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Progressive supranuclear palsy pathology caused by a novel silent mutation in exon 10 of the *tau* gene

Expansion of the disease phenotype caused by *tau* gene mutations

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Summary

Genetic mutations in the *tau* gene on chromosome 17 are known to cause frontotemporal dementias. We have identified a novel silent mutation (S305S) in the *tau* gene in a subject without significant atrophy or cellular degeneration of the frontal and temporal cortices. Rather the cellular pathology was characteristic of progressive supranuclear palsy, with neurofibrillary tangles concentrating within the subcortical regions of the basal ganglia. Two affected family members presented with symptoms of dementia and later developed neurological deficits including abnormality of vertical gaze and extrapyramidal signs. The third presented with dystonia of the left arm and dysarthria, and later developed a supranuclear gaze palsy and falls. The mutation is located in exon 10 of the *tau* gene and forms part of a stem–loop structure at the 5′ splice donor site. Although the mutation does not give rise to an amino acid change in the tau protein, functional exon-trapping experiments show that it results in a significant 4.8-fold increase in the splicing of exon 10, resulting in the presence of tau containing four microtubule-binding repeats. This study provides direct molecular evidence for a functional mutation that causes progressive supranuclear palsy pathology and demonstrates that mutations in the *tau* gene are pleiotropic.

Keywords: progressive supranuclear palsy; frontotemporal dementia with Parkinsonism; corticobasal degeneration; tau; mutations

Abbreviations: BA = Brodmann area; FDG-PET = fluorodeoxyglucose PET; FTDP-17 = frontotemporal dementia with Parkinsonism linked to chromosome 17; PCR = polymerase chain reaction; PSP = progressive supranuclear palsy; RT–PCR = reverse transcriptase–PCR

Introduction

Progressive supranuclear palsy (PSP) (Steele–Richardson–Olszewski syndrome; OMIM 601104) is a relatively rare, progressive neurological disorder characterized by motor and visual symptoms including disturbances in balance and gait, and supranuclear vertical gaze palsy progressing to horizontal gaze palsy. Although behavioural changes and dementia frequently are observed during the course of PSP (Bergeron *et al*., 1998), early severe dementia is rare and, currently, this feature is a mandatory exclusion criterion for the clinical diagnosis of PSP (Daniel *et al*., 1995; Litvan *et al*., 1996). In comparison with Alzheimer's disease, patients with PSP have a more severe executive dysfunction syndrome with explicit learning deficits without retention difficulties (Pillon *et al*., 1995). PSP pathology consists of cerebral atrophy with

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pallor of the substantia nigra and shrinkage of the globus pallidus. Neuropathologically there is neuronal loss and gliosis with abundant neurofibrillary tangles and neuropil threads. The tangles and threads are localized primarily to subcortical regions and are composed of hyperphosphorylated tau (Daniel *et al.*, 1995; Litvan *et al.*, 1996).

The gene encoding the microtubule-associated protein tau has been considered a candidate gene for PSP. An association between a polymorphic dinucleotide repeat and PSP was reported by Conrad and colleagues (Conrad *et al.*, 1997) and this result subsequently has been supported by other studies (Bennett *et al.*, 1998; Higgins *et al.*, 1998; Oliva *et al.*, 1998; Morris *et al.*, 1999*a*). However, the polymorphism was not considered to be biologically significant and it was thought that the marker was in linkage disequilibrium with an undefined mutation within the *tau* gene. More recently, Baker and colleagues showed that an extended haplotype in the *tau* gene was significantly over-represented in PSP individuals (Baker *et al.*, 1999), providing support for the hypothesis that the *tau* gene is associated with PSP.

The *tau* gene previously has been linked to pedigrees with frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) (Foster *et al.*, 1997). FTDP-17 presents clinically with behavioural and personality changes, cognitive decline, deterioration in speech, memory loss and sometimes Parkinsonism in the later stages. The major neuropathological features are severe atrophy of the frontal and/or temporal cortices, basal ganglia and substantia nigra. Neuronal inclusions composed of abnormally hyperphosphorylated tau are present in most cases. Mutations in the *tau* gene have been demonstrated to cause FTDP-17 (Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998*c*).

In contrast to FTDP-17, PSP has been thought of as primarily a sporadic disease. However, a recent study reported 12 pedigrees with PSP, eight with probable autosomal dominant inheritance (Rojo *et al.*, 1999). This study reports the identification of a novel silent mutation, S305S, in exon 10 of the *tau* gene which results in increased splicing of exon 10 and production of tau containing four microtubule-binding repeats. The mutation is present in two affected sisters with differing clinical presentations, one of whom has clinically and neuropathologically confirmed PSP. This mutation defines the first molecular lesion shown to be responsible for PSP. Our data also reveal the clinical heterogeneity of mutations in the *tau* gene and demonstrate that mutations in the *tau* gene can be pleiotropic.

Methods

Pedigree

The proband (III-14) of the PD6 family (see Fig. 1) was recruited as part of a *tau* gene mutation screen. III-14 presented with cognitive impairment at age 55 years. Other family members were recruited when a possible mutation in the *tau* gene was identified. The proband's sister (III-15) presented at age 48 years with dystonia of the left arm which was followed by dysarthria, supranuclear gaze palsy and falls. She died at age 51 years with pathologically confirmed PSP. The proband's mother (II-9) was institutionalized at a psychiatric hospital for 2 years and died at age 56 years. II-11, aged 82 years, is the unaffected maternal aunt of the proband. 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.

Mutation screen and detection

Genomic DNA was isolated from blood or paraffin-embedded tissue. Exons of the *tau* gene 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, Calif., USA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs (deoxynucleoside triphosphates), 5% DMSO (dimethylsulfoxide) and 1.5 U of Ampli*Taq* Gold (PE Biosystems). A 100 ng aliquot of genomic DNA was 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 by a PCR cycle sequencing kit (PE Biosystems) using fluorescent dye terminators on an ABI 377 DNA sequencer.

A PCR-based restriction enzyme digest assay for the detection of the S305S mutation was used to screen additional pedigree members and 100 unrelated control individuals. DNA from family members and control individuals was amplified with primers specific for exon 10 of the *tau* gene, digested with the restriction enzyme *Pf1*M1 and electrophoresed on a 3% agarose gel. The mutant allele is digested by *Pf1*M1 into 164 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 5′ and 3′ intronic flanking sequence, respectively, were amplified by PCR from patient DNA. Mutant *tau* alleles included the S305S mutation and the previously reported +16 mutation (Hutton *et al.*, 1998). 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, 293 and SK-N-MC cell lines were plated on to 10 cm plates 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 3.75 µg of construct DNA using 11.5 µl of Transfast reagent (Promega,

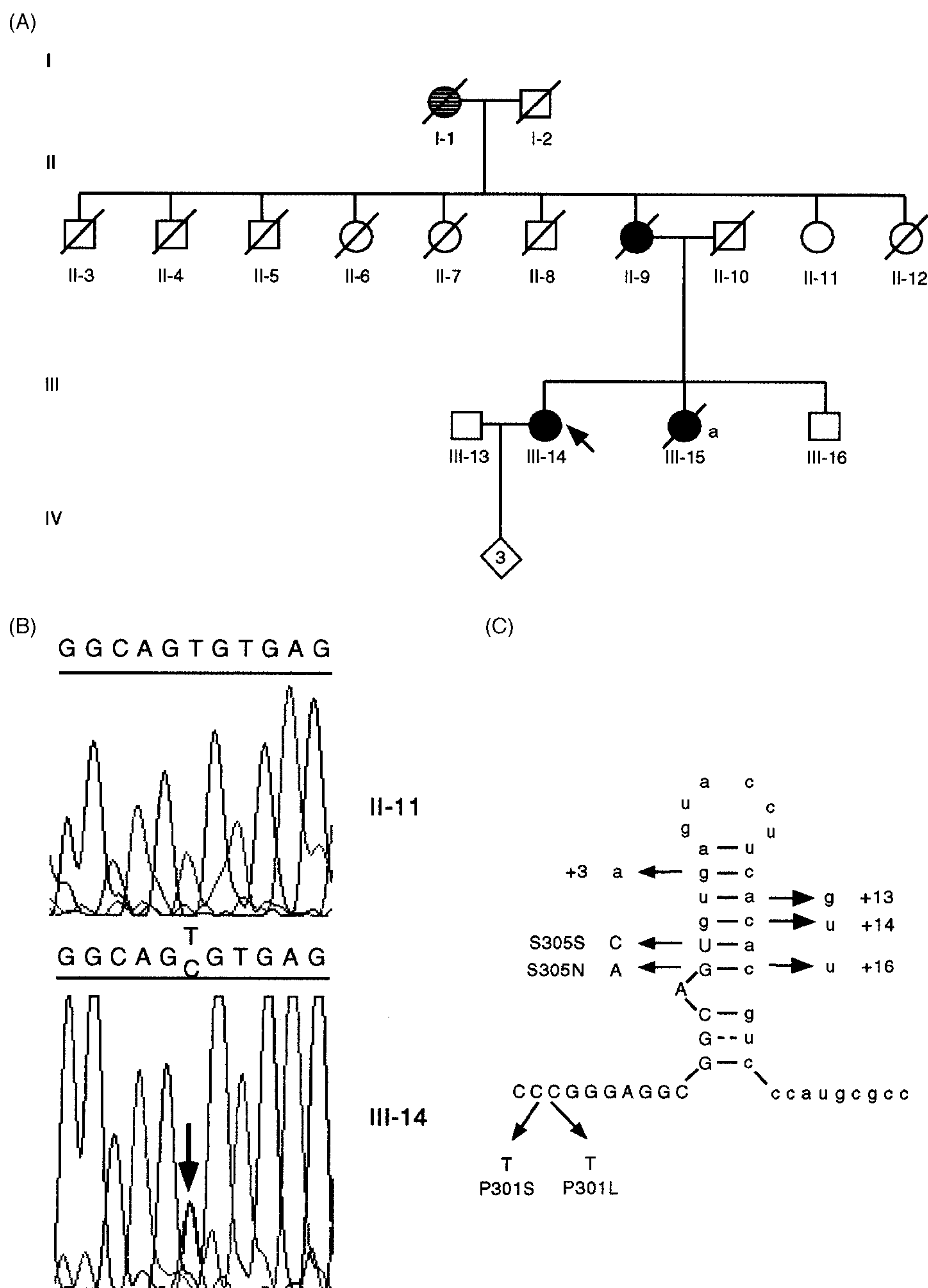

Fig. 1 Identification of a novel silent mutation in the *tau* gene. (**A**) Pedigree PD6. Symbols designating affected patients are shaded in black; the hatched individual is inferred to be affected by family history. Squares = males; circles = females; a = autopsy performed; ↑ = proband. (**B**) DNA sequence electropherograms showing the heterozygous mutation in III-14 (affected) and the normal sequence in II-11 (unaffected). (**C**) The stem–loop structure at the 5′ splice donor site of exon 10 of the *tau* gene. Splice site (P301L, P301S, S305N, S305S) and intronic (+3, +14, +13, +16) mutations described for FTDP-17, corticobasal degeneration and the PD6 (PSP) pedigrees are shown. Exonic sequences are shown in upper case and intronic sequences in lower case.

Foster City, Calif., USA) according to the manufacturer's instructions. SK-N-MC cells were transfected with 4 μg of construct DNA using 32 μl of Lipofectamine reagent (Gibco-BRL) according to the manufacturer's instructions. Transfected cells were harvested 48 h post-transfection. RNA was prepared using the SV Total RNA Isolation System (Promega, Madison, Wis., USA). First strand cDNA was synthesized using 3 μg of RNA with 20 pmol of SA2 primer, specific for the pSPL3 exon-trapping vector, and SUPERSCRIPT II reverse transcriptase. A 0.3 μg aliquot of cDNA, derived from COS-7, 293 and SK-N-MC RNA, was PCR amplified using pSPL3 vector-specific primers dUSA4 and dUSD2. Amplification was carried out over 40 cycles as described above and the PCR products were resolved on a 3% agarose gel.

The relative amount of exon 10 splicing was semiquantitated by reverse transcriptase–PCR (RT–PCR) using 0.3 µg of 293 cDNA with pSPL3-pecific primers dUSD2 (^{33}P-labelled) 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 quantitated using a phosphoimager 445 S1 (Molecular Dynamics, Sunnyvale, Calif., USA).

Haplotype construction and genotype analysis

Individuals were genotyped for the dinucleotide polymorphism between exon 9 and 10 of the *tau* gene as described by Conrad and colleagues (Conrad *et al.*, 1997) where the A0 allele contains 11 TG repeats. Haplotypes extending the *tau* gene were constructed according to the methodology of Baker and colleagues (Baker *et al.*, 1999).

Neuropathology

The brain of patient III-15 was obtained at autopsy with consent and fixed in 15% buffered formalin for 2 weeks. 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 micron sections from each of the following brain regions: the precentral gyrus [Brodmann area (BA) 4], frontal cortex (BA 9), temporal cortex (BA 20), parietal cortex (BA 39), occipital cortex (BA 17 and 18), cingulate cortex (BA 24), hippocampus (at the level of the lateral geniculate nucleus), amygdala, cerebellar vermis and lateral lobe (including the dentate nucleus), anterior and posterior basal ganglia (including the basal forebrain), diencephalon, midbrain, pons, medulla oblongata and the cerebellar vermis and lateral lobe, including the dentate nucleus. Sections were stained with haematoxylin/eosin and modified Bielschowsky silver. Immunohistochemistry for 70–200 kDa neurofilament (MAS330, Sera-lab, Becton, Leicestershire, UK, diluted 1 : 2000/cresyl violet), tau II (T5530, Sigma, St Louis, Mo., USA, diluted 1 : 10 000/cresyl violet), ubiquitin (Z0458, Dako, Glostrup, Denmark, diluted 1 : 200/cresyl violet) and glial fibrillary acidic protein (Z334, Dako, Glostrup, Denmark, diluted 1 : 750/luxol fast blue) was undertaken using peroxidase visualization as previously described (Halliday *et al*., 1995; Harding and Halliday, 1998).

Semiquantitative analysis of the severity of pathology in the frontal, primary motor (left and right), temporal and parietal cortices, and the caudate, putamen, globus pallidus, substantia nigra, subthalamus and thalamus was performed as previously described and validated (Mirra *et al*., 1991; Verny *et al*., 1996). For each area studied, the following pathological features were evaluated in regions of maximal pathology using a four level scale (at ×200 magnification).

Tissue cubes (1 mm) were sampled from the formalin-fixed specimens of anterior cingulate cortex (a region containing neurofibrillary tangles on light microscopic examination), post-fixed in 1% buffered osmium tetroxide, embedded in Spurr's epoxy resin (ProSciTech, Brisbane, Australia), and sectioned on a Reichardt Ultracut 3. Semi-thin sections were stained with toluidine blue. Ultra-thin sections were stained with 2% uranyl acetate and 2.7% lead citrate and viewed using a Joel 100C electron microscope.

Results

Mutation screen analysis

DNA sequence analysis of exon 10 of the *tau* gene and the flanking intronic sequence identified a T to C transition in the third base of codon 305 in a neuropathologically confirmed case of PSP from pedigree PD6 (Fig. 1). This nucleotide substitution introduces a *Pf1*M1 restriction site which allowed confirmation, in parallel with DNA sequencing, of the presence of the mutation in the second affected individual III-15 and the absence of the mutation in an unaffected aunt, II-11. The mutation was not found in 200 unrelated control chromosomes. This silent mutation (S305S) falls in the predicted stem–loop structure at the 5′ splice donor site of exon 10 (Fig. 1C).

Effect of the S305S mutation on tau *exon 10 splicing*

To investigate the functional consequences of the S305S silent mutation on exon 10 splicing of the *tau* gene, exon-trapping was employed. Wild-type and mutant exon 10 alleles of the *tau* gene were subcloned into the exon-trapping vector pSPL3. Because exon 10 is normally alternatively spliced, we expected to see mRNA with and without exon 10 in normal transcripts, corresponding to PCR products of 270 and 177 bp, respectively. Mutant alleles examined consisted of the novel S305S silent mutation and the previously reported +16 mutation which causes an increase in the splicing of exon 10 (Hutton *et al*., 1998). Expression of the S305S allele caused an increase in the splicing of *tau* exon 10 when examined in either COS-7, 293 or the neuronal SK-N-MC cell lines (Fig. 2), corresponding to the presence of the four-repeat isoform of tau. Increased splicing of exon 10 by the S305S mutation was observed in all three cell lines. The S305S mutation resulted in a significant 4.8-fold increase in the ratio of splicing of exon 10 in 293 cells ($P < 0.01$, Student's *t*-test) with even higher levels of four versus three repeat tau seen in COS-7 and SK-N-MC cells (Fig. 2).

Haplotype analysis

The genotype of a polymorphic dinucleotide marker (GenBank accession no. L77209) was determined for individuals II-11 and III-14, and both were homozygous for the A0 allele. Determination of the extended haplotype based

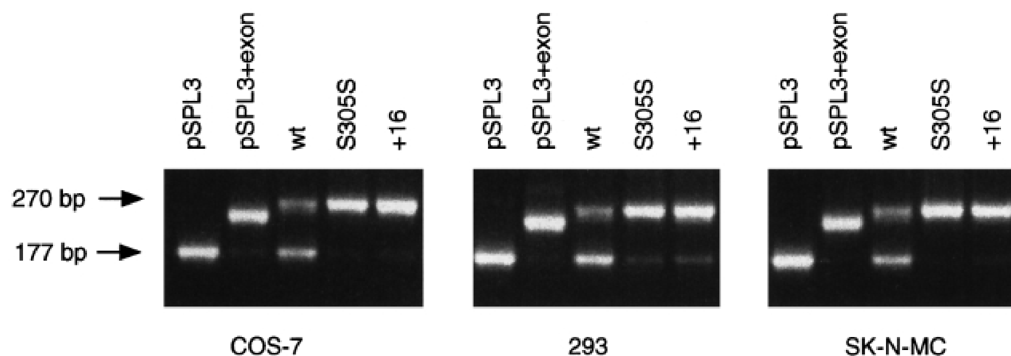

Fig. 2 Effect of the S305S silent mutation on *tau* exon 10 splicing. An ethidium bromide-stained agarose gel of RT–PCR products from splicing assays from COS-7, 293 and SK-N-MC cell lines. The splicing of exon 10 yields a 270 bp band, while its absence results in a 177 bp band. The normal *tau* gene (wt) shows the presence of both exon 10-positive and exon 10-negative mRNA, whereas the S305S and +16 mutations result in a marked increase in exon 10-containing products. The vector-only negative control (pSPL3) reveals only a 177 bp product, while a positive control exon (pSLP3+exon) yields a 244 bp product as expected.

Table 1 *Summary of the clinical signs and symptoms and the stage at which symptoms began during the disease process*

Signs and symptoms	III-14	III-15	II-9
Age at onset (years)	55	48	49
Age at death (years)	–	51	56
Duration (years)	–	4	7
Cognitive decline	Early	–	Early
Falls	–	Mid	Late
Speech disturbances	Mid	Early	Mid
Abnormal vertical gaze	Mid	Early	Mid
Limb involvement	Mid	Early	Mid
	(brisk tendon reflexes)	(rigidity)	(brisk tendon reflex)
Axial rigidity	–	Mid	–

on a further eight single nucleotide polymorphisms, as determined by restriction digests of PCR products from exons 1, 2, 3, 9, 11 and 13 and the presence or absence of a deletion upstream of exon 10 of the *tau* gene, revealed the affected and unaffected individuals had the common H1/H1 haplotype.

Clinical features

Individual III-14

At the age of 55 years, III-14 was referred with concerns about forgetfulness. The family stated that there had been some withdrawal from social and personal interests since her retirement from nursing 2 years earlier. Neurological examination was normal and, although neuropsychological testing revealed no memory impairment, there were deficits in expressive language skills, visuospatial memory and aspects of executive functioning. Retesting 8 months later revealed a decline in frontal executive functions for planning, generating and regulating behaviour. At the age of 58 years, performance on the Mini-Mental State Examination was stable (27/30) but neurological examination was now abnormal, with monotonous voice, mild impairment of voluntary gaze, minimal clumsiness of the left hand, increased tone in the lower limbs and pathologically brisk tendon reflexes with extensor plantar responses (Table 1). She could not perform complex motor sequences. Gait was normal, there was no axial rigidity and she had not had any falls. The deficits at this stage of the disease did not impact on the ability of the patient to live independently at home. Cerebral imaging was performed 2 years after clinical presentation. Mild atrophy of the posterior frontal lobe was detected on MRI of the brain (Fig. 3A), and fluoro-dexyglucose (FDG) PET of the brain demonstrated a marked reduction in cerebral glucose utilization in the frontal and temporal lobes bilaterally (Fig. 3B).

Individual III-15

At the age of 48 years, III-15 presented with a 12-month history of progressive clumsiness of the left hand, slurring of speech, as well as difficulty walking up and down stairs, turning over in bed at night and writing with the right hand. On examination, the left arm was rigid, and held tightly flexed at the elbow. There was marked impairment of fine finger movements. Upward eye movements were restricted. There was no tremor or cognitive impairment (Table 1). Response to levodopa–carbidopa (600/150 mg/day) and bromocriptine was minimal. Further examination at the age of 50 years revealed progressive axial and limb rigidity, with

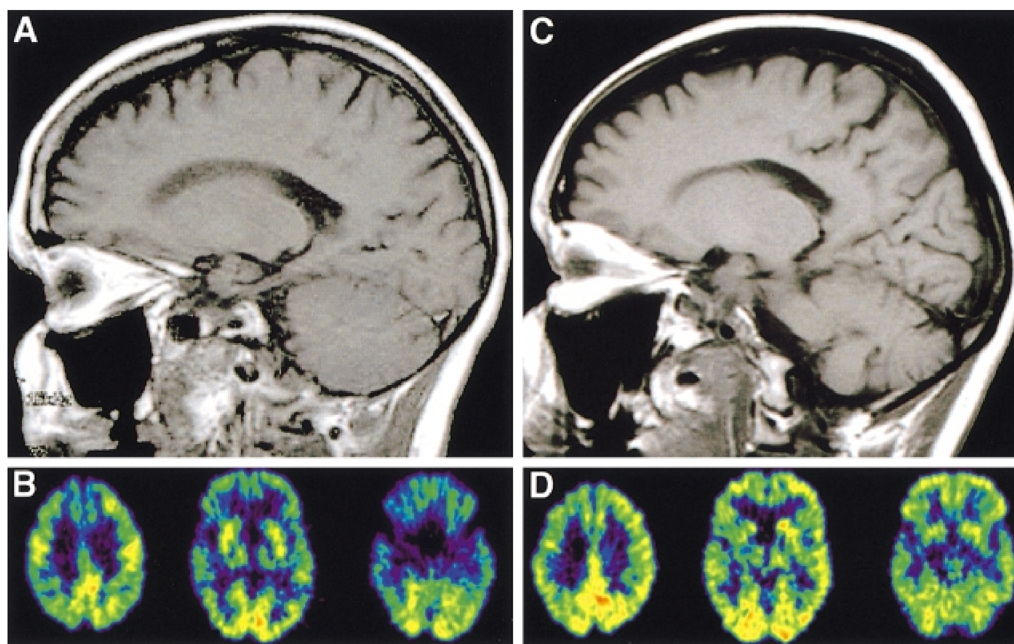

Fig. 3 MRI and FDG-PET imaging of subjects III-14 and III-15. T_1-weighted sagittal MR images show mild frontal atrophy in subjects III-14 (**A**) and III-15 (**C**). Representative transaxial FDG-PET image planes for subjects III-14 (**B**) and III-15 (**D**). There is a marked reduction in glucose metabolism throughout the frontotemporal cortices in III-14 (**B**) in the absence of prominent frontotemporal atrophy on anatomical imaging (**A**). In comparison, in subject III-15 (**D**), the glucose hypometabolism is asymmetric and involves the right striatum, right thalamus and the right mesial and lateral superior frontal cortex. Regions of normal glucose metabolism appear yellow–red and regions of reduced glucose metabolism appear blue–green; the subject's right is on the reader's left.

involuntary grasping movements of the right hand, and prominent supranuclear vertical gaze palsy. There was marked dysphagia and frequent falls, with eventual immobility and dependence for all activities of daily living. Cerebral imaging was performed 3 years after clinical presentation. MRI showed mild atrophy over the vertex of the cerebrum (Fig. 3C), and FDG-PET showed an asymmetric reduction in glucose metabolism in the right superior frontoparietal cortices, the right thalamus and the right striatum (Fig. 3D). Death occurred at age 51 years.

Individual II-9

In 1967, at 54 years of age, the mother of sisters III-14 and III-15 was admitted to a psychiatric hospital with presenile dementia of ~5 years' duration and remained there until her death just before her 57th birthday. On admission, she was childlike and fatuous but was able to give a history. She knew the date, but gave the previous year instead of the current one. She had been incontinent for the previous 9 months. Her illness was marked by memory and language deficits, but she was also recorded as being withdrawn and apathetic, with inappropriate behaviour at times. Late in her illness, she had falls, poor balance and a shuffling gait. Some limitation of upward gaze was noted at one point, together with a fluctuating left sided upper motor neuron facial weakness and an equivocal right plantar response. One year after admission, she had an episode of apparent coma of gradual onset, lasting several hours but with no localizing signs. Three months before she died, she had a further episode of decreased level of consciousness associated with forced grasping of the right hand, brisk, right sided tendon reflexes and an equivocal right plantar response. These resolved after several days; 2 months later she was noted to be wandering about the ward. The next night she was found dead in bed. Autopsy was not performed.

Neuropathology

Individual III-15 died of pneumonia at 51 years of age. On examination after fixation, the brain weighed 1053 g, the leptomeninges were thin and transparent and the vessels at the base of the brain had a normal architectural pattern with no atheroma. There was focal atrophy of the left precentral gyrus and the right precentral and postcentral gyri (Fig. 4A). On the inferior surface of the brain, there was focal atrophy of the anterior inferior temporal and parahippocampal gyri (Fig. 4B). Examination after coronal sectioning revealed additional atrophy of the insular cortex and medial temporal lobe (Fig. 4C) as well as mild loss of the white matter in the body of the corpus callosum and corticospinal tracts.

Cell loss and gliosis was found in areas of atrophy, along with neurofibrillary and glial tangle formation (Fig. 5). Tufted astrocytes, indicative of PSP (Komori, 1999), were observed

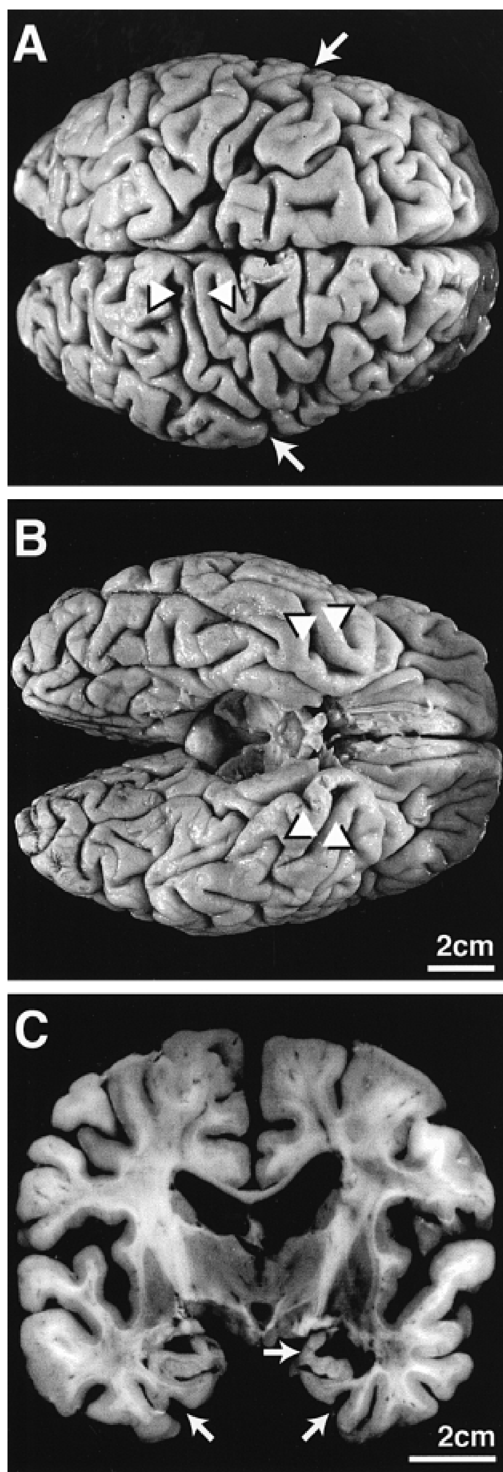

Fig. 4 Gross morphology of the brain of subject III-15. Photographs of the external features of the brain (**A** and **B**, equivalent scales) and a representative coronal section (**C**). Arrows in **A** indicate the central sulcus, arrowheads in **A** and **B** and arrows in **C** indicate regions of atrophy. Focal atrophy was found at the vertex (right side greater than left), particularly in the pre- and postcentral gyri (**A**), and in the anterior medial and inferior temporal lobe (right side greater than left, **B** and **C**).

in all degenerative cortical regions and the amygdala (Table 2). Ballooned neurons were found in the cortical areas of atrophy (Fig. 5D and E) as well as in other frontal regions sampled (Table 2). In contrast, the hippocampus displayed virtually no Alzheimer-type changes with only a few neurofibrillary tangles in the CA1 region and upper layers of the entorhinal cortex, consistent with the age of the patient (Braak and Braak, 1995). Sections through the basal ganglia showed cell loss and gliosis in the globus pallidus, subthalamus and substantia nigra, again with neurofibrillary and glial tangle formation (Fig. 6A–C). Neurofibrillary tangles were also found in the periaqueductal grey region and oculomotor nuclei within the midbrain. No abnormalities were found in the cerebellum. No Lewy or Pick bodies or diffuse, neuritic or astrocytic plaques were observed in any of the sections sampled. Electron microscopic examination of frontal cortex revealed perikarya and cell processes with abundant abnormal filamentous bundles. Neurofibrillary tangles identified in neurons in semi-thin sections were analysed in detail and were largely made up of 10–15 nm twisted and straight filaments (Fig. 6D and E). No paired twisted filaments, indicative of Alzheimer's disease, were observed.

Discussion

The present findings show that the S305S mutation in the *tau* gene causes only mild cortical atrophy and extensive subcortical neurofibrillary tangles, a pathology consistent with the neuropathological diagnosis of PSP. However, of the cases presented, only one of the three had significant movement disorder at onset and this is the only case to have progressed to neuropathological examination. Until recently, because of the scarcity of familial cases, PSP was considered to be largely a sporadic disease. However, PSP recently has been shown to be an autosomal dominant trait and the scarcity of reported familial cases is related to the lack of clinical recognition of the variable phenotypic expression of the disease (Rojo *et al*., 1999).

PSP is, after Parkinson's disease, the most common form of degenerative Parkinsonism. Typically, patients with PSP present with early postural instability and falls, with supranuclear vertical gaze palsy usually occurring 2–3 years after symptom onset (Daniel *et al*., 1995; Litvan *et al*., 1996). Speech disturbances and swallowing difficulties also occur early in the course of the disease, with pyramidal signs eventually developing in at least one-third of patients (Daniel *et al*., 1995; Litvan and Hutton, 1998). Neuropsychological examination reveals an early and prominent executive dysfunction, which is the presenting symptom in at least 8% of PSP patients (Litvan and Hutton, 1998). However, florid frontal lobe symptoms frequently occur throughout the disease, with almost all PSP patients exhibiting concrete thought, difficulty in shifting concepts, decreased word-list generation, preservation, impaired analogical thinking, behavioural disturbances and apathy, as well as disinhibition,

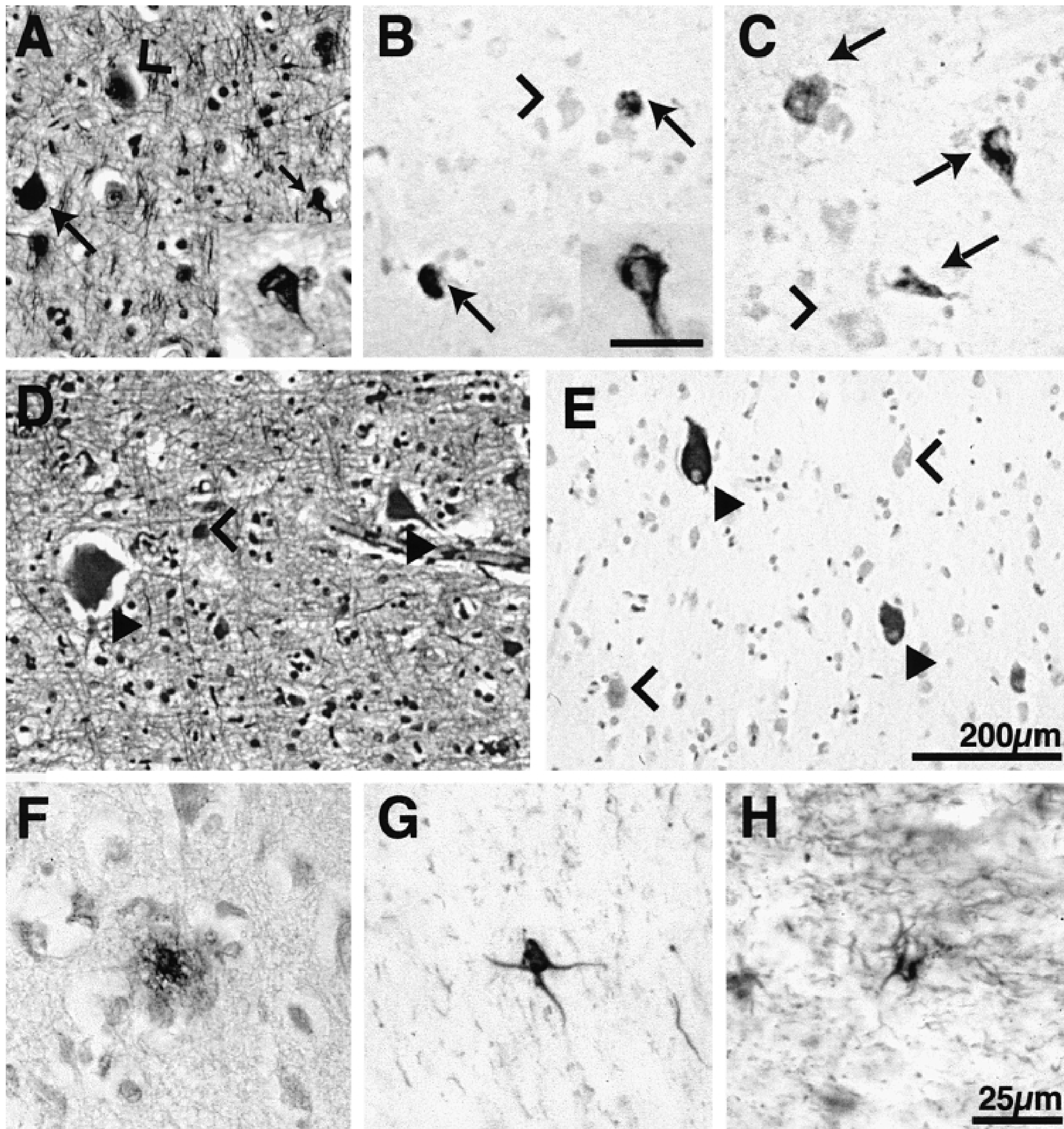

Fig. 5 Microscopic neuropathology of the brain of subject III-15. Photomicrographs of representative cortical (**A**–**F**) and subcortical (**G** and **H**) sections stained with the modified Bielschowsky silver stain (**A** and **D**) or immunohistochemically for tau II (**B**, **C** and **F**–**H**) and phosphorylated 70–200 kDa neurofilaments (**E**). Sections stained immunohistochemically were counterstained with cresyl violet (**B**, **C** and **E**–**G**). The scale in **E** is equivalent to that in **A**–**D** and the insets in **A** and **B** have equivalent scales of 50 μm as indicated by the length of the scale bar in **B**. Examples of normal pyramidal neurons are indicated by open arrowheads, neurofibrillary tangles are indicated by large arrows, a glial tangle is indicated by a small arrow, and ballooned neurons are indicated by closed arrowheads. (**A**, **B** and **C**) A proportion of cortical pyramidal neurons contained silver- and tau-positive neurofibrillary tangles (large arrows and insets). Tangle formation was also found within glial cells (small arrow). These features were concentrated in the regions of atrophy. (**D** and **E**) Ballooned neurons (closed arrowheads) were found throughout the samples of the frontal lobe as well as in atrophic regions. (**F**–**H**) Tau-positive glia were located within the cortex (**F**) as well as in the subcortical white matter (**G**) and basal ganglia (**H**). Tufted astrocytes were observed within the cortex (**F**). These cells had many fine tau-positive appendages within a short distance of their enlarged cell body, giving the impression of a halo around the cell. A fine neuritic network was prominent in the basal ganglia, with some tau-positive coiled bodies evident (**H**). Neuritic pathology- and tau-positive astrocytes were also evident in the subcortical white matter (**H**).

depression and anxiety (Daniel *et al*., 1995; Litvan and Hutton, 1998).

PSP has a distinctive neuropathology which remains the ‘gold standard’ for definitive diagnosis. PSP is characterized by abundant subcortical neurofibrillary and glial tangles with associated neuropil threads (concentrating in the basal

Table 2 *Regional distribution and frequency of microscopic neuropathology in PSP subject III-15*

Region	Neuronal loss/gliosis	Ballooned neurons	Neuritic pathology	Tau-positive glia	Tufted astrocytes
Frontal lobe	1	3	2	1	1
Left motor cortex	2	0	2	3	2
Right motor cortex	3	3	2	3	3
Parietal lobe	1	0	1	2	1
Temporal lobe	1	0	2	0	1
Mean for cortex	1.6	1.2	1.8	1.8	1.6
Caudate/putamen	0	0	0	3	0
Globus pallidus	1	0	3	3	0
Substantia nigra	3	0	3	3	0
Subthalamus	3	0	3	3	0
Thalamus	1	0	3	3	1
Mean for subcortex	1.6	0	2.4	3	0.2

For each area studied, the following pathological features were evaluated in regions of maximal pathology using a four-level scale (at ×200 magnification): neuronal loss/gliosis (0 = absent, 1 = mild, 2 = moderate, 3 = severe); ballooned neurons (0 = absent, 1 = 1/field, 2 = 2/field, 3 = 3+/field); neurofibrillary tangles (0 = absent, 1 = 1–2/field, 2 = 3–5/field, 3 = 6+/field); tau-positive glia (all glial types; 0 = absent, 1 = 1/field, 2 = 2–5/field, 3 = 6+/field); tufted astrocytes (0 = absent, 1 = <1/field, 2 = 1/field, 3 = 2+/field).

ganglia, brainstem and cerebellum) in the absence of β-amyloid deposition (Daniel *et al*., 1995; Litvan, 1997). However, there is considerable variability in the density of lesions found in the subcortical structures (Daniel *et al*., 1995). Ultrastructurally, the tangles comprise predominantly straight filaments composed of abnormally hyperphosphorylated tau. The neuropathology observed in the family studied with the S305S mutation was characterized by neuronal loss in the regulating and output nuclei of the basal ganglia, as well as in focal frontal and temporal cortical regions. Of note was the absence of substantial frontotemporal atrophy. Ballooned neurons were observed in frontal cortical regions, tufted astrocytes were concentrated in cortical regions of atrophy and neurofibrillary and glial tangle pathology were concentrated within the basal ganglia (Table 2). Intracellular filament bundles were composed of many straight and twisted filaments. No paired twisted filaments, indicative of Alzheimer's disease, were observed. In addition, the absence of plaque formation excludes diagnoses of Alzheimer's disease and corticobasal degeneration, while the absence of Lewy or Pick bodies excludes diagnoses of Parkinson's disease, dementia with Lewy bodies and Pick's disease. The absence of gross atrophy and the distribution and type of pathology (tangle formation and tufted astrocytes concentrating in the basal ganglia, amygdala and motor cortices and the absence of astrocytic plaques) are consistent with a diagnosis of PSP (Table 2). Our results agree with those of Komori, who noted that astrocytic plaques are characteristic of corticobasal degeneration and tufts of abnormal fibres are characteristic of PSP (Komori, 1999). A similar distribution of pathology has been observed in cases of FTDP-17 who also have mutations in the *tau* gene (Table 3).

Tau *mutations*

Tau is a microtubule-associated protein that is involved in the neuronal cytoskeleton, in particular the assembly and stability of microtubules. In adult brain, tau consists of six isoforms generated from a single gene by alternative splicing (Goedert *et al*., 1989). The isoforms differ by the presence or absence of 29 and 58 amino acid insertions in the N-terminal half of the protein or by the alternative splicing of exon 10 in the C-terminal half of the protein. The tau protein contains four microtubule-binding repeat motifs which are encoded by exons 9, 10, 11 and 12. The variable splicing of exon 10 generates tau containing either three or four repeat motifs (Goedert *et al*., 1989).

Mutations have been identified in both the exonic and intronic regions of the *tau* gene that lead to FTDP-17 (reviewed in Goedert *et al*., 1998). Intronic mutations (+3, +13, +14 and +16) identified in the 5′ splice donor site of intron 10 cause an increase in splicing by disrupting a stem–loop structure (Hutton *et al*., 1998; Spillantini *et al*., 1998*c*; Goedert *et al*., 1999; Morris *et al*., 1999*b*). The S305S silent mutation which we identified is located in the last codon of exon 10 within the stem–loop (see Fig. 1C). The mutation provides an ideal test of validity of the stem–loop hypothesis since the mutation does not alter the encoded amino acid but causes a nucleotide substitution in the stem–loop structure. Our exon-trapping experiments showed that the S305S mutation caused a significant increase in exon 10 splicing in three different cell lines. Identical conclusions were reached by Grover and colleague (Grover *et al*., 1999). A missense mutation in the same codon (S305N) also caused increased splicing of exon 10 (D'Souza *et al*., 1999; Grover *et al*., 1999; Hasegawa *et al*., 1999; Varani *et al*., 1999), although it was suggested that this mutation did not destabilize the stem–loop structure but rather resulted in the splice donor sequence conforming more closely to the consensus splice donor sequence of constitutively spliced exons [AGgu(a/g) agu] (D'Souza *et al*., 1999). However, the S305S mutation results in a sequence change (GCgugagu) that differs from the consensus donor sequence yet still causes increased

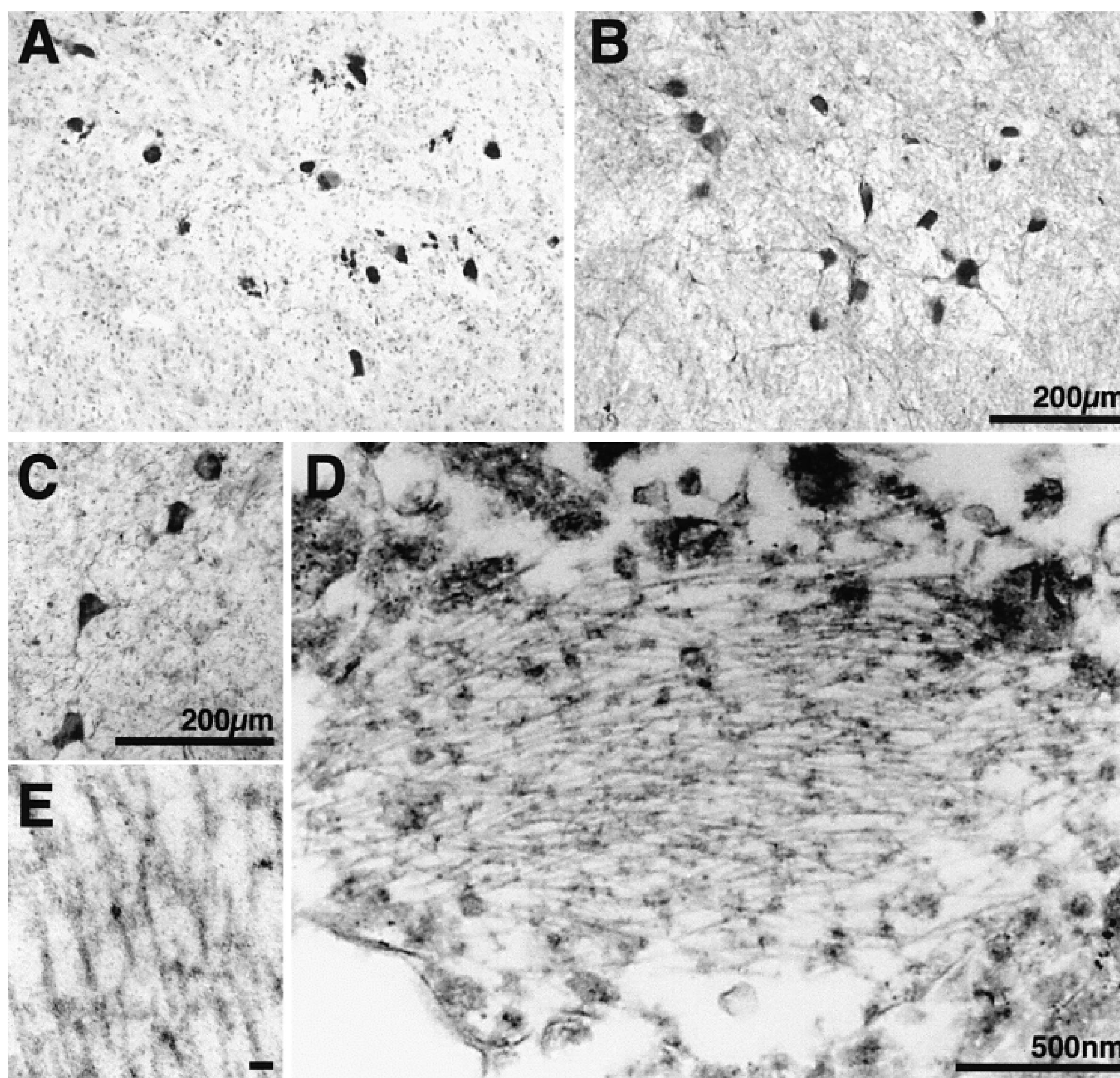

Fig. 6 Microscopic subcortical neuropathology and electron microscopic analysis of the brain of subject III-15. Micrographs of representative regions of the basal ganglia stained with cresyl violet (A) or with tau immunohistochemistry (**B** and **C**), and electron micrographs showing the structure of the intracellular filaments (**D** and **E**). The scales for **A** and **B** are equivalent and the bar in **E** equals 25 nm. (**A** and **B**) Significant depigmentation and neuronal loss were found in the ventral tier of the substantia nigra pars compacta. Gliosis and pigment-laden macrophages were evident (**A**). Many of the remaining neurons were intensely immunoreactive for tau II (**B**). (**C**) Extensive tau immunoreactivity was observed within the neurons and the neuropil of the subthalamic nucleus (**C**) and the globus pallidus (not shown). (**D**) Intracellular bundles of filaments were found in cortical neurons. (**E**) High magnification of a filamentous bundle within a neuron composed of both straight and twisted filaments of ~10 nm in width.

splicing of exon 10. This supports the stem–loop structure as a mechanism regulating alternative splicing in the *tau* gene.

Mutations in exon 10 (see Fig. 1C) (Clark *et al.*, 1998; Hutton *et al.*, 1998; Poorkaj *et al.*, 1998; Spillantini *et al.*, 1998*c*; Bugiani *et al.*, 1999; D'Souza *et al.*, 1999; Iijima *et al.*, 1999; Mirra *et al.*, 1999) either reduce the rate of microtubule assembly or binding or, like the intronic mutations, alter the splicing of exon 10 to generate more four-repeat tau (Table 3). Both the S305S and S305N mutations increase splicing of exon 10 by destabilizing the stem–loop structure. The N279K mutation creates a splicing enhancer element and the L284L mutation is thought to destroy a splicing silencer element (D'Souza *et al.*, 1999), both leading to an increased splicing of exon 10. The reduction in microtubule binding and assembly caused by the P301S and P301L mutations leads to an increase in unbound four-repeat tau which may gain a toxic function with excess free protein available for assembly into filaments (Goedert *et al.*, 1998). The G272V mutation in the microtubule-binding domain of exon 9 also causes a reduction in microtubule binding and assembly in all six isoforms of tau (Hasegawa *et al.*, 1998; Hutton *et al.*, 1998). The V337M mutation in exon 12 (Poorkaj *et al.*, 1998) and the R406W mutation in exon 13 (Hutton *et al.*, 1998) both reduce

Table 3 *Summary of* tau *gene mutations, mechanism of action and neuropathology.*

Mutation	Effect on exon 10 splicing	Microtubule assembly and binding	F/T atrophy	Filament inclusions	Plaque pathology	Tufted astrocytes	Pathology distribution	References*
S305S	Increased	–	Mild	NFT	Absent	Present	PSP-like	
S305N	Increased	–	Mild	CBI	Absent	Absent	CBD-like	1, 2
N279K	Increased	No effect	Mild	PBI, CBI and NFT	Largely absent	Absent	PSP-like	1, 3, 4
P301S	–	Reduced	Severe	CBI and PBI	Absent	Present	FTDP-17 or CBD-like	5
P301L	–	Reduced	None to severe	None or NFT and CBI	Largely absent	Absent	FTDP-17 or PSP-like	6, 7, 8 9
L284L	Increased	–	Moderate	NFT	Present	–	FTDP-17	10
G272V	–	Reduced	Severe	PBI, CBI and NFT	Absent	Absent	FTDP-17 9	6, 7
+3	Increased	–	Mild to severe	NFT	Absent	Present	FTDP-17 or CBD-like or PSP-like	11
+13	Increased	–	–	–	–	–	–	12
+14	Increased	–	Mod to severe	Some CBI and NFT	Largely absent	Absent	PSP-like or FTDP-17	12, 13, 14
+16	Increased	–	Mod to severe	PBI and NFT	Absent	Present	FTDP-17 or PSP-like or CBD-like	12, 15
V337M	–	Reduced	Mod to severe	NFT	Absent	–	FTDP-17	7, 16
R406W	–	Reduced	Mild	NFT	Absent	Present	PSP-like	7, 12, 17

F/T = frontotemporal, NFT = classic and globose neurofibrillary tangles, CBI = corticobasal-like inclusions, PSP = progressive supranuclear palsy, CBD = corticobasal degeneration, PBI = Pick body-like inclusions, FTDP-17 = frontotemporal dementia with Parkinsonism linked to chromosome 17. For the distribution of pathology, FTDP-17 cases are defined by having frontotemporal atrophy with involvement of the amygdala. *1 = Hasegawa *et al*. (1999); 2 = Iijima *et al*. (1999); 3 = Reed *et al*. (1998); 4 = Delisle *et al*. (1999); 5 = Bugiani *et al*. (1999); 6 = Heutink *et al*. (1997); 7 = Hasegawa *et al*. (1998); 8 = Bird *et al*. (1999); 9 = Spillantini *et al*. (1998*b*); 10 = D'Souza *et al*. (1999); 11 = Spillantini *et al*. (1998*c*); 12 = Hutton *et al*. (1998); 13 = Sima *et al*. (1996); 14 = Hong *et al*. (1998); 15 = Goedert *et al*. (1999); 16 = Spillantini *et al*. (1998*a*); 17 = Reed *et al*. (1997).

microtubule binding and assembly by altering the biochemical properties of all six isoforms of tau (Hasegawa *et al*., 1998; Hong *et al*., 1998; Dayanandan *et al*., 1999).

Mutations in *tau* that cause PSP have been suspected from the association of a polymorphic dinucleotide marker (Conrad *et al*., 1997; Bennett *et al*., 1998; Higgins *et al*., 1998; Oliva *et al*., 1998; Morris *et al*., 1999*a*) and an extended haplotype (Baker *et al*., 1999) with the disorder. The a0 polymorphism occurs in ~55% of the population and is present in both affected and unaffected individuals in our pedigree with the S305S mutation. Rojo and colleagues reported the inheritance of the a0 polymorphism by affected and unaffected individuals in two pedigrees with familial PSP that failed to show linkage to chromosome 17 (Rojo *et al.*, 1999). Baker and colleagues reported an over-representation of the H1 haplotype in PSP individuals, suggesting an association between *tau* and PSP (Baker *et al.*, 1999). However, haplotype analysis shows that both affected and unaffected individuals in this study carry the H1 haplotype.

Genetic and phenotypic correlates

Descriptions of the clinical presentations of families with characteristics of PSP have been infrequent until recently. However, a feature of these families is the variability of clinical presentations which often only progress towards more typical signs of PSP after several years of disease (de Yébenes *et al*., 1995; Rojo *et al*., 1999). Documented atypical presentations include dementia, tremor, dystonia and tics (Rojo *et al*., 1999). The family studied here fits this profile, with members presenting with either dementia or motor abnormalities. Variable clinical presentations are observed consistently in the FTDP-17 families with *tau* mutations (Foster *et al*., 1997; Heutink *et al*., 1997; Bird *et al*., 1999; Nasreddine *et al*., 1999). FTDP-17 begins with either behavioural or motor problems. Early cognitive problems include speech disturbances and disorders of executive function (Foster *et al*., 1997). Motor disturbances are corticospinal and/or extrapyramidal (Foster *et al*., 1997), with eye signs usually preserved (Spillantini *et al*., 1998*a*). The behavioural disturbances appear to be the most characteristic, with disinhibition, apathy, defective judgement, poor impulse control, repetitive, stereotypic, compulsive behaviour, psychosis, aggression, alcoholism, hyperorality, early loss of personal awareness and neglect of personal hygiene typifying the disorder (Foster *et al*., 1997; Spillantini *et al*., 1998*a*). The members of the present family do not have the typical behavioural features of FTDP-17. In both FTDP-17 and familial PSP, it has been noted that the disease is difficult to recognize and distinguish clinically from other similar syndromes because of the wide-ranging variability in clinical presentation (Spillantini *et al*., 1998*a*; Bird *et al*., 1999; Rojo *et al*., 1999). Our results suggest that these disorders have similar genetic causes, with the need for clinical separation warranting review. It will be important to determine the pathophysiology underlying such variability.

Our findings also indicate considerable variability in the neuropathology of families with *tau* mutations (reviewed in Table 3). Such mutations do not always produce gross frontotemporal atrophy as expected from the FTDP-17 literature. In many of these cases, the distribution of pathology is similar to that in PSP. However, the type of cellular tau deposition often differs, with most cases having tau pathology similar to that observed in corticobasal degeneration (Table 3). It should be noted that in some FTDP-17 cases the pathology described appears similar to that observed in the present study (Table 3) and would similarly fulfil international criteria for the neuropathological diagnosis of PSP (Daniel *et al*., 1995; Litvan, 1997). A feature observed in the affected individual in the present study, but not highlighted in previous studies, was the presence of tufted astrocytes, a feature now thought to aid in the differential diagnosis of PSP (Feany *et al*., 1996; Bergeron *et al*., 1997). Asymmetrical clinical disease was related to asymmetrical pathology in the motor cortices (Fig. 5), a pathological association also highlighted previously in a number of PSP cases (Barclay and Lang, 1997; Bergeron *et al*., 1997). It will be important to determine the cellular mechanisms influencing such variable neuronal vulnerability in patients with *tau* mutations.

Historically, neurodegenerative diseases have been defined by clinical presentation and neuropathology. Mutations in the *tau* gene can cause either FTDP-17 (Foster *et al*., 1997), corticobasal degeneration (Bugiani *et al*., 1999), progressive subcortical gliosis (Goedert *et al*., 1999), multiple system tauopathy dementia (Spillantini *et al*., 1998*c*) and, now, PSP (Delisle *et al*., 1999; this study), thus demonstrating pleiotropy. The clear variability in both clinical and neuropathological parameters in patients with diseases caused by mutations in the *tau* gene necessitates rethinking this issue. That members of the same family can have diverse clinical presentations (Bird *et al*., 1999; Bugiani *et al*., 1999; Nasreddine *et al*., 1999) or variable neuropathology (Table 3) suggests that additional genetic and/or environmental factors influence the regional, tissue-specific and temporal expression of the disease. It will be important to determine these factors in order to understand the disease process further.

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