**Tau Gene Mutation G389R Causes a Tauopathy with Abundant Pick Body-like Inclusions and Axonal Deposits**

**JILL R. MURRELL, PHD, MARIA GRAZIA SPILLANTINI, PHD, PAOLO ZOLO, MD, MARIO GUAZZELLI, MD, MICHAEL J. SMITH, MASATO HASEGAWA, PHD, FRANCESCO REDI, MD, R. ANTHONY CROWTHER, PHD, PIETRO PIETRINI, MD, PHD, BERNARDINO GHETTI, MD, AND MICHEL GOEDERT, MD, PHD**

**Abstract.** Exonic and intronic mutations in *Tau* cause familial neurodegenerative syndromes characterized by frontotemporal dementia and dysfunction of multiple cortical and subcortical circuits. Here we describe a G389R mutation in exon 13 of *Tau*. When 38 years old, the proband presented with progressive aphasia and memory disturbance, followed by apathy, indifference, and hyperphagia. Repeated magnetic resonance imaging showed the dramatic progression of cerebral atrophy. Positron emission tomography revealed marked glucose hypometabolism that was most severe in left frontal, temporal, and parietal cortical regions. Rigidity, pyramidal signs, and profound dementia progressed until death at 43 years of age. A paternal uncle, who had died at 43 years of age, had presented with similar symptoms. The proband’s brain showed numerous tau-immunoreactive Pick body-like inclusions in the neocortex and the fascia dentata of the hippocampus. In addition, large numbers of tau-immunoreactive Pick body-like inclusions were present in axons in the frontal, temporal, and parietal lobes. Immunoblot analysis of sarkosyl-insoluble tau showed 2 major bands of 60 and 64 kDa. Upon dephosphorylation, these bands resolved into 4 bands consisting of three- and four-repeat tau isoforms. Most isolated tau filaments were straight and resembled filaments found in Alzheimer disease and some frontotemporal dementias with tau mutations. A smaller number of twisted filaments was also observed. Biochemically, recombinant tau proteins with the G389R mutation showed a reduced ability to promote microtubule assembly, suggesting that this may be the primary effect of the mutation. Taken together, the present findings indicate that the G389R mutation in *Tau* can cause a dementing condition that closely resembles Pick’s disease.

**Key Words:** Axonal tau deposits; Brain metabolism; Frontotemporal dementia; Pick body-like inclusions; Tauopathy.

**INTRODUCTION**

Abundant neurofibrillary lesions made of the microtubule-associated protein tau constitute a defining neuropathological characteristic of Alzheimer disease (AD) (1). Filamentous tau protein deposits are also the defining neuropathological characteristic of other neurodegenerative diseases, many of which are frontotemporal dementias (2), primary progressive aphasia, or movement disorders that have been subsumed under the heading of “Pick complex” (3). Recent work has shown that mutations in *Tau* cause familial frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (4–19). Known *Tau* mutations are either intronic mutations located close to the splice-donor site of the intron following exon 10 or missense, deletion, and silent mutations in the coding region.

Six tau isoforms are produced in the adult human brain by alternative mRNA splicing from a single gene (1). They differ from each other by the presence or absence of 29- or 58-amino acid inserts located in the aminoterminal half and an additional 31-amino acid repeat located in the carboxy-terminal half. Inclusion of the latter, which is encoded by exon 10, gives rise to the 3 isoforms with 4 repeats each (20); the other 3 isoforms have 3 repeats each. The repeats and some adjoing sequences constitute the microtubule-binding domains of tau (21, 22). Similar levels of three-repeat and four-repeat tau isoforms are found in normal cerebral cortex (23), and the tau filaments from AD brain contain all 6 tau isoforms in a hyperphosphorylated state (24).

Four intronic mutations have been described in FTDP-17 at positions +3, +13, +14, and +16 of the intron following exon 10 of *Tau* (with the first nucleotide of the splice-donor site taken as +1) (5, 6). The S305N mutation in exon 10 is also located close to this splice-donor site (10). All 5 mutations destabilize a stem-loop structure located at the exon 10-intron boundary, leading to inclusion of exon 10 in the final tau transcript and resulting in the overproduction of tau isoforms with 4 repeats (5, 6, 12, 15, 25–28). Where analyzed, these mutations lead to a tau pathology consisting of wide twisted ribbons made of four-repeat tau isoforms that are present in nerve cells and glial cells (12, 18, 29, 30). Coging region mutations are missense, deletion, or silent mutations. Experimental studies have shown that most of these mutations reduce the ability...
of tau to interact with microtubules (25, 31, 32), whereas others influence splicing in of exon 10 (8, 15, 18, 26, 27). Moreover, several missense mutations also stimulate heparin-induced assembly of tau into filaments (33, 34). Coding region mutations are concentrated in the microtubule-binding repeat region, where they are located in exons 9 (G272V), 10 (N279K, L284L, Q280, P301L, P301S, S305N) and 12 (V337M). The mutations in exon 10 that reduce the ability of tau to interact with microtubules lead to a pathology made of narrow twisted ribbons that consist predominantly of four-repeat tau isoforms and are found in nerve cells and glial cells (2, 31, 35). By contrast, the missense mutations located outside exon 10 that have been examined lead to a neuronal tau pathology made of Alzheimer-type paired helical and straight filaments that consist of all 6 tau isoforms (19, 36).

To date, only 1 mutation (R406W) has been described in exon 13, which is located outside the microtubule-binding repeats (5, 19). We report here an additional missense mutation in exon 13 of Tau in a familial form of frontotemporal dementia. It changes glycine residue 389 to arginine (G389R). Neuropathologically, this familial tauopathy is characterized by large numbers of Pick body-like inclusions and an extensive filamentous tau pathology in axons. Tau filaments are straight or twisted and contain three- and four-repeat tau isoforms. The straight filaments closely resemble the straight filaments found in AD and in tauopathies with the V337M and R406W mutations. By contrast, the twisted filaments differ from the paired helical filaments of AD. Like most other missense mutations, the G389R mutation markedly reduces the ability of recombinant tau to promote microtubule assembly.

MATERIALS AND METHODS

Pedigree and Clinical Evaluation of Proband

The family of the proband, identified here as "family F" derives from central Italy (Tuscany). We were able to reconstruct a pedigree consisting of 23 members over 4 generations (Fig. 1). The proband (subject III-2) developed neurological symptoms at 38 years of age. In spring 1992, he presented neurological disturbances affecting activities of daily living. He underwent neurological, psychiatric, and neuropsychological examinations. Electroencephalography (EEG), magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), and positron emission tomography (PET) were carried out. PET examination was performed to determine regional cerebral glucose metabolic rates in the resting state following published protocols (37-39). A second SPECT and a second PET scan were done in 1993 and a second MRI in 1995. An all-night sleep EEG was recorded on 2 separate occasions 9 months apart. The patient died in 1997 at age 43 and a brain autopsy was performed.

Fig. 1. Pedigree of Family "F." Three male subjects (I-3, II-6 and III-2), shown by symbol □, had been affected with dementia. The proband is indicated by an arrow. Deceased members are indicated by a.

Neuropathology

Neurohistology: At the time of the proband’s autopsy, a few blocks were selected for neuropathological studies. From the remaining parts of the brain, some were fixed and stored in formalin and others were frozen and stored at −70°C. For the neuropathological and immunohistochemical studies, we processed blocks from multiple areas of the cerebral cortex, including frontal, cingulate, temporal, insular, parietal and occipital gyri, hippocampus, caudate nucleus, putamen, globus pallidus, basal forebrain, amygdala, thalamus, hypothalamus, cerebellar cortex, dentate nucleus, midbrain, andpons. Eight 𝜇m-thick sections were obtained from formalin-fixed, paraffin-embedded tissue and stained with hematoxylin and eosin, the Heidenhain-Woelcke method for myelin, the Bodian method for neurofibrils, as well as Congo red and thioflavin S for amyloid. In addition, blocks of formalin-fixed tissue were frozen in liquid nitrogen and sectioned on a cryostat at −20°C. Frozen sections were cut at 10 𝜇m and stained with oil red O to demonstrate tissue lipids.

Immunohistochemistry: Polyclonal antibodies raised against glial fibrillary acidic protein (GFAP) (Biogenex Laboratories) (1:50), α-b crystallin (Vector Laboratories) (1:750), a synthetic peptide corresponding to residues 117–131 of human α-synuclein (1:300) and ubiquitin (Dako) (1:1,000) were used, as were monoclonal antibodies against the amyloid β protein (AB) [1D5 (Athena Neurosciences) (1:100)] and the phosphorylated and nonphosphorylated high-molecular weight neurofilament subunit [SMI 31 and SMI 32, Sternberger Monoclonal (1:1,000)]. Phosphorylation-dependent (p), phosphorylation-independent (p) and dephosphorylation-dependent (dd) anti-tau monoclonal antibodies were used. From the first group (p), we used AT270 (Innogenetics) (1:400), AT8 (Innogenetics) (1:200), AT100 (Innogenetics) (1:400), AT180 (Innogenetics) (1:400), 12E8 (Athena Neurosciences) (1:5,000), PHF-1 (donated by P. Davies) (1:400) and AP422 (1:2,000). These antibodies recognize phosphorylated (p) Thr181 [AT270] (40), pSer202/pThr205 [AT8] (41), pThr212/pSer214 [AT100] (42), pThr231 [AT180] (40), pSer262 and/or pSer356 [12E8] (43), pSer396/pSer404 [PHF1] (44), and pSer242 [AP422] (45). From the second group (p), we used AT250 (donated by P. Davies) (1:250), E10 (donated by A. Delacourte) (1:500), BR134 (1:500),...
PICK BODY-LIKE INCLUSIONS, AXONAL DEPOSITS AND TAU G389R

BR304 (1:500) and BR189 (1:500). A1250 preferentially recognizes a conformation of assembled tau protein (46). E10 is largely specific for tau isoforms with 4 repeats (47). BR134 was raised against amino acids 428–441 of human tau (1). BR304 and BR189 were raised against amino acids 45–73 and 76–87 of human tau; they are specific for the amino-terminal 29- and 58-amino acids inserts of tau, respectively (24). From the third group (24), we used Tau-1 (donated by P. Davies) (1:100), which recognizes an epitope including residues 189–207 of tau (48).

Double labeling studies using anti-GFAP and anti-tau (AT8, AT100, AT180, AT270) antibodies or an anti-neurofilament (SMI 32) antibody and anti-tau antibodies were carried out to demonstrate the location of the tau inclusions present in the white matter and to establish whether they were axonal or glial. Polyclonal anti-GFAP antibody was detected using avidin-biotin, with goat anti-rabbit immunoglobulin as secondary antibody, and horseradish peroxidase-conjugated streptavidin visualized with diaminobenzidine as chromogen. Monoclonal antibody AT8 was revealed by avidin-biotin, with goat anti-mouse immunoglobulins as secondary antibody, and streptavidin conjugated with alkaline phosphatase.

Electron Microscopy: Tissue from cerebral cortex and white matter specimens was fixed in 4% formaldehyde, post-fixed in 1% osmium tetroxide, and embedded in Epon. One-μm-thick sections were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with a Philips 300 electron microscope. For immunoelectron microscopy, formalin-fixed specimens (1 mm²) of frontal cortex were washed in distilled water and placed in Tris-buffered saline, pH 7.6. Fifty-μm-thick sections were cut using a vibratome. Immunogold labelling with AT8 (1:5) was carried out using 10 nm colloidal gold particles (Goldmark Biologicals) conjugated to goat anti-mouse IgG at a dilution of 1:10. Sections were postfixed in 2.5% glutaraldehyde, followed by 1% osmium tetroxide, dehydrated through graded alcohols and embedded in epoxy resin. Semithin sections were stained with toluidine blue. Ultrathin sections were contrasted with lead citrate and uranyl acetate, and scanned with the electron microscope.

DNA Extraction and Mutational Analysis

Informed consent from the next of kin was obtained for DNA studies. DNA from the proband was extracted from peripheral leukocytes, as described (49). Sequencing of the prion protein gene (PRNP) was carried out while the patient was still alive, as described (50). Tau exons were sequenced when it became clear that the patient had a tauopathy. Primers from the intronic sequences surrounding the Tau exons were used, so that the entire exon sequences and the corresponding splice signals could be analyzed. Polymerase chain reaction (PCR) amplification was done with 20ng/μl of genomic DNA, followed by gel purification of the amplified products. To generate single-stranded template for sequencing, asymmetric amplification was performed as described (13). The amplified products were subjected to Qiagen PCR purification spin columns (Qiagen) that remove remaining primers and deoxynucleotides. Dideoxynucleotide sequencing was performed using the US Biochemicals Sequenase kit, [α³²P]dATP (Amersham) and modified T7 DNA polymerase (Sequenase Version 2.0, US Biochemicals/Amersham). To test for the mutation, NcoI or MspI restriction enzyme digestion was done using the exon 13 amplified product using primers 5'-CTTTCTCTGACACCTCATC3' and 5'-CGAGCAAAGCGCATTGAC-3'. The extended Tau haplotype for the proband was determined as described (51). It was confirmed by direct sequencing of Tau exons 1, 2, 3, 9, 11, and 13.

Tau Extraction, Dephosphorylation, and Immunoblotting

Sarkosyl-insoluble tau was extracted from frontal and occipital cortices of subject III-2, as described (24). Sarkosyl-insoluble tau extracted from frontal cortex of an AD patient was used as a control. Dephosphorylation was done as described (24). For the extraction of soluble tau, 200 mg of frontal cortex was dounce homogenized in 0.5 ml 2.5% perchloric acid, as described (23). Dephosphorylation and immunoblotting were done as described (23). The following anti-tau antibodies were used: BR133 (1:1,000), BR134 (1:1,000), BR304 (1:500), BR189 (1:500), and PHF1 (1:500). BR133 was raised against amino acids 1–16 of human tau (1).

Electron Microscopy of Dispersed Filaments

Aliquots of dispersed filament preparations were placed on carbon-coated, 400-mesh grids and stained with 1% lithium phosphotungstate, and micrographs were recorded at a nominal magnification of ×40,000 on a Philips EM 208S microscope, as described (52). Anti-tau serum BR134 and antibody AT8 were used at a dilution of 1:100. Procedures for immunoelectron microscopy were as described (52).

Microtubule Assembly

Site-directed mutagenesis was used to change G389 to arginine in the three-repeat 381 amino acid isoform and in the four-repeat 412 amino acid isoform of human tau, in the numbering of the 441 amino acid isoform of human tau (1), expressed from cDNA clones htau37 and htau46, respectively. Wild-type and mutant tau proteins were expressed in Escherichia coli BL21 (DE3), as described (23). Tau proteins were purified as described (31) and their concentrations determined by densitometry. Purified recombinant and mutant htau37 (0.5 mg/ml) and htau46 (0.1 mg/ml) were incubated with bovine brain tubulin (1mg/ml, 20 μM, Cytoskeleton) in assembly buffer at 37°C, as described (53). The assembly of tubulin into microtubules was monitored over time by a change in turbidity at 350 nm. In all experiments, wild-type and mutant tau proteins were expressed and purified in parallel.

RESULTS

Family History

The pedigree is shown in Figure 1. Only limited information was available on most family members. Subjects I-1, I-2, and II-2 died of unknown causes. Subject I-4 died of hepatic cirrhosis. Subject I-3 died demented at age 41; information on the age at the time of death of subject I-4 was not available. Subject II-3, the mother of the proband, died of hepatic cirrhosis at 60 years of age. Subject II-4, the proband’s father, died of a
stroke at 59 years of age. Subject II-5 died of a renal disease at age 59.

Subject II-6, the proband's paternal uncle, complained of anxiety, asthenia, and restlessness when 41 years old. These symptoms were followed by expressive aphasia with episodes of nominal aphasia, progressive loss of graphic abilities and emotional incontinence. One year later, the neurological examination showed aphasia with dysprosody, nominal aphasia, severe dysgraphia, ideomotor apraxia, buccofacial apraxia and mild right side hemineglect. Pneumoencephalography revealed a moderate and symmetric ventricular dilatation. Blood and cerebrospinal fluid tests were normal. The patient no longer worked, became more and more withdrawn and apathetic, and spent his days following his wife around. He deteriorated rapidly and, by 43 years of age, language was limited to "yes" and "no" and to isolated monosyllabic expressions. No motor deficits were observed. EEG showed a slowing of the base rhythm. A repeated pneumoencephalography showed a worsening of the ventricular dilatation. A brain biopsy of the right frontal lobe was performed, but the results could not be retrieved. The patient died in 1969 at age 43.

Subject II-9 died at 23 years of age following appendicitis. Subject III-1, the sister of the proband, died at 5 years of age with a clinical diagnosis of "Schilder's disease." Subject III-4 presented with congenital absence of the fingers bilaterally. Subject IV-1 had a congenital stenosis of the pulmonary artery. The proband's children, IV 1, IV 2, and IV 3, who are below 21 years of age, are clinically normal.

Clinical History of Proband

Subject III-2: At 38 years of age, this previously healthy Caucasian male showed initial signs of cognitive dysfunction and was seen by a neurologist (April 1992). Prior to that time, he had conducted an uneventful life, working as a carpenter. He was married and had 3 children. The initial complaints were memory disturbance related to his professional activities and language disturbance manifesting as anomia. There were no other detectable cognitive deficits or focal neurological signs. Over the ensuing months, the language difficulties became more severe and behavioral deterioration, mainly characterized by apathy, indifference, and inertia, became apparent. By the end of 1992, the proband had stopped working, even though he continued to go to his shop every day.

Neuropsychology: Early in the course of the disease, the patient underwent neuropsychological testing. On the Mini-Mental State Examination (MMSE) (54), the patient's score was significantly lower than the normal range (10/30). At the time of the PET scan of December 1992, the patient's MMSE score was 7/30. He also showed lower than normal scores on the Wechsler Adult Intelligence Scale (WAIS) (55). Language abilities were evaluated with the Aachener Aphasic Test (AAT) and the patient's deficits were classified as Wernicke aphasia with prevalent disturbances in comprehension and naming. Repetition was relatively well preserved. There was a severe deficit of verbal fluency in the analysis of semantic categories with semantic intrusions and perseverations. Short-term memory was tested in order to assess verbal and visual-spatial memory. The Wechsler Memory Scale (WMS) gave a score of 62/100 and the Buschke-Fuld test (56) for verbal learning showed a complete lack of performance. Visual-spatial learning tested by the Rey complex figure (57) showed the following scores: 28/36 for simple copying, 12/36 for memory reproduction and 42/100 for recall. Perception tested with Benton's visual discrimination test (58) showed a borderline performance with a score of 26/32.

Neuroradiology: The MRI of June 1992 (Fig. 2A, B) showed a moderate atrophy localized to the left frontal and temporal operculum and the insular cortex, with focal dilatation of the sylvian fissure. It also showed a hypodensity of the left insular subcortical white matter, a moderate bilateral atrophy of hippocampal formations and a dilatation ex vacuo of the temporal horn of the left ventricle. In addition, moderate atrophy of the anterior third of the left parahippocampal gyrus and the inferior medial and superior temporal gyri was observed. The third ventricle and the lateral ventricles were moderately dilated. The atrophy progressed and was very severe by 1995 (Fig. 2C, D).

In Vivo Brain Metabolic Studies: The SPECT of July 1992 showed a marked reduction of perfusion in the temporal lobes, which was more severe on the left side. Six months later, SPECT examination showed a marked reduction of perfusion in the left frontal, temporal, and parietal regions, as well as a milder and more localized reduction in the right frontal and parietal regions. The PET scan of December 1992 showed a reduction in glucose metabolism in frontal and temporal neocortical association areas, with the most severe reduction in the frontal cortex. Glucose utilization in subcortical structures, including the thalamus and the cerebellum, was in the normal range (Fig. 3, top). At a follow-up examination in August 1993, there was a more dramatic impairment of cerebral glucose utilization throughout neocortical association areas of the frontal and temporal lobes. At this time, the thalamic nuclei were also compromised (Fig. 3, bottom). Cerebral glucose metabolism was preserved in occipital and parietal cortical regions, as well as in primary sensory cortex and cerebellum.

Clinical Course: In the summer of 1993, after a period of aimless hyperactivity, the patient became completely aphasic and his behavior deteriorated dramatically. A neuropsychological evaluation was repeated at which no verbal production could be elicited. The activities of daily
living were reduced to a few routines, such as self-dressing and sphincteric control. The patient showed perseverations, hyperphagia, and nonfinalized hyperactivity. In December 1993, he was still ambulatory and could walk for long distances without getting lost on known paths. An all-night sleep EEG was recorded in December 1992 and was within the normal range, while the recording of August 1993 showed an alteration of the sleep pattern with a reduction of the rapid eye movement (REM) phase. Therefore, in order to rule out a diagnosis of fatal familial insomnia, the open reading frame of the PRNP gene was sequenced. No mutation was found. An additional MRI, performed in April 1995 (Fig. 2C, D), showed a severe bilateral symmetric atrophy of the temporal and frontal lobes and of the hippocampal formation, with dilatation of the Sylvian and perihippocampal fissures, as well as a knife edge appearance of the cerebral gyri in several regions and an increased depth of cerebral sulci. A dilatation ex vacuo of the lateral ventricles and the third ventricle was present, as was a marked atrophy of the corpus callosum. The MRI also showed a hyperintensity of the frontal insular and temporal subcortical

Fig. 2. Two MRI scans of the proband’s brain illustrate the progression of the cerebral atrophy over a 3-year period. The first MRI (A and B) was carried out when the patient was 38 years old, whereas the second (C and D) was obtained when the patient was 41 years of age. (L) indicates left.
white matter, the posterior arms of the internal capsules and the cortico-spinal tracts down to the brain stem with atrophy of the pyramids (Wallerian degeneration).

The patient continued to be followed with periodical neurological examinations. In spring 1995, he showed marked rigidity, hyperreflexia of all 4 limbs, clonus of the wrists, persistent contracture of the masseters with bruxism, postural and kinetic tremor of upper limbs, bilateral grasping following palmar stimulation, and incontinence. By January 1996, the rigidity had worsened and the patient presented with automatic movements of the limbs and bruxism. On subsequent examinations, he showed spastic hypertonus with clonus of wrists, patella, and ankles. Tendon reflexes were hyperactive with clonus and there was a Babinski sign bilaterally. In December 1996, the patient showed accentuation of the spasticity, opisthotonus, dysphagia, and continuous bruxism. By August 1997, he was tetraplegic with hypothonus on the right and spastic hypothonus on the left. The patient died in August 1997.

**Neuropathology**

**Gross Findings:** The proband's brain showed a severe atrophy of the frontal and temporal lobes with a knife edge atrophy of the convolutions. The atrophy also involved the white matter of the centrum semiovale and corpus callosum. There was atrophy of the subcortical nuclei, which was most prominent at the level of the caudate nucleus. Hippocampus and amygdala were reduced in volume. The lateral and third ventricles were severely dilated. In the midbrain, the substantia nigra showed loss.
of pigmentation. The pons was moderately atrophic and the medulla was unremarkable.

**Light Microscopy:** Neuronal loss was severe in frontal, cingulate, temporal, and insular cortices, with relative sparing of parietal and occipital cortices. Spongiosis was present in the second cortical layer and in the lower layers of the temporal cortex. A separation of the upper from the lower layers of the cortex could be seen. Neuronal loss was also seen in caudate nucleus, putamen, and globus pallidus. In cerebellum, moderate loss of Purkinje cells was observed, while the dentate nucleus was relatively spared. In the substantia nigra, there was prominent neuronal loss with abundant neuromelanin pigment dispersed in the neuropil or contained in macrophages, which were located in the parenchyma or adjacent to blood vessels. Gliosis was present in grey matter areas in which neuronal loss and spongiosis were most pronounced. In the neocortex, it was most marked in the first, second, fifth, and sixth layers. In the white matter, gliosis was diffuse. It was particularly severe in frontal and temporal lobes. No amyloid deposits were seen in thioflavin S and Congo red preparations.

The fibrillary changes were analyzed using the Bodian silver stain, as well as single- and double-labeling immunohistochemistry (Figs. 4–7). By Bodian silver stain, the most prominent changes were Pick body-like inclusions in neurons of the neocortex (Fig. 4A, B). They were particularly prominent in the second, fifth, and sixth layers of the frontal, cingulate, temporal, and insular cortices, while they were rare in occipital cortex. Some neurons of the cortex showed discrete tangle-like, silver-positive deposits. Pick body-like inclusions were also abundant in the fascia dentata. Argentophilia of the cytoplasm of glial cells was only rarely noticed in neocortex and subcortical white matter. Argentophilic neuronal inclusions were infrequent in caudate nucleus, putamen, and thalamus. In the amygdala, numerous nerve cells had intracytoplasmic deposits. In the cerebellum, some Purkinje cells showed axonal torpedoes (Fig. 7D). No argentophilia was seen in neurons of the substantia nigra, while in the pons, argentophilic inclusions were seen in the raphe nuclei and occasionally in the pontine grey nuclei.

In subcortical white matter of the centrum semiovale, the internal, external, and extreme capsules, as well as within grey matter of subcortical nuclei, argyrophilic elongated structures ranging between 4 and 40 µm were observed (Fig. 4C–E). They appeared either as long threads or focal axonal swellings, or as dystrophic axons. With the Heidenhain-Woelcke method for myelin, extensive loss of stain was seen. The most severe pallor, resulting from an almost complete loss of myelin, was observed in white matter of the temporal lobe, whereas the pallor observed in the frontal lobe, corpus callosum, and in the internal, external, and extreme capsules was also severe. No loss of myelin was detected in the occipital lobe. In the pons, loss of myelin was severe at the level of the basis pontis and appeared to involve mostly the descending fibers of the cortico-spinal and cortico-bulbar tracts, while sparing the transverse bundles of ponto-cerebellar fibers.

**Immunohistochemistry:** Immunohistochemical studies showed no immunolabeling using a monoclonal antibody against Aβ (10D5) or a polyclonal antibody against α-synuclein. In contrast, strong cytoplasmic immunopositivity was seen in nerve cells using anti-tau antibodies (Figs. 5A–C, 6, 7A, B). The latter also stained axonal inclusions in grey matter structures and in the white matter (Figs. 5D, E, 7C). When comparing adjacent sections that were either immunolabeled with anti-tau antibodies or stained with the Bodian silver stain, immunopositive neurons were more numerous than those showing silver deposits. Many neurons were immunopositive with antibodies to the phosphorylated high-molecular weight neurofilament subunit, whereas only few neurons were stained by a polyclonal antibody to α-B crystallin.

Using antibody AT8, tau immunopositivity was strong in many regions of the grey and white matter (Figs. 5–7). In the cerebral cortex, especially in frontal, insular, and temporal regions, the majority of neurons in layer 2 and many neurons in layers 5 and 6 showed strong tau immunoreactivity. In layer 2, neurons showed either a diffuse cytoplasmic immunopositivity or the presence of round, well-circumscribed, densely immunolabeled inclusions, resembling Pick bodies (Fig. 5A, B). In layers 5 and 6, there were numerous neurons with Pick body-like...
inclusions and others with diffuse cytoplasmic immunopositivity, which occasionally extended into the main dendritic process (Fig. 5C). Immunopositive neurons with Pick body-like inclusions were sometimes seen in parietal and occipital cortices. Occasionally, tangle-like inclusions were observed. Pick body-like inclusions were also present in the fascia dentata of the hippocampus. Some Pick body-like inclusions were labeled using antibodies to ubiquitin. Tau immunopositive neurons were numerous in the amygdala, while they were only occasionally seen in caudate nucleus, putamen, and globus pallidus. No Pick body-like inclusions were seen in these brain regions. Anti-tau immunopositive neuronal perikarya were also present in brainstem, particularly the pons. A small number of neurons in the raphe (Fig. 7B) and pontine nuclei were diffusely tau-positive. Tau immunopositivity of glial cell perikarya was only rarely seen.

Besides perikaryal accumulations of hyperphosphorylated tau, a most striking change was the presence in white matter of fine, elongated, thread-like structures that were reactive with anti-tau antibodies (Figs. 5D, E, 7C). They were of uneven thickness and sometimes had a beaded appearance. They were strongly labeled with antibody AT8 and were most numerous in the lowest cortical layers, as well as in the adjacent white matter, where they ran tangentially to the cortex. They were abundant in white matter of the centrum semiovale and particularly numerous in white matter of the temporal lobe. They were also present within the internal, external, and extreme capsules. Only a few of these structures were found in white matter of the cerebellar folia and the pons. They were also present in grey matter structures, including cortex and subcortical nuclei. Among the latter, they were particularly prominent in the putamen, globus pallidus, and thalamus.

Anti-tau antibodies AT270, AT100, AT180, I2E8, PHF1, AP422, AT50, BR134, BR304, BR189, and Tau-1 all recognized neurons in a pretangle state, as well as Pick body-like inclusions (Fig. 6A–H), tangles and axonal inclusions. AT270, AT100, AT180, I2E8, PHF1, AP422, BR134 and BR304 showed staining similar to AT8, whereas BR189 stained some neurons in a pretangle state and Pick body-like inclusions, but only few axonal inclusions. To determine the cellular origin of the tau-positive inclusions, white matter was

Fig. 6. Sections of the frontal cortex of the proband showing Pick body-like inclusions immunolabeled with various anti-tau antibodies. A: AT50, B: AT8, C: AT100, D: AT180, E and F: I2E8, G: PHF1, H: AP422. Magnification: A–H, ×1,700.
studied by double-labeling immunohistochemistry using AT8 and SMI 32 or AT8 and the GFAP antibody. In some instances, elongated processes were labeled by both AT8 and SMI 32 (Fig. 5F). In most of these, a thin segment labeled with SMI 32 was seen in continuation with a widening segment labeled with AT8. Only in rare instances was AT8 immunopositivity seen in association with GFAP-positive cells.
Electron Microscopy

Pick body-like inclusions contained round aggregates of filamentous material that was decorated by gold particles after immunolabeling with antibody AT8 (Fig. 8A, B). The filaments were straight or twisted. Since individual filaments were oriented in a haphazard fashion, it was not possible to identify segments of filaments that were long enough to establish their periodicity with accuracy. The width of twisted filaments varied between about 8 and 21 nm. The straight filaments were approximately 13 nm wide. Neurons that appeared free of inclusions at the light microscopic level sometimes showed small wisps of filaments that were immunogold labelled for tau. In white matter, rare glial cells contained filamentous material with characteristics similar to that seen in neurons. The thread-like structures (Fig. 9A, B) were numerous in white matter and showed strong labeling with AT8, which was particularly evident at the edges. They had an elongated, irregular, often wavy shape, when cut in the longitudinal plane, whereas they appeared as round or oval clusters when cut transversely. The thread-like structures were composed of filaments with characteristics identical to those of the intraperikaryaial filaments. In some instances, bundles of immunolabeled filaments were seen in close apposition to amorphous material on one side and a myelin sheath on the other, indicating an axonal location (Fig. 9C).

Genetic Analysis

Sequencing of the proband’s genomic DNA showed wild-type sequence in all exons of Tau, with the exception of exon 13, where a G to C transversion was present at codon 389. It changes glycine residue 389 to arginine (G389R) (Fig. 10A). This change was not seen in DNA of 50 individuals from the Indiana University DNA Bank and in DNA of 30 individuals from the region of Italy that the proband originated from (MGS and S. Sorbi, unpublished). The nucleotide change creates NciI and MspI restriction sites. When the amplified exon 13 product from subject III-2 was digested with NciI, 4 bands of 338, 236, 102, and 89 base pairs (bp) were observed. Only 2 bands of 338 and 89 bp were seen in controls. When MspI was used for digestion, 5 bands of 179, 159, 102, 89, and 77 bp were observed in the proband’s DNA. Three bands of 179, 159, and 89 bp were seen in controls (Fig. 10B). The proband was homozygous for the more common H1 Tau haplotype, which is significantly over-represented in patients with progressive supranuclear palsy (51, 59). He did not have the rare single nucleotide polymorphism in exon 9 (9iii) that is associated with the H1 haplotype.

Tau Extraction, Dephosphorylation, and Immunoblotting

We extracted sarkosyl-insoluble tau from frontal and occipital cortices of subject III-2. By immunoblotting with anti-tau serum BR134, it resolved into 2 major bands of 60 and 64 kDa apparent molecular mass and 2 minor bands of 68 and 72 kDa (Fig. 11). Antiserum E10 labeled the 64 kDa band. Antiserum BR304 stained the 64 kDa band, whereas antiserum BR189 failed to label the sarkosyl-insoluble tau bands. When compared with sarkosyl-insoluble tau extracted from AD brain, the 68 and 72 kDa bands were much weaker. After alkaline phosphatase treatment, sarkosyl-insoluble tau from subject III-2 resolved into 4 major bands that probably corresponded to the three-repeat tau isoforms of 352 and 381 amino acids and the four-repeat tau isoforms of 383 and 412 amino acids (Fig. 11). The 410 and 441 amino acid isoforms of human brain tau were not clearly seen. Following alkaline phosphatase treatment, the shortest tau band failed to align with the 352 amino acid recombinant tau isoform, probably because it co-run with alkaline phosphatase. A similar phenomenon has previously been observed for sarkosyl-insoluble tau extracted from AD brain (24). Soluble dephosphorylated tau extracted from the frontal cortex of patient III-2 resolved into 6 bands, with a pattern that was indistinguishable from that of soluble tau extracted from the frontal cortex of controls (not shown).

Electron Microscopy of Dispersed Filaments

Electron microscopy of preparations of extracted, sarkosyl-insoluble filaments showed filaments with 2 morphologies. The major species (Fig. 12A–C) was a straight filament with a twisted stranded sub-structure very similar in appearance to straight filaments in AD (52) or in the V337M and R406W tauopathies (19, 36). The minor species (Fig. 12D–F) was an open-looking, low contrast twisted filament with a cross-over spacing of about 120 nm and a projected width varying between about 6 and 23 nm. In the wider parts, the image showed a strong central white stain-excluding axial line flanked by 2 somewhat weaker stain-excluding lines. Both kinds of filament were strongly labeled by antibodies directed against nonphosphorylated and phosphorylated epitopes of tau (Fig. 12). No examples of standard Alzheimer-type paired helical filaments were observed.

Microtubule Assembly

Recombinant three-repeat httau37 and four-repeat httau46 with the G389R mutation showed a markedly reduced ability to promote microtubule assembly, when compared with the corresponding wild-type proteins (Fig. 13A, B). Thus, the G389R mutation in httau37 led to a 60% reduction in the rate of microtubule assembly when expressed as the optical density at 2 min (Fig. 13C). This reduction was 35% for httau46 with the G389R mutation (Fig. 13C).
Fig. 8. Electron micrographs of a neuron containing a Pick body-like inclusion. A: Neuron showing the nucleus and the Pick body-like inclusion. B: Filaments immunolabeled with antibody AT8 within the Pick body-like inclusion. Magnification: A, ×13,500; B, ×72,500.
Fig. 9. Electron micrograph of a thread-like axonal structure. A: The thread-like structure is seen in longitudinal view. B: Straight filaments of the thread-like structures immunolabeled with antibody AT8. Immunolabeling is most evident on the edges, probably because of the dense packing of filaments. C: Electron micrograph of an axon shows transversely cut filaments labeled with antibody AT8. Nonlabeled osmiophilic axoplasm is seen in the central part. A portion of the myelin sheath is seen at the periphery. Magnification: A, ×13,700; B, ×64,800; C, ×70,000.
DISCUSSION

The present study, which combines molecular genetic analysis, in vivo longitudinal brain functional and neuroradiological assessments, as well as postmortem neuropathological and biochemical studies, shows that the G389R mutation in Tau causes a syndrome of frontotemporal dementia. It begins in the fourth decade of life and...
is characterized by a rapidly progressing decline of cognitive and behavioral abilities, marked metabolic functional impairments, and severe frontal and temporal atrophy. Histologically, this syndrome is characterized by large numbers of filamentousPick body-like and axonal inclusions that are made of hyperphosphorylated tau protein.

Based on the available information, it is difficult to determine the pattern of inheritance of the disease in family F. The study of families with mutations in Tau has shown that frontotemporal dementia is inherited in an autosomal-dominant manner (35). The proband was the only subject of family F to be analyzed genetically. Neither of his parents had suffered from frontotemporal dementia. However, one of the proband's paternal uncles had died at age 43 from a rapidly progressing syndrome characterized by frontotemporal dementia and brain atrophy. Based on the similarities of the clinical presentations and the ages of onset of symptoms, it appears likely that the proband and his uncle had suffered from the same genetic disease. The fact that neither of the proband's parents had early-onset frontotemporal dementia leads us to consider the possibility of either incomplete penetrance of the genetic defect in the proband's father, or of nonpaternity. Since no genomic DNA was available from either the proband's parents or his uncle, it was not possible to distinguish these possibilities.

The disease of the proband and his uncle was characterized by personality changes, deterioration of social skills, apathy, aphasia, hyperactivity, memory disturbance, apraxia, and other features of dementia that were accompanied by extrapyramidal and pyramidal signs. The presenting symptoms were consistent with damage to the prefrontal and temporal cortices (60). Shortly after the onset of symptoms, neuroradiological and in vivo brain metabolic studies revealed atrophy and severe hypometabolism of the frontal and temporal regions. The MRI performed 2 months after the initial clinical evaluation revealed atrophy of the frontal and temporal structures that was more prominent on the left side, being particularly evident at the level of the superior temporal and parahippocampal gyri. Focal atrophy with predominance of the left temporal lobe has been known ever since the first descriptions of Pick's disease (61, 62).

Asymmetry of the atrophy is observed in approximately 60% of these patients, with the atrophy being usually more conspicuous on the left (63). The in vivo PET measurements of cerebral glucose utilization at rest, an index of neuronal synaptic activity (37, 64), that were obtained a few months after the onset of the symptoms, showed a reduction of metabolism in the frontal lobes that was more severe on the left side. The metabolic lesions also involved the temporal lobes and the thalamus on the follow-up evaluation 9 months later. This pattern of hypometabolism, which is distinct from that found in patients with AD (37, 38, 64), is consistent with the progression of the clinical symptoms and the neuropsychological dysfunction of the patient. The clinical, neuropsychological, and brain functional and structural imaging investigations of the proband are consistent with a diagnosis of Pick's disease. An intriguing finding was the severe alteration of the sleep pattern with marked shortening of the REM stage, which led us
initially to consider a diagnosis of fatal familial insomnia. However, this diagnosis was ruled out by the absence of a mutation in the open reading frame of the PRNP gene. Interestingly, thalamic glucose metabolism was abnormally low on the second PET scan, coincident with the disruption of the sleep pattern.

The gross neuropathologic findings of the proband's brain showed severe frontal and temporal lobar atrophy, consistent with the brain imaging data and with a clinical diagnosis of Pick's disease. The histopathological findings would also have fulfilled the criteria for this diagnosis, since the Bodian stain revealed a large number of argentophilic intracytoplasmic inclusions that were indistinguishable from Pick bodies. The clinico-pathologic concept of Pick's disease is being re-evaluated following the discovery of Tau mutations (4–6). Familial diseases that had previously been identified as hereditary Pick's disease have now been subsumed under the heading of FTDP-17 (5, 65–68). So far, more than 15 different mutations have been found in exons 9, 10, 12, and 13 of Tau, as well as in the intron following exon 10 (4–19). Neuropathologically, Pick body-like inclusions have been seen in some families with FTDP-17 (12, 13, 16, 17), especially in Dutch family 2 with the G272V in exon 9 of Tau (5, 68). The concept of "Pick body" (69) has evolved recently, largely due to the availability of phosphorylation-dependent anti-tau antibodies, such as AT270, AT8, AT100, 12E8, PHF-1, and AT100. These antibodies stain both Pick bodies and the neurofibrillary pathology of AD. The only exception is antibody 12E8, which stains neurofibrillar lesions, but not Pick bodies (70, 71). In the present case, the intracytoplasmic inclusions were indistinguishable from Pick bodies when using the Bodian stain and many anti-tau antibodies. However, unlike Pick bodies, they were immunoreactive with 12E8. Previously, the Pick body-like inclusions in Dutch family 2 were found to be 12E8-negative (68), like the Pick bodies of Pick's disease (70, 71).

Besides Pick body-like inclusions, a second major histological feature of the proband's brain was the presence in white matter of a large number of inclusions of variable length, irregular shape and variable width. These inclusions were argyrophilic and strongly immunoreactive with phosphorylation-dependent anti-tau antibodies. Antibody BR189, which recognizes tau isoforms with the 58 amino acid amino-terminal insert (24), only labelled a small number of the white matter inclusions. Using double immunolabeling, these inclusions were also labeled with anti-neurofilament antibody SMI 32. By electron microscopy, they were surrounded by a myelin sheath supporting their axonal nature. Interestingly, in recent years, tau-positive neuropil threads of axonal origin have been recognized as an essential characteristic of Pick's disease (70, 72). We speculate that the axonal inclusions belonged to axons derived from neuronal perikarya containing Pick body-like inclusions. In the proband, Pick body-like inclusions were mostly present in the second, fifth, and sixth cortical layers. The neurons of the second layer are small pyramidal cells from which cortico-cortical fibers originate; these fibers may have contained the axonal inclusions found subcortically in the vicinity of the lowest cortical layers. The neurons of the fifth and sixth cortical layers provide cortico-spinal fibers, as well as cortico-cortical, callosal, and cortico-thalamic fibers. These fibers may have contained the axonal inclusions found in deeper white matter and in the corpus callosum.

Immunoblotting of sarkosyl-insoluble tau extracted from cerebral cortex of the proband showed 2 strong bands of 60 and 64 kDa and 2 weak bands of 68 and 72 kDa. This pattern is reminiscent of that found in Pick's disease (73) and differs from the pattern of AD (24, 47) by the low levels of the 68 and 72 kDa bands. Following dephosphorylation, the sarkosyl-insoluble tau resolved into 4 bands corresponding to the 352 and 381 amino acid tau isoforms with 3 repeats, and the 383 and 412 amino acid isoforms with 4 repeats. The 410 and 441 amino acid isoforms of tau were not seen. This pattern differs from that of Pick's disease, where only three-repeat tau isoforms are present (74). By contrast, in AD, all 6 brain tau isoforms are found in sarkosyl-insoluble tau (24). It is unclear whether the absence of the 410 and 441 amino acid tau isoforms merely reflected the normally low levels of these isoforms, or whether they were truly excluded from pathological tau.

Tau filaments isolated from cerebral cortex of the proband showed 2 distinct morphologies. The major species was a straight filament and the minor species a twisted filament. Similar filaments were seen in tissue sections by immunoelectron microscopy, although it was not possible to define their exact morphologies. The straight filament had the same morphology as the straight filament of AD (52). However, in the latter disease, straight filaments account for only about 10% of isolated filaments, with paired helical filaments constituting the major species. Alzheimer-type paired helical filaments were not present in sarkosyl-insoluble tau prepared from the proband's cerebral cortex. Instead, small numbers of open-looking twisted filaments with a width of 6–23 nm and crossover spacing of 120 nm were present. Isolated filaments with this morphology have so far not been described in either sporadic tauopathies or in familial cases with mutations in the Tau gene (35). Electron microscopy on tissue sections from Pick's disease brain has shown a large number of straight filaments and a smaller number of twisted filaments (72, 75–77), further underlining the similarities between Pick's disease and the pathology resulting from the G389R mutation in Tau.
Comparison with the characteristics of other cases of FTDP-17 shows that the G389R mutation leads to a unique neuropathological phenotype. Mutations in exon 10 and in the intron following exon 10 produce a neuronal and glial tau pathology consisting of twisted ribbons that are made predominantly of tau isoforms with 4 repeats (30, 68). By contrast, mutations located in exons 9, 12, and 13 lead to a largely neuronal tau pathology. Where analyzed, the tau filaments are paired helical and straight filaments that are made of all 6 brain tau isoforms. This has been shown for the V337M mutation in exon 12 and the R406W mutation in exon 13 (19, 36).

Biochemically, most coding region mutations lead to a reduced ability of tau to promote microtubule assembly (9, 16, 31). The same was found here for the G389R mutation, suggesting that a reduced ability of mutant tau to interact with microtubules may be the primary effect of this mutation. Consistent with its location in exon 13, the G389R mutation produced a largely neuronal tau pathology. However, unlike the V337M and R406W mutations (19, 36), the tau filaments and the pattern of sarkosyl-insoluble tau bands differed from those found in AD and resembled most closely the characteristics of Pick's disease. These findings indicate that depending on the positions of Tau missense mutations in exons 12 and 13, and perhaps the nature of these mutations, a filamentous tau pathology ensues that resembles either that of AD or of Pick's disease. The mechanism underlying this exquisite specificity remain to be established. Meanwhile, the present findings indicate that the G389R mutation in Tau in the proband causes a dementia disease that is similar to Pick's disease in its clinical, neuroradiological, neuropathological and biochemical characteristics.

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