Mutations in the tau gene that cause an increase in three repeat tau and frontotemporal dementia

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Summary
The majority of cases with frontotemporal dementia (FTD) have no tau deposition in the brain, yet mutations in the tau gene lead to a similar clinical phenotype with insoluble tau depositing in neuropathological lesions. We report two tau gene mutations at positions +19 and +29, in the intronic sequences immediately following the stem loop structure in exon 10, which segregate with FTD. Exon-trapping experiments showed that these gene mutations alter the splicing out of exon 10 and produce an increase in tau isoforms with three microtubule binding domains (three repeat tau). Mutagenesis experiments demonstrated that the +19 mutation was responsible for the increase in three repeat tau, possibly by altering an intron silencer modulator sequence element found at this region of the gene. Microtubule binding experiments revealed a significant decrease in microtubule assembly with increasing amounts of three and decreasing amounts of four repeat tau. Brain autopsy was available in one case. Analysis of the type of soluble tau isoforms revealed an increase in three repeat tau and an absence of tau isoforms with exon 3 inserts. No insoluble tau was isolated in the tissue fractions, consistent with the absence of tau-positive histopathology. There was also an increase in tau degradation products suggestive of increased proteolysis. This increase in tau breakdown products was associated with TUNEL- and activated caspase-3-positive neurons identified histologically. These studies show that increases in soluble three repeat tau can be responsible for FTD in cases with tau gene mutations in the intronic region immediately adjacent to the stem loop in exon 10. These cases of FTD have tau isoforms (without exon 3 inserts) that do not form abnormal aggregates and appear more prone to proteolysis. The increase in tau proteolysis was associated with increased evidence of apoptosis. This mechanism of neurodegeneration may be more applicable to the majority of FTD cases, which do not accumulate insoluble tau deposits.

Keywords: apoptosis; four repeat tau; frontotemporal dementia; tau gene mutation; three repeat tau

Abbreviations: 3R = three repeat; 4R = four repeat; FTD = frontotemporal dementia; H & E = haematoxylin and eosin; ISM = intron silencer modulator sequence element; PCR = polymerase chain reaction; SPECT = single photon emission computerized tomography; TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling

Introduction
Frontotemporal dementia (FTD) is the second most common form of early-onset dementia (Ratnavalli et al., 2002) with the majority of cases having no tau deposition in the brain (Mann et al., 2000; Snowden et al., 2002). However, FTD cases with tau gene mutations have insoluble tau deposits in neuropathological lesions (Hodges and Miller, 2001; McKhann et al., 2001; Snowden et al., 2002). Tau gene mutations account for disease in 10–50% of families with FTD, with more than 20 exonic and intronic pathogenic mutations described (Houlden et al., 1999; Kowalska et al., 2001; Morris et al., 2001; Poorkaj et al., 2001a). The tau gene generates six

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48–67 kDa tau isoforms by the alternative splicing of exons 2, 3 and 10. The alternative splicing of exon 10 generates tau isoforms containing either three or four repeats of 31 or 32 amino acids (Goedert and Jakes, 1990). These repeats constitute the microtubule binding domains of the protein (Goedert et al., 1989). Tau is one of the most abundant axonal microtubule-associated proteins having a major role in the assembly of tubulin into microtubules and the stability of microtubules, and therefore overall axonal structure (Garcia and Cleveland, 2001). All six tau protein isoforms are required for normal axonal functions, with microtubule binding being regulated by the number of binding repeats and the phosphorylation state of the protein (Buée et al., 2000; Forman et al., 2000; Lee et al., 2001).

Analysis of mutations in the tau gene has shown a diversity of mechanisms responsible for the characteristic pattern of neurodegeneration (Garcia and Cleveland, 2001; Lee et al., 2001). To date, two classes of tau gene mutations have been described (Garcia and Cleveland, 2001; Lee et al., 2001): missense mutations which alter the microtubule binding properties of tau protein and mutations that alter the splicing of exon 10 to produce an increase in tau mRNA with exon 10 inserts. Missense mutations are located in or around the microtubule binding domains and act via decreasing microtubule assembly, leading to filament destabilization and an increase in cytosolic tau (Buée et al., 2000; Crowther and Goedert, 2000; Garcia and Cleveland, 2001; Lee et al., 2001). All tau gene mutations producing splicing defects increase the levels of four repeat tau isoforms that accumulate as insoluble aggregates in the brain (Buée et al., 2000; Crowther and Goedert, 2000; Garcia and Cleveland, 2001; Lee et al., 2001). These mutations are clustered in the 5′ splice donor site of exon 10. The present study describes two mutations in the tau gene that work via a novel mechanism to increase the ratio of three rather than four repeat tau.

Methods

Pedigrees and linkage analysis

The probands of both pedigrees (Pedigree A, IV-4 and Pedigree B, II-1, see Fig. 1) were recruited as part of a tau gene mutation screen. Both probands presented with cognitive impairment and behavioural change at age 52 years. Other family members were recruited when a possible mutation in the tau gene was identified. Studies were approved by the Human Ethics Committees of the University of Sydney and the University of New South Wales, and informed consent was obtained.

Pedigree A has eight affected family members over four generations, all presenting with profound executive dysfunction and memory problems (Fig. 1A). Linkage analysis was performed using the MLINK program (Badenhop et al., 2001). Pedigree B had no additional family members diagnosed with a dementia syndrome; however, the proband’s brother (III-3, Fig. 1B), father (II-8, Fig. 1B) and grandfather (I-1, Fig. 1B) are said to be alcoholics with gambling addictions.

Mutation screen and detection

Genomic DNA was isolated from blood. All exons of the tau gene (exons 1, 2, 3, 4, 4A, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14) were amplified by polymerase chain reaction (PCR) from genomic DNA using primers derived from 5′ and 3′ intronic sequences (Baker et al., 1997). PCR was performed in a 30 μl reaction volume, using 30 μM of each primer, 1× reaction buffer (PE Biosystems, Foster City, CA, USA), 1.5 mM MgCl₂, 0.2 mM dNTPs, 5% DMSO, and 1.5 units of AmpliTaş Gold (PE Biosystems). One hundred nanograms of genomic DNA were denatured for 12 min at 94°C followed by 35 cycles of amplification (30 s at 94°C, 30 s at 55°C and 30 s at 72°C). PCR products were purified by gel electrophoresis on 2% low-melting agarose gels. Bands were excised and purified using a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) and used for double-stranded sequencing. Fragments were sequenced with a PCR cycle sequencing kit (PE Biosystems) using fluorescent dye terminators on an ABI 377 DNA sequencer (PE Biosystems).

PCR-based restriction enzyme digest assays for the detection of the +19 and +29 mutations were used to screen 100 unrelated control individuals. DNA from control individuals was amplified with primers specific for exon 10 of the tau gene. PCR products were digested with the restriction enzymes PmlI and PstI (New England Biolabs, Beverly, Massachusetts).
MA, USA) and electrophoresed on a 3% agarose gel. The +19 mutant allele was digested by PmlI into 160 and 55 bp fragments. The +29 mutant allele was digested by PstI into 184 and 36 bp fragments.

Exon-trapping analysis of tau exon 10 splicing
Normal and mutant alleles of exon 10 of the tau gene, together with 38 and 69 bp of the 5' and 3' intronic flanking sequence, respectively, were amplified by PCR from patient DNA. Mutant tau alleles included the +19 and +29 mutants and the previously reported S305S and +16 mutations (Hutton et al., 1998; Stanford et al., 2000). PCR products were subcloned into the exon-trapping vector pSPL3 (GIBCO BRL, Life Technologies, Gaithersburg, MD, USA). Mutant and wild type constructs were confirmed by DNA sequence analysis.

COS-7 and 293 cell lines (American Type Culture Collection, Rockville, MD, USA) were plated onto six well dishes at ~70% confluency for 24 h prior to transfection with mutant and wild type tau exon 10-pSPL3 exon-trapping constructs. COS-7 and 293 cells were transfected with 2 μg construct DNA using 6 μl FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Basel, Switzerland) according to manufacturer’s instructions. Transfected cells were harvested 48 h post-transfection. RNA was prepared using a SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA). First strand cDNA was synthesized using 3 μg RNA with 20 pmol SA2 primer (GIBCO, BRL), specific for the pSPL3 exon-trapping vector and SUPERSCRIPT II reverse transcriptase (RT) (GIBCO, BRL). cDNA (0.3 μg) derived from COS-7 and 293 RNA was PCR-amplified using pSPL3 vector-specific primers dUSA4 and dUSD2. Amplification was carried out over 40 cycles and PCR products were resolved on a 3% agarose gel.

The relative amount of exon 10 splicing was semi-quantified by RT–PCR using 0.3 μg of cDNA with pSPL3 specific primers dUSD2 (32P-labelled) (Amersham Bioscience, Little Chalfont, UK) and dUSA4. PCR was performed for 28 cycles and PCR products were resolved on a 6% acrylamide gel. The relative intensity of the bands was quantified using a phosphoimager 445 S1 (Molecular Dynamics, Sunnyvale, CA, USA).

Mutagenesis
Oligonucleotide-directed mutagenesis based on the method described by Kunkel (1985) was used to introduce mutations into the DNA encoding exon 10 of the tau gene. This method requires two rounds of PCR amplification and four different primers. Two mutagenic primers containing the required mutation are the reverse complement of each other; two flanking primers are also needed. The PCR template (tau exon 10) was subcloned into pGEM-T-Easy (Promega Corporation) and the flanking primers were vector specific to T7 and Sp6 primers (Beckman Instruments, Sydney, Australia), respectively.

In the first round of PCR amplifications, two sets of amplifications were performed with the forward mutagenesis and Sp6 primer together, and the reverse mutagenesis and T7 primers together. The +19 normal C residue (+19C) was mutated to an adenine and to a thymine to generate mutants +19A and +19T, respectively (Fig. 4B). The first residue of codon 304 (GGC) in exon 10 of the tau gene was mutated to a cytidine (CGC), which encodes an amino acid change from glycine to arginine to form the G304R mutant (Fig. 4B). Mutagenesis of the 304 codon was also performed on the +19 mutant allele (+19G) to generate the double mutant, +19G/G304R. PCR products were purified by gel electrophoresis on 2% low-melting agarose gels. Bands were excised and purified using a Qiagen Gel Extraction Kit and used in a second round of PCR amplification. Both fragments are combined and re-amplified using primers T7 and Sp6 to generate a PCR product with the desired mutation. Mutagenesis PCR amplification was carried out on a Corbett thermal cycler (Corbett Instruments, Sydney, Australia) using the following profile: 1 cycle of 12 min at 94°C; 5 cycles of 30 s at 94°C, 30 s at 37°C and 30 s at 72°C; 30 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C; and 1 cycle of 5 min at 4°C. Reactions were carried out in 30 μl using 10× PCR Buffer II (PE Biosystems) as described above. Purified PCR products were subcloned in the exontrapping pSPL3 vector and DNA sequence verified by cycle sequencing.

The effect of each mutant construct (+19A, +19T, G304R, +19G/G304R) on splicing of exon 10 of the tau gene was examined by exon-trapping in COS-7 and 293 cells as described above. The normal exon 10 allele and the +16 mutant allele were included as controls.

Brain preparation and tau protein extraction and analysis
The brain of the proband from Pedigree A (IV-4, Fig. 1A) was obtained at autopsy; the posterior part of the right occipital lobe removed and frozen at −80°C, and the remaining brain fixed in 15% buffered formalin for 2 weeks. Crude preparations of soluble and insoluble tau protein were prepared (Lee et al., 1999) from the frozen brain tissue of proband IV-4 from Pedigree A (Fig. 1A) as well as from frozen brain tissue of a well-characterized patient with sporadic Alzheimer’s disease (positive disease control, 75 years old, disease duration of 14 years, homozygous for ApoE4). Briefly, 1 g of cortical grey matter was homogenized in 1.5 ml of buffer containing 0.75 M NaCl, 100 mM 2-(N-morpholino) ethanesulphonic acid, 1 mM EGTA, 0.5 mM MgSO4, 2mM dithiothreitol at pH 6.8, supplemented with protease inhibitors. Homogenates were incubated at 4°C for 20 min and centrifuged at 11 000g for 20 min at 4°C. The supernatant was then removed and recentrifuged at 100 000g for 60 min at 4°C. The resulting
supernatants contained soluble tau, whereas insoluble tau remained in the pellets. For isolation of insoluble tau, the pellets were combined and resuspended in 10 ml (1:10w/v) of extraction buffer (10 mM Tris, 10% sucrose, 0.85 M NaCl, 1 mM EGTA, pH7.4) and spun at 15 000g for 20 min at 4°C. After centrifugation, the pellets were re-extracted in the same fashion and the supernatant was treated with 1% sarkosyl for 1 h at room temperature prior to a final centrifugation at 100 000g for 30 min. The resulting pellet containing crude insoluble tau was resuspended in 50 mM Tris (pH 7.4, 0.2 ml per gram of starting tissue).

Dephosphorylation of the tau proteins was carried out using alkaline phosphatase (Roche Molecular Biochemicals, Basel, Switzerland) at 67°C for 2 h. Samples were run on 10% sodium dodecyl sulphate–polyacrylamide gels and electrophoretically transferred overnight at 4°C to Biotrans™ polyvinylidene difluoride membranes (ICN Bio Medical Products, Costa Mesa, CA, USA). Residual protein sites were blocked by incubation with 5% semi-fat dried milk for 30 min at room temperature followed by overnight incubation in primary antibody at room temperature (Ab-2 Clone TAU-5; NeoMarkers, Fremont, CA, USA; diluted 1:200). All antisera were diluted in 2.5% semi-fat dried milk containing 0.05% Tween-20. Following a 3 × 10 min wash in Tris-buffered saline (TBS) the membrane was incubated in a goat anti-mouse horseradish peroxidase conjugated secondary antibody for 2 h at room temperature (goat anti-mouse IgG peroxidase conjugate; Sigma, St Louis MI, USA; diluted 1:5000). A final 3 × 10 min wash in TBS was carried out prior to visualization using Supersignal® West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions.

Full length cDNA clones encoding six human tau protein isoforms (Goedert and Jakes, 1990) were transformed into BL21(DE3) Escherichia coli for expression. Approximately 1 µl of each isoform was pooled and used as a recombinant tau ladder for western blots.

Tissue histopathology
The cerebellum and brainstem were separated from the cerebrum and the volume of the cerebrum determined prior to being embedded in 3% agar and sectioned at ~3 mm intervals in the coronal plane using a rotary slicer. Microscopic examination was performed on paraffin-embedded 10 µm sections from each of the following brain regions: frontal cortex (Brodman area (BA) 9), cingulate cortex (BA 24), the precentral gyrus (BA 4), parietal cortex (BA 39), temporal cortex (BA 20), occipital cortex (BA 17 and 18), amygdala, hippocampus (at the level of the lateral geniculate nucleus), anterior and posterior basal ganglia (including the basal forebrain), diencephalon, midbrain, pons, medulla oblongata, cerebellar vermis and lateral lobe (including the dentate nucleus).

For diagnostic pathology, sections were stained for haematoxylin and eosin (H & E), modified Bielschowsky silver and Gallyas silver. Immunohistochemistry for 70–200 kD neurofilament (MAS330, Sera-lab, UK, diluted 1:2000/cresyl violet), Tau II (T5530, Sigma, USA, diluted 1:10 000/cresyl violet), ubiquitin (Z0458, Dako, Denmark, diluted 1:200/cresyl violet) and GFAP (Z334, Dako, Denmark, diluted 1:750/luxol fast blue) was undertaken using peroxidase visualization as previously described (Stanford et al., 2000).

For analysis of cell death mechanisms, the in situ Cell Death Detection Kit (Boehringer Mannheim, Mannheim, Germany) was used according to the manufacturer’s specifications to detect DNA fragmentation by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) method as described previously (Broe et al., 2001) and immunohistochemistry for activated caspase-3 enzyme (clone CPP32, Pharmingen, USA, diluted 1:700/cresyl violet) using peroxidase visualization. Ten micron formalin-fixed paraffin-embedded sections taken from the frontal and temporal cortex of proband IV-4 and human colon tissue (positive control) were used in all experiments. The fluorescent secondary label for TUNEL was photo-converted to a standard peroxidase reaction and enhanced using 1% nickel sulphate. The peroxidase sections were counterstained with cresyl violet. Specificity of the TUNEL reaction was tested by omitting the terminal deoxynucleotidyl transferase enzyme from the reaction mixture. Specificity of the immunohistochemistry test was by omitting the primary antibody. No reaction was observed in test sections.

Results
Mutation screen and linkage analysis
DNA sequence analysis of the tau gene exons and the flanking intronic sequences identified two point mutations at positions +19 and +29 in the 5’ splice donor region of exon 10 (Fig. 2). The G→A nucleotide change at position +29 was identified in proband IV-4 from Pedigree A, and in affected relatives IV-1 and III-12, but not in two unaffected relatives III-4 and IV-5 (Figs 1A and 2A). The +29 mutation co-segregated with the disease phenotype throughout multiple generations [logarithm of the odds (LOD) score of 1.02]. The C→G nucleotide change at position +19 was identified in proband III-1 from Pedigree B (Figs 1B and 2B). This nucleotide substitution was absent in the unaffected mother (Figs 1B and 2B). A control population cohort was screened for the presence of the +19 and +29 nucleotide substitutions by PCR-based restriction digest assays with PmlI and Pf/M1, respectively. The +19 mutation was not present in 200 control chromosomes screened. The +29 mutation was identified in one of 200 control chromosomes screened (1/200; 0.5%).

Clinical features
Detailed analysis of the disease phenotype could only be conducted on the current generations within the pedigrees
In Pedigree A, two affected members agreed to participate (IV-1 and IV-4, Fig. 1A), while in Pedigree B, there is only one affected member (III-1, Fig. 1B).

**Individual IV-1, Pedigree A**

This individual was referred for clinical examination at age 52 years for problems with planning and executive function, and anxiety. She had recently stopped working as a salesperson because of her inability to perform necessary tasks. At assessment, she was noted to be anxious and tearful. Neurological examination was normal. She scored 20/30 on the Mini-Mental State Examination. CT scan was normal. Over the following 3 years, her dementia progressed with increasing anxiety and inertia affecting all self-care activities. Her speech became incomprehensible, tone significantly increased in all limbs, and an intermittent tremor in all limbs developed. She was admitted to a nursing home 4 years after symptom onset, aphasic, incontinent and wheelchair-bound. She is presently 59 years old.

**Individual IV-4, Pedigree A**

This individual was referred for clinical examination at age 52 years for problems with speech, planning and executive function, and motivation. He had recently stopped working as a truck driver and road worker because of a back condition. At assessment, he was noted to be unkempt, disinhibited and impulsive, with a fatuous and sometimes impulsive affect. Neurological examination was normal apart from a left palomental reflex. Neuropsychological assessment showed poor attention, concentration and psychomotor speed, with disorientation in place and time, poor calculation, intact verbal memory for meaningful material but impaired new learning ability for both structured and complex information. He scored 20/30 on the Mini-Mental State Examination. CT scan and EEG were normal. Dementia was rapidly progressive with noticeable inertia 8 months later affecting all self-care activities, and marked weight loss. Speech was decreased and difficult to understand, and he developed L-dopa resistant parkinsonian features (tremor in the left hand, a shuffling festinating gait, a tendency to fall backwards and an inability to turn over in bed). He became mute and immobile, and was admitted to a nursing home, where he died at age 55 years of an acute urinary tract infection 3 years after presentation.

**Individual III-1, Pedigree B**

This individual was referred for clinical examination at age 52 years because the family had noted a behavioural change. He had recently been dismissed from his job as a porter because of erratic and inappropriate behaviour, he was no longer attending to his personal hygiene and he had become generally less communicative. He was treated with a selective serotonin reuptake inhibitor (SSRI) for suspected depression, and his mother thought this led to slight improvement. Over the following months, he became uncharacteristically selfish, irritable and abusive to people in the street and as a passenger in a motor car would urge the driver to run pedestrians over. He spoke to family members about sexual matters, which had not been his custom previously. He overate and talked more. On re-examination 6 months later, he was well oriented in time and place, and could repeat six digits forwards and four in reverse. He recalled only one of four words after a 5 min delay. He generated a total of 31 words beginning with the letters F, A or S in 3 min, and had difficulty performing the serial three hand manoeuvre of Luria. There was prominent pout reflex and bilateral paloments. Lower limb reflexes were absent. A CT scan showed atrophy more prominent frontally (Fig. 3A). A single photon emission computer tomography (SPECT) scan showed frontal hypoperfusion,
particularly on the left (Fig. 3B). His clinical progress was characterized by increasing inertia and apathy. His hygiene deteriorated further and he gained weight. It became evident that he was quite unable to care for himself and he was placed in a long-stay psychiatric facility. Repeat MRI 2 years later showed marked frontal atrophy and repeat SPECT showed marked frontal hypoperfusion.

**Exon-trapping**

As exon 10 of the tau gene is normally alternatively spliced, we expected to see mRNA with and without exon 10 in normal transcripts, corresponding to PCR products of 270 bp and 177 bp, respectively. Expression of the +19 and +29 alleles caused a decrease in the splicing of tau exon 10 (Fig. 4A and B). This corresponded to a decrease in 4R tau isoforms. Expression of the S305S allele and +16 allele caused an increase in the splicing of tau exon 10 (Fig. 4A and B) as previously reported (Stanford et al., 2000). This corresponded to an increase in the 4R tau isoforms. Decreased splicing of tau exon 10 by the +19 and +29 point mutations and increased splicing by the S305S and +16 mutations was observed in both COS-7 and 293 cells (Fig. 4A and B).

In both 293 and COS-7 cells, the +19 mutation resulted in a significant 15–18 fold decrease in the ratio of splicing of exon 10 (Fig. 4C and D, \(P < 0.005\), 2-tailed \(t\)-test, \(n = 5\) per group). Similarly, the +29 mutation resulted in a lesser, but still significant, 2–3 fold decrease in exon 10 splicing in both cell lines (Fig. 4C and D, \(P < 0.05\), 2-tailed \(t\)-test, \(n = 5\) per group). These results contrast with our positive mutation controls (Fig. 4C and D), +16 and S305S, which resulted in over a 2.5 fold increase in the ratio of splicing in both cell lines (\(P < 0.0005\), 2-tailed \(t\)-test, \(n = 5\) per group).

**Mechanism of alternative splicing by the +19 mutation**

The +19 point mutation in the 5′ splice donor site of exon 10 of the tau gene causes a decrease in exon 10 splicing. The +19 point mutation is a C→G nucleotide change at the base of the stem-loop structure in the 5′ splice donor site of exon 10 (Fig. 2). The +19 normal nucleotide was mutated to an adenine (+19A) and a thymine (+19T) to destabilize the stem loop (Fig. 4E). A double mutant (+19G/G304R) was also created to restore the Watson–Crick base pairing and to stabilize the stem loop structure by mutating codon 304 in exon 10 from GGC to CGC. If the stem loop structure was the crucial mechanism in determining the efficiency of exon 10 splicing, the double mutation should re-stabilize the stem loop structure and restore wild type splicing.

The naturally occurring +19G mutation caused a significant reduction in exon 10 splicing (\(P = 0.02\), 1-tailed \(t\)-test). The +19G/G304R double mutant caused a 1.8-fold decrease in exon 10 splicing (Fig. 4F and G). This contrasts with the single G304R mutant that resulted in a 1.5-fold increase in exon 10 splicing (Fig. 4F and G). Expression of the +19A and
+19T mutants resulted in a small non-significant increase in exon 10 splicing compared with the normal exon 10 +19C allele (Fig. 4F and G).

**Tau protein analysis and histopathology**

The brain of proband IV-4 from Pedigree A was collected at autopsy and the posterior part of the right occipital lobe removed and frozen prior to fixation of the remaining tissue. Soluble and sarkosyl insoluble tau was extracted from the fresh brain tissue of proband IV-4 and analysed by western immunoblotting using the phosphorylation independent antibody tau-5. Fresh brain tissue from a well-characterized Alzheimer’s disease case was used as a disease control. Following alkaline phosphatase treatment, soluble tau was detected as four major bands which migrated with apparent molecular weights of 59, 54, 52 and 48 kDa (Fig. 5F) from both proband IV-4 and the Alzheimer’s disease case. The four bands aligned with recombinant tau containing three repeat (3R) tau without exons 2 and 3 (48 kDa), four repeat (4R) tau without exons 2 and 3 (52 kDa), 3R tau with exon 2 (54 kDa) and 4R tau with exon 2 (59 kDa). Both the 54 and 52 kDa bands were stronger than that of the 59 and 48 kDa bands in both diseased brain samples (Fig. 5F). However, the intensity of the 3R 54 kDa band was much greater in IV-4 compared with the Alzheimer’s disease case, despite equal amounts of protein being loaded (Fig. 5F). In addition to these well-characterized full length tau isoforms, lower molecular weight isoforms were visible in both cases (Fig. 5F). These bands migrated with apparent molecular weights of between 15–30 kDa. The intensity of these bands was greater in IV-4 compared with the Alzheimer’s disease case (Fig. 5F).

Sarkosyl insoluble tau was clearly visible in the Alzheimer’s disease case and migrated as three bands with apparent molecular weights of 67, 62 and 59 kDa (Fig. 5F). Upon dephosphorylation using alkaline phosphatase, these
separated into all six isoforms (data not shown) corresponding to previously described classical paired helical filament tau (Goedert et al., 1992). No sarkosyl insoluble tau was visible in IV-4, despite comparable protein concentrations being loaded onto the SDS-PAGE gels (Fig. 5F).

Examination of the fixed brain specimen of proband IV-4 from Pedigree A revealed gross knife-edged atrophy of the frontal lobes, with sparing of the motor and premotor regions (Fig. 5A–C) and severe atrophy of the right temporal lobe sparing the medial parahippocampal and hippocampal regions (Fig. 5C–E). The brain specimen weighed 1062 g. Examination after coronal sectioning revealed a severe loss of frontal grey matter, atrophy and discolouration of the head of the caudate nucleus, and excessively enlarged lateral ventricles (Fig. 5D, E).

**Fig. 5** Gross morphology of the brain of individual IV-4 from Pedigree A and western immunoblots of tau isoforms from brain extracts from proband IV-4 and an Alzheimer’s disease case. (A–C) Photographs of the external features of the brain at equivalent scales. Knife-edged atrophy was found in the frontal lobes (A–C) and right temporal lobe (C*). (D, E) Representative coronal sections of the brain at equivalent scales. Knife-edged atrophy was confirmed in the frontal lobes (A–C) and right temporal lobe (C–E*), largely sparing the medial parahippocampal and hippocampal regions (E). Internal atrophy was severe in the caudate nucleus (D), mammillary bodies (E arrow), and right amygdala (E) along with excessive enlargement of the right lateral ventricle (E*). (F) Western immunoblotting using the phosphorylation independent tau-5 antibody on brain extract from IV-4 (lanes 3 and 4) and an Alzheimer’s disease (AD) case (lanes 5 and 6). Each sample lane was loaded with 25 µg of total protein. Lanes 1 and 2 correspond to recombinant 4R and 3R tau isoforms, respectively. The intensity of the 3R 54 kDa band was much greater in IV-4 (lane 3). High intensity low molecular weight tau isoforms were also present in IV-4 (lane 3 arrowheads) and to a lesser extent in AD (lane 5 arrowheads). No sarkosyl insoluble tau was visible in IV-4 lane 4, although classic PHF tau bands were observed in AD (lane 6).
Fig. 6 Photomicrographs illustrating specific pathologies in the brain of proband IV-4. The scale bar in (D) is also appropriate for (B–D). The scale bar in (H) is also appropriate for (G) and that in (M) for (J–M). (A) H & E stained section demonstrating widespread cell loss, gliosis and microvacuolar change in the left frontal cortex. (B) H & E stained section showing a cortical ballooned neuron with enlarged cytoplasm and an eccentrically placed nucleus in parietal cortex. (C, D) Ubiquitin- and neurofilament- immunoreactive ballooned neurons in parietal cortex. (E) H & E stained section showing severe neuronal loss in the caudate nucleus. (F) H & E stained section showing severe neuronal loss in the mammillary bodies. (G, H) Sections stained with Gallyas silver and counterstained with cresyl violet demonstrating limited left sided (G) but prominent right-sidedcell loss and microvacuolar changes (H) in the hippocampus. No silver-positive cellular inclusions were found in these or any other sections from this case. (I) Gallyas silver stained section through the hippocampus of the sporadic Alzheimer’s disease case showing numerous silver-positive neurofibrillary tangles. (J–M) TUNEL staining (J, L) and activated caspase-3-immunoreactivity (K, M) showing positive nuclei in the frontal cortex. Condensed TUNEL-positive chromatin and no nucleolus (J, L) with activated caspase-3-positive cytoplasm (K, M) is consistent with irreversible apoptotic mechanisms of degeneration.

tricles (Fig. 5D). The hypothalamus and medial thalamic regions (including the anterior and pulvinar regions) were also severely atrophic with very little tissue remaining in the mammillary bodies (brown and severely shrunken, Fig. 5E). The right superior, middle and inferior temporal gyri were severely atrophic with little underlying white matter (Fig. 5C–E). The right amygdala was severely atrophic, although the hippocampus was relatively spared, and the right anterior horn of the lateral ventricles was asymmetrically enlarged (Fig. 5E).

Cell loss, gliosis and microvacuolar degeneration was particularly severe in the frontal (Fig. 6A and B) and right temporal cortices with some ballooned neurons remaining. Prominent right-sided cell loss and microvacuolar changes were also found in the hippocampus and amygdala, with limited left-sided involvement (Fig. 6G and H). Cell loss was also observed in the relatively intact parietal cortex along with a high density of ballooned neurons (~5/200× field). Ballooned neurons were immunoreactive with neurofilament and ubiquitin (Fig. 6C and D), but not tau antibodies. Sections...
through the basal ganglia and diencephalon showed severe neuronal loss in the caudate nucleus (Fig. 6E) and mammillary bodies (Fig. 6F), with more mild cell loss in surrounding structures. No significant abnormalities were found in the brainstem or cerebellum. No neurofibrillary tangles (positive control in Fig. 6I), Pick bodies, Lewy bodies, tau or ubiquitin positive cellular inclusions or diffuse, neuritic or astrocytic plaques were observed in any of the sections sampled. The distribution and pattern of cell loss, the presence of ballooned neurons and microvacular changes, and the absence of plaques, tangles or Lewy bodies are consistent with a diagnosis of frontotemporal lobar atrophy.

Neurons displaying TUNEL-positive nuclei and activated caspase-3-immunoreactive cytoplasm were abundant throughout the cortex of IV-4 (Fig. 6J–M). A proportion of the TUNEL-positive nuclei was morphologically normal with no typical apoptotic features such as chromatin condensation or cytoplasmic shrinkage. However, numerous caspase-positive neurons were observed. These were distributed throughout the frontal cortex and, to a lesser extent, within the temporal and hippocampal cortices. Upon close examination, these neurons exhibited chromatin condensation and cytoplasmic shrinkage with no discernibly discernible nucleolus (Figs. 6J–M), features consistent with apoptosis (Broe et al., 2001). These neurons did not clump together, as is often observed in necrosis, and were not associated with infiltrates of inflammatory cells.

Discussion

We describe two families with point mutations in the 5′ splice donor region of exon 10 of the tau gene at positions +19 and +29 that cause the typical clinical features of FTD and gross frontotemporal atrophy. The +19 mutation has not previously been described, although the +29 mutation has been reported as a rare polymorphism (D’Souza et al., 1999; Poorkaj et al., 2001a). Our functional experiments showed that the +29 mutation has a similar biological consequence to that shown for the +19 mutation and segregation analysis showed that the +29 mutation co-segregates with the disease phenotype throughout multiple generations (LOD score of 1.02) in Pedigree A, suggesting it is pathogenic. While the +19 mutation was detected in only one individual (III-1, Pedigree B), no polymorphism has been observed at this site. In addition, the patient has all the characteristic features of FTD. The recent identification of tau gene mutations in individuals without a strong family history of disease (Pickering-Brown et al., 2000) also supports the pathogenic nature of this mutation.

The clinical features of our cases with the +29 and +19 mutations are relatively typical of the behavioural presentation of FTD (McKhann et al., 2001). All affected members had initial behavioural problems progressing rapidly to inertia and apathy. Cases presented at the same age (52 years old) and had relatively similar disease courses with rapid deteriorations requiring full care within 1–3 years. These features are similar to those observed in cases with the +13 mutation, but differ from the presentation of disinhibition and restless overactivity observed in cases with the +16 mutation (Pickering-Brown et al., 2002). The similar presentation and disease course of these cases probably reflects a similar pattern and timing of atrophy and degeneration. In one of our cases (IV-4, Pedigree A), frank parkinsonism was a subsequent feature, as described for similar intronic mutations in this region of the tau gene (McKhann et al., 2001; Pickering-Brown et al., 2002). This clinical feature strongly suggests the involvement of basal ganglia circuits, as seen in our case.

Our exon-trapping and mutagenesis experiments suggest that these mutations cause FTD via a novel exon 10 splicing effect. Almost all of the previously characterized intronic mutations in the tau gene have been shown to upregulate the splicing of exon 10 with a corresponding increase in 4R tau (Buée et al., 2000; Crowther and Goedert, 2000; Garcia and Cleveland, 2001; Lee et al., 2001). We have demonstrated that both the +19 and +29 intronic mutations caused a marked decrease in exon 10 splicing, to generate more 3R tau. This effect is similar to that observed with the ΔK280 deletion in exon 10, which also causes a decrease in exon 10 splicing to generate more 3R tau (D’Souza et al., 1999). Previous splicing assays in COS-7 cells on the +29 mutation did not show this effect (D’Souza et al., 1999). In addition, the shorter genomic fragments used previously (33 bp and 51 bp of 5′ and 3′ flanking sequence, respectively, by D’Souza et al. 1999 compared with 59 bp and 70 bp of 5′ and 3′ flanking sequence, respectively, in the present study) may explain some of the differences between results.

As the +19 and +29 mutations are located at the base and outside the stem loop structure, the decreased splicing of exon 10 by these mutations suggests that this splicing is controlled by an alternative mechanism to the stem loop structure. Recent genomic sequencing of the mouse and human tau gene has revealed substantial sequence conservation and provided evidence for numerous regulatory sequences controlling tau gene expression and alternative splicing (Poorkaj et al., 2001b). In particular, regulation of the alternative splicing of exon 10 involves at least five distinct exon splicing silencer and enhancer elements in the intron immediately flanking exon 10 (D’Souza and Schellenberg, 2000; Poorkaj et al., 2001b). The +19 residue has been associated with an intron silencer modulator sequence element (ISM; CCCATGCG) the deletion of which abolishes the splicing of exon 10 (personal communication, Ian D’Souza and Gerard D. Schellenberg, University of Washington, Seattle, USA). Our mutagenesis experiments to restore the Watson–Crick base pairing, in conjunction with those reported for other mutations within the stem loop structure, suggest that both the stem loop and the ISM are involved in the control of the alternative splicing of exon 10 of the tau gene. The stem loop acts as a structural mechanism and the ISM as a sequence specific motif that regulates the alternative splicing of exon 10. It is through the ISM that the +19 mutation probably exerts its effect.
The normal function of tau depends on its ability to remain soluble for rapid interactions with cellular kinases, phosphatases, membranes and the cytoskeleton (Bué et al., 2000; Garcia and Cleveland, 2001). The splicing mutations that increase 4R tau are thought to saturate the microtubule binding sites, leaving an increase of unbound 4R tau to aggregate and cause cell death via filament formation (van Slegtenhorst et al., 2000). As 3R tau is far less efficient at microtubule assembly, an increase in unbound 3R isoforms should cause insoluble tau aggregates if pathogenesis is via a similar mechanism. Soluble fractions from the +29 brain contained both 3R and 4R tau isoforms with increased levels of 3R tau, but tau precipitation was not a feature of this case. A comparison between soluble tau isoforms in other FTD cases with tau gene mutations is likely to be informative concerning potential functional differences, particularly as recent work suggests that it is the increase in soluble intracellular tau protein levels that is important for pathogenesis (Gotz, 2001; Mack et al., 2001). Soluble tau isoforms in cases with the P301L mutation are mainly those lacking exon 3 inserts and, in some cases, there is a similar reduction in 4R soluble and an increase in 3R soluble tau (Hong et al., 1998; Rizzu et al., 2000). Similar findings have also been reported in cases with the +16 mutation (Goedert et al., 1999).

Few other studies report the types and quantities of tau isoforms in the soluble tissue fraction, concentrating on the insoluble abnormal fraction. It is of interest that in our case (+29 mutation) and the cases with soluble tau reported (P301L and +16 mutations), there is a consistent reduction in the longest tau isoforms with exon 3 inserts in the soluble cellular fraction. This may confer more instability in axons requiring these tau isoforms, i.e. the long projection, large diameter axons (Bué et al., 2000). The similarity in this change in cytosolic tau between cases with potentially diverse pathogenic mutations may explain the similarity in the anatomical site of pathology and the similarity in clinical features and functional decline observed.

The mutations reported here cause a decrease in exon 10 splicing generating more 3R tau mRNA. This resulted in an increase in soluble 3R tau in proband IV-4 and no insoluble tau deposition. The majority of patients with FTD (64%) are characterized neuropathologically by frontotemporal neuron loss with no specific diagnostic lesion (Mann et al., 2000; Snowden et al., 2002). Therefore, the distribution and type of degeneration observed in proband IV-4 was typical for FTD, as no insoluble tau was detected or tau-positive intracellular inclusions observed. However, this distribution and type of pathology is unusual for cases with tau gene mutations, as the majority have filamentous pathology composed of hyperphosphorylated tau (Bué et al., 2000; Crowther and Goedert, 2000; Garcia and Cleveland, 2001; Lee et al., 2001). Only one other FTD family has been identified with linkage to chromosome 17, no exonic mutations in the tau gene and no abnormal tau deposition (Zhukareva et al., 2001). In these cases, there is a significant reduction in all tau isoforms in both areas of degeneration and those relatively spared by the disease, despite no change in neuronal tau mRNA (Zhukareva et al., 2001). This differs significantly from current animal models where the transgenic expression of human tau causes increased intracellular tau and neurodegeneration without aggregate formation (Gotz, 2001; Wittmann et al., 2001). A reduction rather than increase in intracellular tau protein has been suggested to be the main pathogenic mechanism for the majority of FTD cases with no specific diagnostic lesions (Zhukareva et al., 2001), although this has already been challenged (Adamec et al., 2001). Of interest is the more recent identification of a number of lower molecular weight bands indicating the presence of tau degradation products (Adamec et al., 2001). In the present study, tau breakdown products were very prominent in proband IV-4 compared with the case of sporadic Alzheimer’s disease. Both foetal (short 3R) and adult tau isoforms are rapidly proteolyzed by calpain in vitro (Bué et al., 2000). The increase in 3R tau isoforms and the lack of stable aggregates are likely to increase tau degradation by proteolysis. This may account for the observed decrease in tau protein in some FTD cases.

The proteolysis of tau and other proteins occurs during apoptosis and in the brain tissue of proband IV-4 apoptotic neurons were observed. This is consistent with previous findings showing colocalization of caspase-3 immunoreactivity and TdT labelling in neurons and astrocytes in FTD cases (Su et al., 2000). Transgenic mice expressing the P301L mutation have TUNEL-positive neurons in addition to those expressing phosphorylated tau (Gotz et al., 2001). Recent brain gene expression profiling on these mice has revealed abnormalities in genes contributing to the inhibition of apoptosis and intracellular transport (Ho et al., 2001). These data suggest that apoptosis is a mechanism of cell death in FTD. Tau is a substrate of the proteases calpain-1 and caspase-3 during apoptosis (Canu et al., 1998; Yen et al., 1999) and tau fragments generated during apoptosis act as effectors to further apoptosis generating a positive feedback intracellular loop (Fasulo et al., 2000). Some mutant tau proteins are either more or less susceptible to proteolysis by calpain-1 (Pickering-Brown et al., 2000) suggesting that some types of tau may be particularly prone to degradation. Our results indicate that an increase in 3R tau leads to cell death via the apoptotic pathway.

Overall, our study demonstrates that an increase in 3R tau is pathogenic via the apoptotic pathway and not through the accumulation of insoluble tau deposits. As the majority of sporadic and familial FTD cases do not have insoluble tau deposits, it is possible that these cases are caused by changes in 3R tau isoforms.

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