicing patterns derived from the expression of both mutant minigene constructs carrying -15 and +4 mutations, suggest a novel Exon 10 alternative splicing effect, that gives rise to a higher increase of mRNAs transcripts lacking Exon 10 (E10-) and an altered isoforms ratio.

The pattern of *MAPT* Exon 10 splicing and the Tau4R to Tau3R ratio was successively assessed in *brain tissue* study on total RNA extracted from different patient's brain areas. Semi-quantitative RT-PCR analysis showed a 1.6 fold-increase of Tau3R mRNAs to Tau4R ratio in different brain areas, except for cerebellum where the ratio was almost normal. The higher increase of mRNAs transcripts lacking Exon 10 (E10- or Tau3R) observed in the index patient is in contrast with data reported in literature for the

mutations in intron 9 as wells as in intron 10 and exon 10. These mutations, except those at codon 301 (P301L and P301S), are considered splicing regulation mutations that disturb the normal splicing balance of exon 10, usually resulting in more inclusion of exon 10 with an increased 4R/3R tau ratio as the final outcome (**Buee et al, 2000; Spillantini et al. 2000; Crowther and Goedert, 2000; Garcia and Cleveland, 2001; Lee et al., 2001**). Only the +19 and +29 mutations have been reported in literature to reduce exon 10 splicing leading to an increase of 3R tau (**Stanford PM et al, 2003**). The +29 change was reported as a rare polymorphism, before being considered a mutation (**D'Souza I et al, 1999; Stanford PM et al, 2003**). However, this family has been found to have a *Progranulin* mutation (**Pickering-Brown SM et al, 2006**), establishing that +29 is a benign polymorphism.

Immunohistochemical analyses performed on the index patient's *brain tissue* revealed the presence of abundant neuronal and glial inclusion mostly made of 3R tau isoforms. Overall an altered tau3R to tau 4R ratio give rise to a tau protein dysfunction. Tau protein is known to bind to microtubules and to promote microtubule assembly; tau isoforms with four repeats being better at binding to microtubules and at promoting microtubule assembly than isoforms with three repeats (**Goedert, M. & Jakes, R; Goode, B. L. & Feinstein, S. C.,1994; Butner, K. A. & Kirschner, M. W.,1991; Gustke, N et al, 1992; Lee, G. & Rook, S. L. 1992**). It is possible that tau isoforms with three repeats and isoforms with four repeats bind to distinct sites on microtubules thus an equal ratio tau3R to tau4R is crucial (**Goode, B. L. & Feinstein, S. C.,1994**).

As known tau is a natively unfolded protein that is believed to become structured upon binding to microtubules (**Schweers, O et al, 1994 and Goode, B et al, 1997**) and it is unlikely that tau would assemble into filaments while bound in structured form to microtubules. An increase in four-repeat tau isoforms may lead to an excess in protein over available binding sites, thus increasing the time four-repeat tau spends in its natively unfolded state in the cytoplasm. Over time, this may lead to the hyperphosphorylation of four-repeat tau isoforms, rendering them completely unable to bind to microtubules. Interaction with other factors, such as sulfated glycosaminoglycans, could be also important in nucleation and filament formation. It is

known that tau deposits are immunoreactive for heparan sulfate (**Spillantini, M. G et al, 1997**). As 3R tau is far less efficient at microtubule assembly, an increase in unbound 3R isoforms should cause also insoluble tau aggregates with a similar mechanism.

Thus, a changed ratio in the levels of tau isoforms appears to be sufficient to lead to the formation of neurofibrillary tangles (NFTs) and cell death. NFTs are a hallmark of 'tauopathies'. They include progressive supranuclear palsy, corticobasal degeneration, Pick's disease and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTPD-17) (**Delacourte and Buee 2000; Spillantini et al. 2000**).

Biochemical analysis on sarkosyl insoluble and soluble tau extracted from the index patient brain showed both 3R and 4R tau isoforms, with increased levels of shortest 3R tau fetal isoform (Tau 3R0N).

Tau 3R0N is the predominant tau splicing isoform produced in the human fetal brain. Recently, it has been demonstrated that there is a transient expression of 0N3R tau isoform during adult neurogenesis (**Bullmann et al, 2007**), suggesting the presence of regulatory factors suppressing the tau exon inclusion expressed in the fetal brain.

Considering that the two mutations are located in splicing regulatory region surrounding exon 10, we could speculate a possible reactivation of factors which are functional in the fetal period. Recently, using the expression cloning method, the *trans*-regulatory factor SRp54 was identified as a repressor for tau exon 10 inclusion.

The high level of expression of SRp54 factor in the fetal brain and its reduced expression in the adult brain, correlate with the Tau exon 10 alternative splicing pattern changes, supporting a role of the endogenous SRp54 protein in regulating tau exon 10 splicing during development. It is not clear how SRp54 and other expression in the human brain changes during neural development or whether SRp54 expression varies in different brain regions (**Jiang et al., 2003; Jane Y Wu et al, 2006**).

In addition to trans-acting factors (**Broderick et al., 2004; Gao et al., 2000; Hartmann et al., 2001; Jiang et al., 2003; Kondo et al., 2004; Wang et al., 2004, 2005; Andreadis A. 2005; Jiang Z. et al. 2003, Jane Y Wu et al, 2006; D'Souza et al, 2006;**), in recent years, several groups have analyzed the regulation of exon 10 alternative splicing and evidenced that exon 10 is regulated by a finely tuned balance of

cis-acting sequences (**D'Souza et al., 1999; D'Souza et al, 2000; D'Souza et al, 2002; Gao et al., 2000; Grover et al., 1999; Hutton et al., 1998; Stanford et al., 2003; Wang et al., 2004**).

Considering that the E10+4 mutation inserts a C into position +4 of the RNA stem loop structure, considered crucial for the quantitative regulation of exon 10 alternative splicing **(M. Hutton et al, 1998; M. Spillantini et al, 1998**), we hypothesized that this sequence variation could be important for the increased expression of 3RTau observed either in minigene constructs than in *brain tissue* studies.

NMR, UV melting and exon trapping experiments have demonstrated that most of *MAPT* intronic mutations downstream exon 10 located in the upper part of the stem loop structure destabilize this structure and give rise to an increase of E10 inclusion and 4RTau **(L. Varani et al, 1999)**. As a consequence, this region of pre-mRNA becomes less or more available for interaction with the U1 small nuclear ribonucleoprotein (snRNP) or other splicing factors, with a corresponding increase of E10 inclusion and 4RTau expression. Moreover, recent studies have also demonstrated that stem loop variants that increase the stability of the stem loop result in decreased inclusion of exon 10 and increased expression of 3Rtau (**Christine P. Donahue et al, 2006**).

Our data, based on bioinformatic prediction of the stem loop sequence thermostability and Ultraviolet (UV) melting experiments, demonstrate a strong increasing of stability in the stem-loop structure carrying the E10+4 mutation. We have also compared these results with those from RNA oligonucleotides *wild type* and containing the E10+3 mutation (a G to A transition), the first mutation correlated to the FTD pathology resulting from the higher expression of tau4R isoform **(Spillantini MG and Murrel JR et al 1998**; **M. Tolnay et al, 2000; Neumann M et al 2005; Spina S. et al 2008)**. Similarly to that reported in literature the presence of E10+3 mutation gives rise to a destabilization of the stem loop structure.

We designed also a minigene construct carrying the E10+3 mutation to investigate the splicing patterns of cotrasfection with *wild type* minigene, in order to simulate the heterozygous condition of the FTD-17 patients carrying the E10+3 mutation. We obtained a ~2 fold increase of E10 inclusion transcripts, similarly to that observed in

brain tissue studies by Neumann et al (**Neumann M et al 2005**), but much less than that observed in exon trapping experiments (**L. Varani et al, 1999**).

Overall, although the role of such structural elements in regulation of pre-mRNA splicing remains understood, the emerging picture implies that pre-mRNA sequences may harbour structural elements *in vivo*. In many cases, it has been reported that RNA secondary structure also influences the outcome of pre-mRNA splicing (**Buratti,E et al 2004**). Conserved stem-loop regions within introns have been recently shown to play a role in the pre-mRNA splicing processes of human Spinal Muscolar Atrophy (*SMN*) exon 7 (**Singh,N.N et al, 2007**), human COL2A1 exon 2 (**McAlinden,A et al 2005**), the internal splicing enhancer region in the fibronectin gene (**Buratti,E. and Muro,A.F et al, 2004**) or to silencer regions in the presenilin 2 gene (**Higashide S et al, 2004**). Interestingly, RNA secondary structures have also been proposed to play a role in helping the splicing machinery to distinguish between real exons and pseudoexon sequence (**Zhang,X.H et al, 2005**). There are several mechanisms through which RNA secondary structure can impact splicing: it can present binding sites for auxiliary splicing factors, it can bring widely separated cis-elements into juxtaposition, or it can directly affect accessibility of the splice sites.

However, we cannot exclude other effects due to the E10+4 mutation, that can increase or disrupt the interaction of different *trans*-acting splicing factors with the proximal downstream intron of exon 10. This *cis*-regulatory sequences contains not only the putative stem loop but also two regulatory elements: an intronic splicing silencer (residues 11-18) and an intronic splicing modifier directly downstream of the ISS (residue 19-26) (**D'Souza I, Schellenberg GD et al, 2002**). Investigation of dementia pedigrees have established that mutations in the downstream region of exon 10 result in a misregulation of tau exon 10 and cause disease. Although many regulators involved were unknown, a recent study based on minigene construct showed that the SR protein 9G8 directly interacts whit the proximal downstream intron of exon 10 and inhibit inclusion of exon 10. 9G8 is the first splicing factor involved in the regulation of this region, which is critical to both the splicing regulation and the disease causing potential of the tau protein (**Lei Gao et al, 2007**).

Hypothesizing a compound molecular effect of the [T(-15)C] and E10+4 mutations on the increased splicing of E10, we investigated the condition of the patient's parents, heterozygous for either of the mutations, and phenotipically and cognitively healthy.

Cotrasfection experiments of mutant and *wild-type* minigenes demonstrate that if only one mutation is present in heterozygous condition, the [t(-15)c] in the father or the E10+4 in the mother, the ratio of E10 including/E10 excluding transcripts is quite normal. Thus we established that [T(-15)C] or E10+4 mutation alone were not able to alter alternative splicing of exon 10. Both mutations are important for the resulting altered tau4R to tau3R ratio.

In summary, we demonstrated that the two novel mutation lead to an overproduction of 3R tau isoforms altering the Tau 3R: Tau4R ratio as a final outcome. Although the immunohistochemical and biochemical analysis evidenced abundant neuronal and glial tau inclusions exclusively composed of 3R tau isoforms we cannot completely associate the altered alternative splicing to the neurodegenerative process considering that the younger compound heterozygous sister of the index patient presents only with very slight cognitive troubles that have to be taken into account for a clinical follow-up but are insufficient for a clear diagnosis.

On the basis of our findings we hypothesize a *trans*-acting regulatory effect of the both mutations, with known or unknown splicing factors, which might have contributed to the very atypical clinical and pathological FTD phenotype of the sporadic FTD index patient. Understanding how these alternative splicing events are regulated by *cis*-acting sequence elements and *trans*-acting splicing factors is a major challenge in functional genomic studies. Identification of critical regulators for tau exon 10 alternative splicing not only helps to understand the regulatory mechanism underlying the alternative splicing, but also provides useful information for designing future therapeutic approaches to correct the tau splicing defects. The activation or inhibition of specific factors expression in the adult brain may have therapeutic potential in patients carry tau splicing mutation that cause excessive Exon 10 inclusion or exclusion respectively.

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