Selective Deposition of Mutant Tau in the FTDP-17 Brain Affected by the P301L Mutation

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Abstract. Frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) is a familial neurological disorder exhibiting autosomal dominant inheritance. Linkage analyses have led to the identification of many exonic and intronic mutations in the tau gene in affected families. Because FTDP-17 causes extensive neuronal loss and intracellular tau deposits in affected regions, investigation of this disease should provide an important insight into the significance of tau deposits leading to neurodegeneration. Using site-specific antibodies that distinguish between wild-type and mutant tau, we have analyzed the proportions of wild-type and mutant tau in the soluble and insoluble fractions of the P301L brain. Western blotting showed that mutant tau was selectively deposited in the Sarkosyl-insoluble fraction. Consistent with this, immunocytochemistry showed that intraneuronal tau deposits consisted exclusively of mutant tau. In one case in which abundant senile plaques occurred, in addition to mutant tau, small amounts of wild-type tau were also deposited. On the other hand, the protein levels of mutant tau in the soluble fraction were selectively decreased despite no detectable decrease in the levels of mutant tau mRNA.

Key Words: FTDP-17; Mutation; Neurofibrillary tangle; P301L; R406W; Senile plaque; Tau.

INTRODUCTION

Neurofibrillary tangles (NFTs), bundles of unit fibrils called paired helical filaments (PHFs), are a major neuropathological hallmark of Alzheimer disease (AD) and many other “tauopathies.” Because the areas in which NFTs are formed match precisely the areas exhibiting neuronal loss in AD, the formation of NFTs is probably involved in a pathway leading to neuronal death (1, 2). Tau, a microtubule (MT)-associated phosphoprotein, has previously been identified as composing the framework of PHFs. Thus, the current question is how tau is involved in the process of neurodegeneration and/or neuronal death as well as in the formation of NFTs.

A group of non-Alzheimer-type dementias were recently categorized as frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) (3–7). This entity is characterized neuropathologically by extensive neuronal loss that is predominant in the anterior part of the cortex, the basal ganglia, and the midbrain. Furthermore, it is also characterized by the presence of PHFs or PHF-like fibrils in subsets of neurons, and sometimes in glial cells, particularly in oligodendrocytes. Further analysis of FTDP-17 families has led to the identification of more than 20 exonic and intronic pathogenic mutations in the tau gene (3–7). This disease therefore should provide an important insight into the relationship between tau and neuronal death, a longstanding, as yet unsolved, enigma. Most exonic mutations have been claimed to significantly decrease the capacity of tau for promoting MT assembly, which may explain at least some parts of the pathogenesis of FTDP-17 (8–11). However, some of the exonic and all intronic mutations lead to an increase in the splicing-in of exon 10, which encodes the second repeat in the MT-binding domain (4, 10, 12–15). As a result, in contrast to most exonic mutations, the levels of the 4-repeat (4R) tau isoform increase, resulting in enhanced ability to promote MT assembly. Thus far, this contradiction has not been appropriately explained.

Among FTDP-17 mutations, the proline to leucine substitution on position 301 (P301L) (numbered according to the 441-residue isoform) is the most frequently encountered and is well known for its aggressive clinical phenotype (early onset and short duration) (5, 16–19). This P301L mutation is located in exon 10, and has no effect on the alternative splicing of exon 10 (4, 10). Several in vitro experiments have indicated that P301L tau has the least potential to promote MT assembly and the highest tendency to form tau filament (10, 20–24). Recently, it has been reported that the transgenic mice harboring human P301L tau showed progressive motor deficits and distinct neuropathological changes including tau inclusions (25, 26). This strongly suggests a gain of toxic function in P301L mutant tau.

One prevailing speculation is that the P301L mutation reduces the affinity of tau for MTs, leading to their destabilization. The resultant cytosolic free tau, preferentially mutant tau, becomes highly phosphorylated and aggregates into PHF-like fibrils, which may in turn exert...
neurotoxicity. If so, it is important to study the proportion of wild-type to mutant tau in the affected brain, which may deviate from the expected ratio of 1:1. Thus we have raised site-specific antibodies that distinguish between wild-type and mutant tau, and show here that P301L mutant tau is selectively deposited in the Sarkosyl-insoluble fraction of the brain and is an exclusive component of the tau deposits in the P301L brain. Immunocytochemical examination using the same antibodies has substantiated the above conclusion. In addition, we show that the levels of P301L tau are selectively decreased in the soluble fraction of P301L brains, despite no decrease in its mRNA levels.

MATERIALS AND METHODS

Autopsy

Frozen brain tissues from 3 P301L patients (patient 1, aged 52 yr; patient 2, aged 64 yr; and patient 3, aged 76 yr) and formalin-fixed, paraffin-embedded tissue sections from 5 P301L patients (patients 1 to 3; patient 4, aged 60 yr; and patient 5, aged 66 yr) and from 2 patients affected by the R406W mutation (the arginine to tryptophan substitution on position 406) in the same family (patient 6, aged 70 yr; and patient 7, aged 71 yr) were obtained from the Netherlands Brain Bank (NBB) (average postmortem delay 6 hours [h]). Patients 1 and 3 were a cousin and an aunt of another patient, respectively; patients 6 and 7 were brother and sister, respectively; the other patients were unrelated. Patients 1 and 3–5 were clinically diagnosed as atypical Pick disease. Small pieces of the brain tissues were taken from various areas following the dissection protocol and either fixed in 10% formalin or stored at −80°C until use.

The brains from patients 1 and 3 were at Braak stage 1 (1). Patient 2 was diagnosed as “atypical” presenile AD because the pattern and density of NFTs in the hippocampus and the parahippocampal gyrus suggested Braak stage 2, but those in the neocortex suggested Braak stage 4, and because of the fact that presenile AD brains usually show Braak stage 5 or 6. Patients 6 and 7 were diagnosed as having presenile AD with typical NFT distribution and showed the Braak stages 5 and 6, respectively. Braak stages for β-amyloid were B and B/C, respectively: the amyloid load appeared to be relatively low. Heu- tink and van Swieten and coworkers found the P301L mutation in the tau gene of patient 2 and the R406W mutation in patients 6 and 7. Frozen AD brains were kindly provided by Dr. Dennis J. Selkoe.

Tissue Fractionation

Brain tissues were homogenized in Tris-saline (TS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.6) containing a cocktail of protease inhibitors as described previously (27). The homogenates were centrifuged at 540,000 × g for 20 minutes (min) and the supernatants (soluble fractions) were obtained. The supernatants from P301L brains were heated at 95°C for 5 min and cleared by centrifugation. Crude tau, after precipitation with 50% saturated ammonium sulfate, was treated with 10 U/ml E. coli alkaline phosphatase (type III, Sigma, St. Louis, MO) at 67°C for 3 h in 50 mM Tris-HCl (pH 8.3) containing protease inhibitors. The Sarkosyl-insoluble pellets were prepared from the TS-insoluble pellets as described previously (Sarkosyl-insoluble fraction) (27). When dephosphorylation was required, the pellets were solubilized in 6 M guanidine hydrochloride, which was replaced by dialysis with 50 mM Tris-HCl (pH 8.3) containing 2 M urea.

Recombinant Human Tau

Wild-type or P301L mutant 1N4R (presence of 1 amino-terminal and the second repeat) tau cDNA cloned into the pRK172 vector, was kindly provided by Dr. M. Goedert (MRC, Cambridge, UK) (28). Each tau species was expressed in E. coli (BL21 [DE3], Novagen, Inc., Madison, WI) and purified as described previously (8). Obtained tau was carboxymethylated after reduction and further purified on an Aquapore RP300 column 2.1 × 30 mm (Applied Biosystems, Foster City, CA) by reversed phase high-performance liquid chromatography (Model 1090M; Hewlett-Packard, Waldbronn, Germany). Protein concentrations were determined by bicinchoninic acid method (Pierce, Rockford, IL).

Antibodies

Site-specific polyclonal antibodies against wild-type and mutant tau were raised against synthetic 14mer peptides conjugated with KLH: DNIKHVPGGGSVQC (AP301) and DNIKHLVG-GGSVQC (AL301). For Western blotting, the antiserum (1 μl) were preabsorbed with 10 nmol counterpart peptide at 37°C for 30 min. For immunostaining of tissue sections, AP301 or AL301 antibody was affinity-purified using the corresponding antigenic peptide. The phosphorylation-independent and -dependent tau antibodies used were HT7 (epitope; residues 159–163) (Innogenetics, Zwijndrecht, Belgium), tau 1 (nonphosphoSer-199 and—202) (Chemicon, Temecula, CA), AT8 (phosphoSer-202 and phosphoThr-205), AT100 (phosphoThr-212 and phosphoSer-214) (Innogenetics), M4 (phosphoThr-235), C5 (phosphoSer-396) (29), and polyclonal AP422 (phosphoSer-422) (30). TM2, a phosphorylation-independent monoclonal antibody, was raised against the cyanogen bromide fragment (residues 251–419) of tau purified from fetal rat brain, and its epitope (residues 368–386) was determined with the Novotope system (Novagen) according to the manufacturer’s instructions. Antibody 4G8 (specific for amyloid β-protein (Aβ) 17–24) was purchased from Senetec PLC (Napa, CA).

Western Blotting and Enzyme-Linked Immunosorbent Assay (ELISA)

Western blotting was performed as described previously (31). Bound antibodies were detected by enhanced chemiluminescence (ECL; Amersham, Buckingham, UK). Semiquantitative Western blotting was performed by exposing the blot several times to ECL film to equalize the signal intensity for the same amount of authentic recombinant tau. ECL bands of interest were quantified with a model GS-700 imaging densitometer with Molecular Analyst® Software (Bio-Rad Laboratories, Hercules, CA). The ELISA was performed as follows. Each well of the ELISA plates (Nunc-Immunoplate; Nunc A/S, Roskilde, Denmark) was coated with approximately 0.01–0.1 μg recombinant wild-type or mutant tau, and loaded plates were incubated with appropriately diluted antisera. Bound antibodies were reacted with peroxidase-conjugated anti-rabbit IgG and
RT-PCR

Two μg of total RNA were obtained from each brain tissue sample by an SV total RNA isolation system (Promega, Madison, WI) and subjected to reverse transcription using random hexamers. The reverse-transcribed cDNA equivalent to 0.025 μg of original total RNA was used as the PCR template, and the cDNA of interest was amplified with the following primer set: 5’-GTGCAGATAATTAAGAAGCTGGATCTT-3’ for sense and 5’-CGTGGGTGATATTGTCCGGGACCACATCT-3’ for antisense. Twenty μl of PCR reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, template cDNA, 0.2 μM of each primer, and 1U Taq polymerase (Takara Shuzo, Shiga, Japan). PCR was performed for 24 cycles with each consisting of 30 s at 94°C, 1 min at 65°C, and 1 min at 72°C. This PCR cycling was confirmed to be within an exponential range of amplification (data not shown). The PCR products were digested withMsp I to distinguish between wild-type and P301L tau cDNA. After separation on a 6% acrylamide gel, the bands were visualized with SYBR Green dye (Molecular Probes, Eugene, OR) and quantified using a FluorImager (Molecular Dynamics, Sunnyvale, CA). Under these conditions, reliability of quantification was assessed using varying doses of the template and varying ratios of mutant to wild-type tau templates (data not shown). To compare the levels of mRNA of wild-type and mutant tau, each signal intensity was normalized by the size of the product.

Immunocytochemistry and Other Histological Methods

To obtain optimal staining with TM2, AP301, and AL301, but not with AT8, deparaffinized tissue sections were subjected to autoclaving at 120°C for 7 min, and then treated with 95% formic acid for 4 min. The sections were then incubated in 10% goat serum in phosphate-buffered saline containing 10 μg/ml affinity-purified AP301 or AL301 antibody. The bound antibodies were detected with Vectastain ABC Elite kit according to the manufacturer’s instruction. After the 3’-diaminobenzidine (DAB) chromogen was developed, sections were treated with 1% CuSO₄ to enhance the color of DAB precipitates (32), and then counterstained with hematoxylin. The numbers of TM2-positive tau deposits including the intracellular NFTs, extracellular NFTs (both classified as fibrillar tangles), and pretangles (stage 0 tangles [33], diffuse tangles [34], or presumed group 1 tangles [35], each of which is defined by diffuse, somewhat granular, cytoplasmic staining) were counted and averaged in 4 nonselected areas of 3.2 mm². The numbers of senile plaques were also counted and averaged over the same areas. The extent of senile plaque formation was estimated by Bielschowsky stain and rated as follows: −, none; +, 1 to 5; ++, 6 to 20; ++++, more than 20 per 3.2 mm².

RESULTS

Selective Accumulation of Mutant Tau in the Insoluble Fraction and Its Selective Decrease in the Soluble Fraction of P301L Brains

To distinguish between wild-type and mutant tau, we raised paired antibodies which specifically recognized either wild-type or P301L mutant tau (Fig. 1A). Western blotting and ELISA clearly showed that AP301 and AL301 exclusively recognized wild-type and P301L mutant tau, respectively, following preadsorption with each counterpart peptide (Fig. 1B, C). Because the epitopes for AP301 and AL301 occur within the exon 10-encoded second repeat, these antisera labeled only 4R isoforms (see the right-most lane in Fig. 1B).

Using these antibodies, we first investigated the tau in the soluble (cytosolic) and insoluble fractions of P301L brains. The Sarkosyl-insoluble fractions were prepared from an AD brain and from various cortices, hippocampi, and cerebella of 3 P301L patients, and subjected to Western blotting. TM2, a pan-tau antibody, labeled 2 major bands at 64 and 68 kDa and a smear in the Sarkosyl-insoluble fraction of P301L brains (Fig. 2A). AL301 clearly labeled these 2 bands and a smear in the same fractions. In contrast, AP301 did not label the 2 bands in the medial frontal gyrus or the angular gyrus from patient 1 or in medial frontal gyrus, angular gyrus, or the occipital cortex from patient 3. However, weak AP301-labeling was definitely discernible in angular gyrus and the hippocampus from patient 2, and barely seen in the parahippocampus gyrus from patient 1 and medial frontal gyrus from patient 2 (Fig. 2A). These results were reproducible in 2 independent experiments. This strongly suggests that the deposition is initiated by mutant tau and followed by wild-type tau.

To examine isoform composition, the Sarkosyl-insoluble fractions from an AD brain and FTDP-P301L brains were subjected to dephosphorylation and Western blotting with HT7. Only the 3 bands corresponding to 2N4R (although very faint), 1N4R, and 0N4R were observed in the specimens from P301L patients (Fig. 2B). The amounts of P301L tau in the Sarkosyl-insoluble fraction varied substantially among the patients as well as among brain regions (Fig. 2). In all 3 patients, no tau immunoreactivity was observed in the insoluble fractions of occipital cortex and cerebellum, the regions least affected by FTDP-17 (Fig. 2 and see below). These results indicate that P301L tau, but barely wild-type tau, is deposited in the Sarkosyl-insoluble fraction in the affected regions of P301L brains. The insoluble tau from P301L brains was labeled by several phosphorylation-dependent antibodies including AT8, AT100, M4, C5, and AP422, indicating that the insoluble P301L tau was hyperphosphorylated just like the tau in the Sarkosyl-insoluble fraction of AD brain (data not shown).

Unexpectedly, in the soluble fraction of P301L brains, mutant tau gave consistently weaker signals than wild-type tau when the signal intensities of the antibodies were normalized using authentic recombinant tau (see Materials and Methods) (Fig. 3A). This decrease in the levels of soluble P301L tau was observed across the various affected regions including the medial frontal gyrus and angular gyrus from all 3 patients. Similar attenuation of
mutant tau was also observed in the occipital cortex and cerebellum (Fig. 3A) where no Sarkosyl-insoluble tau was detected (Fig. 2 and data not shown). Densitometric measurement provided the ratios of mutant to wild-type tau: 7.0 ± 0.2% for frontal cortex; 7.5 ± 1.1% for angular gyrus; 14.7 ± 2.7% for occipital cortex; and 20.5 ± 3.3% for cerebellum (Fig. 3A). This result raises the possibility that the mRNA level for each tau species differs among regions, possibly due to differences in its stability. Thus, we quantified the mRNA levels for wild-type and mutant tau by RT-PCR. However, there was no difference in the expression levels between wild-type and mutant tau in both cortices and cerebella of P301L patients (Fig. 3B and data not shown).

Exclusive Incorporation of Mutant Tau into Pretangles in P301L Brain

Frontal and temporal cortices from all 5 P301L patients exhibited severe atrophy, which was easily recognizable by the naked eye. The cortical laminar architecture was
Fig. 2. Selective deposition of mutant tau in the Sarkosyl-insoluble fraction of P301L brains. A: Sarkosyl-insoluble fractions were prepared from frontal cortex (f) of AD brains and medial frontal gyrus (mf), angular gyrus (a), parahippocampal gyrus (ph), hippocampus (h), and occipital cortex (so) of P301L brains. The proteins derived from 0.5 mg wet weight of tissues were subjected to Western blotting with AP301, AL301, and TM2. The 2 major bands at 64 and 68 kDa were labeled with AL301 (arrowheads) and TM2 (arrows) in 3 P301L brains, but the same bands were not labeled with AP301, except at (mf), (a) and (h) of patient 2, and possibly at (ph) of patient 1. The latter presented barely discernible bands (arrowheads). Asterisks indicate nonspecific labeling, which was unlabeled with any other tau antibodies. B: To confirm isoform composition, those Sarkosyl-insoluble fractions from an AD brain and FTDP-P301L brains were subjected to Western blotting with HT7, with or without prior dephosphorylation. The right lane contained 6 recombinant tau isoforms. Six isoforms after dephosphorylation of PHF-tau are indicated to the right of lane 2. The upper 2 bands, which represent 2N4R and 2N3R isoforms, are faintly discernible. Arrowheads on the right indicate 2N4R, 1N4R, and 0N4R isoforms.

barely preserved and extensive neuronal loss and cortical and subcortical gliosis were obvious. Neuronal loss was usually most remarkable in the first to the third layers. Ballooned neurons were scattered throughout the affected cortex, but no Pick bodies were found. Minimal (patients 4 and 5) to slight (patients 1 and 3) senile changes were found in the hippocampus and parahippocampus. However, patient 2 exhibited numerous NFTs, neuropil threads
Fig. 3. Selective decrease of mutant tau in the soluble fraction of P301L brain (A) and no decrease of mRNA for mutant tau (B). A: TS-soluble fractions were prepared from 1.0 mg wet weight of medial frontal gyrus (mf), angular gyrus (a), occipital cortex (so), and cerebellum (cb) of 3 P301L patients. Lanes 1–3 represent patients 1–3, respectively. The soluble fractions were treated with alkaline phosphatase and subjected to Western blotting with AP301 and AL301, as described in Materials and Methods. Four ng (lane 4) and 20 ng (lane 5) of wild-type or P301L recombinant tau were loaded to estimate roughly the levels of tau in each specimens. Arrows indicate three 4R tau isoforms labeled by these antisera. Bands are barely discernible at the top arrow; these represent the longest form of tau (2N4R). B: Quantitative RT-PCR was performed as described in Material and Methods. Msp1 digests of the PCR products were subjected to 6% acrylamide gel electrophoresis and visualized with SYBR Green. The signal intensity of the wild-type fragment (167 bp) was compared with P301L (247 bp) from 24-cycled PCR products. Data were reproducible in 3 independent experiments.
the hippocampus, where patient 2 exhibited abundant NFTs and neuritic plaques (see below). Numerous tau deposits were observed in granule cells of the dentate gyrus in 4 of the 5 patients (patients 2–5). Extensive tau deposition as pretangles was also observed in entorhinal and parahippocampal cortices in 2 of 5 patients (patients 3 and 4) (Table and data not shown).

In R406W brains, AT8 and TM2 labeled to similar extents abundant NFTs and NTs in the temporal cortex. TM2 labeled innumerable tau deposits in CA1 region, while AT8 barely labeled. In general, a larger number of AT8-positive deposits and TM2-positive tangles in the CA1 region of R406W brains. Patient 2 showed a large number of TM2-positive tau deposits in the CA1 region.

Because our immunohistochemical study showed selective deposition of mutant tau in the insoluble fraction, we next analyzed tau deposits immunocytochemically in the P301L brains using the same site-specific antibodies. As expected, AP301 but not AL301 labeled innumerable NFTs and NTs in the AD brain (Fig. 4A, B). In P301L brains, AL301 stained crescent or ring-like perinuclear deposits within the cytoplasm, whereas AP301 did not stain any tau deposits except in patient 2 (see below) (Fig. 4C–F). High magnification with differential interference contrast (DIC) showed clearly that the tau deposits in the P301L brains represent pretangles with diffuse, mostly perinuclear, nonfibrillar cytoplasmic staining (33–35), which contrasted sharply with typical fibrillar NFTs observed in R406W brains or in AD brain (Fig. 5).

### Coexistence with AD Pathologies

Patient 2 exhibited unusual neuropathological characteristics among the P301L patients (Table; Fig. 6): the presence of numerous senile plaques, as well as tau deposits, in the temporal cortex, entorhinal cortex, subiculum, and hippocampus. Antibody 4G8 visualized massive Aβ deposition, including both mature and diffuse plaques. Mature plaques were located exactly in the disorganized neuropil clustered with dystrophic neurites (Fig. 6A, C). The entorhinal cortex exhibited numerous NFTs and senile plaques in a distinct manner (Fig. 6E, F). Such distribution of both NFTs and senile plaques in the brain of patient 2 strongly suggests the concomitant presence of AD pathology. In view of the high prevalence of AD, it is reasonable to assume that this P301L brain is complicated by AD pathologies.

AP301 labeled many pretangles, as well as numerous NFTs and dystrophic neurites in CA1, as seen in AD (Fig. 6A). In contrast, AL301 stained numerous pretangles and a small number of NFTs and dystrophic neurites in CA1, the area that was less affected in other P301L patients. Immunostaining of an adjacent section showed that there were similar numbers of AT8-positive deposits and TM2-positive tangles in CA1 (Fig. 6D; Table; and see below). Unexpectedly, most of dystrophic neurites were barely labeled with AT8.

To further confirm the above finding, we counted the numbers of pretangles and NFTs stained with AP301 or AL301 in the adjacent sections of the hippocampus and temporal cortex. In the hippocampus, AP301 labeled 25 ± 9 NFTs and 24 ± 5 pretangles per 3.2 mm² (average of 4 unselected fields), whereas AL301 labeled 3 ± 1 NFTs and 35 ± 2 pretangles. In the temporal cortex, AP301 labeled 29 ± 11 NFTs and 8 ± 2 pretangles, and
AL301 labeled 11 ± 5 NFTs and 35 ± 2 pretangles. Thus, the 2 antibodies have contrasting staining characteristics in these regions: AP301 preferentially labeled NFTs, and AL301 preferentially labeled pretangles.

DISCUSSION

Although previous reports showed that 4R tau is selectively or preferentially deposited in the Sarkosyl-insoluble fraction of the P301L brain, we have clearly shown here the selective deposition of mutant P301L tau in that fraction of the brains of patients 1 and 3 (Fig. 2A and see below). This is consistent with the immunocytochemical finding of selective incorporation of mutant tau into pretangles in patients 1 and 3 as well as patients 4 and 5. These observations contrast with those on the R406W brain where both wild-type and mutant tau were accumulated in an approximately 1:1 ratio in the Sarkosyl-insoluble fraction and were incorporated into NFTs (36). This may be partly explained by the differential capacity of the mutant proteins for in vitro filament formation. P301L tau has the strongest tendency to aggregate into filaments, while R406W tau has the least (8).

The presence of wild-type tau in the Sarkosyl-insoluble fraction appears to correlate with the levels of mutant tau: when levels of mutant tau are relatively high, trace amounts of wild-type tau appear (Fig. 2A). This strongly suggests that the initial deposition is initiated by mutant tau and followed by wild-type tau. Thus, it can be speculated that in significantly affected regions, both wild-type and mutant tau can be found in the Sarkosyl-insoluble fraction, although the latter is predominant. There are several reports showing that 3R and 4R tau coexist in the Sarkosyl-insoluble fraction of the P301L brain (5, 9, 19, 37). These results may be explained by differences in the regions sampled or as individual variability among or within families. However, in our hands the Sarkosyl-insoluble fractions examined contained no 3R tau. Thus, at least the initial process of FTDP-P301L does not involve 3R tau, and its incorporation in the Sarkosyl-insoluble fraction may be a rather late event.

One may argue that the immunochemical and immunocytochemical observations presented above are somewhat contradictory: although robust AP301-staining of NFTs and dystrophic neurites can be observed in the tissue sections from patient 2 (Fig. 6), the same antibody provided only faint signals on the Western blot compared with AL301 (Fig. 2A). It should be noted that the levels of mutant and wild-type tau were carefully semiquantified on the Western blot using these site-specific antibodies, but this principle cannot be applied to the tissue section. The antibody reactivities on the tissue section depend on the preservation of antigenicity after formalin fixation. Normal, cytoplasmic tau is highly susceptible to fixatives (38) and readily loses its antigenicity, whereas the tau in NFTs and NTs is resistant to fixatives and can retain a strong antigenicity. By analogy, it would be reasonable to postulate that the antigenicity of tau in NFTs is better preserved during fixation than is that in pretangles. Thus, the levels of wild-type and mutant tau cannot be even semiquantitatively estimated from the immunocytochemical data. This consideration further raises the possibility that the number of AL301- and AP301-labeled pretangles is underestimated in the tissue sections. Combined with the biochemical data, it is reasonable to conclude that the mutant tau is predominantly deposited in P301L brains.

Few reports refer to the interplay between Aβ and tau deposition in FTDP-17. Thus, P301L patient 2 provides an invaluable opportunity to observe the rare interaction of FTDP-17 and AD pathological processes. The predominant staining of pretangles with AL301 leads to the reasonable assumption that AL301-stained pretangles represent the FTDP-17 process. Similarly, the preferential staining of fibrillar NFTs with AP301 suggests that AP301-stained NFTs largely represent the AD process. As the hippocampus, which is known to be relatively resistant to FTDP-17, was most affected in terms of tau deposition in patient 2, this finding suggests that coexisting AD pathologies, in particular Aβ deposition (Fig. 6E, F), may have a significant impact on the initiation and development of FTDP-P301L (Fig. 2A). This is supported by a recent report on the tau pathology in transgenic mice overexpressing V717F amyloid precursor protein (39). Another possibility would be that neurodegeneration or neuronal dysfunction caused by tau mutation induces senile plaque formation similar to that in traumatic head injury (40).

Despite numerous AT8-positive pretangles, much smaller numbers of tau deposits were labeled with TM2 in the P301L brains, as compared with the R406W brain (Table). This is likely attributed to differential affinities of these antibodies for various tau deposits: pretangles, intracellular tangles, and extracellular tangles. Lower affinity of TM2 for pretangles may be caused by higher susceptibility of nonphosphorylated epitopes to fixatives. Regarding NFT, the local concentrations of the remaining TM2 epitope may be sufficient for strong immunoreactivity as found in R406W brains. In contrast, scarce AT8 immunoreactivity of extracellular tangles may be explained by processing of amino-terminal portion of the tau containing Ser-202 and Thr-205. Thus, P301L brains are characterized by a larger number of AT8-positive deposits and a smaller number of TM2-positive ones, while R406W and AD brains are characterized by a larger number of TM2-positive deposits and a smaller number of AT8-positive deposits or by similar numbers of TM2- and AT8-positive deposits. The CA1 region of P301L patient 2 has the latter rather than former characteristics (Table). It is likely that tau deposits in P301L brain are not so matured as found in R406W or AD brain.
Fig. 4. Immunostaining of P301L tissue sections with AP301 and AL301. Paraffin-embedded tissue sections from the frontal cortex of an AD patient (A, B) and the temporal cortex (C, D) and hippocampus (E, F) of P301L patient 3 were immunostained with AP301 (A, C, E) and AL301 (B, D, F). An AP301-stained AD section (A) provides a profile very similar to anti-tau-stained section (data not shown). This indicates that the AP301 recognizes specifically the tau epitope on the tissue section, although this
Most interestingly, the levels of P301L tau were selectively reduced in the soluble fraction of P301L brains. Because its mRNA levels did not differ significantly from those of wild-type tau, differential instability of mRNA can be excluded. It is also unlikely that mutant tau is more vulnerable to proteases during the postmortem period, because incubation with the cytosolic fraction from tau-deficient mouse brain did not show preferential degradation of mutant tau (data not shown). Nor can this decrease be explained by selective deposition of mutant tau that could occur concomitantly, because significant decreases in the levels of mutant tau were already noted even in apparently intact regions and in the regions where Sarkosyl-insoluble tau was undetectable. Possible explanation for these observations are that because mutant tau is less tightly bound to MT or tubulin oligomers (8, 9), the resulting free mutant tau could be more susceptible to proteases than bound tau, and the steady-state levels of the mutant tau are decreased in the neuron. This is supported by a recent finding that the mutant tau is displaced from MTs by wild-type in co-transfected cells (41).

Only recently, Rizzu et al have published a paper in which they describe similar findings in frozen brain specimens from P301L patients using another set of site-specific antibodies they raised: selective deposition of mutant tau in the Sarkosyl-insoluble fraction and decrease in the Sarkosyl-soluble fraction (37). Thus, their major findings are duplicated here and further substantiated and extended by detailed immunocytochemical observations. Our data may also support the view that the levels of mutant tau in the soluble fraction are inversely correlated with the extent labeled additional nonspecific bands on the blot (Fig. 2A). While innumerable NFTs and NTs stained only for AP301 in the AD brain, numerous tau deposits were labeled with AL301, but not with AP301, in the P301L brain. Asterisks indicate the same vessel for each pair of adjacent sections in all panels. Scale bar = 100 μm.
Fig. 6. Coexistence of NFTs and senile plaques in patient 2. The tissue sections containing the hippocampus from P301L patient 2 were immunostained with AP301 (A, E), AL301 (B), 4G8 (C, F), and AT8 (D). Fibrillar NFTs (thick arrow and inset in A) and dystrophic neurites (arrowhead in A) in CA1 were intensely immunostained with AP301. Pretangles (thin arrow and inset in B), but not NFTs, in CA1 were labeled with AL301. Dystrophic neurites were barely stained with AL301 (arrowhead in B). In an adjacent section, 4G8 intensely stained senile plaques the locations of which exactly corresponded to those of dystrophic neurites (C). All tau deposits were labeled with AT8 (D). The entorhinal cortex from P301L patient 2 exhibited abundant NFTs (E) and senile plaques (F). Asterisks (A–D) and arrows (E, F) indicate the same vessels in adjacent sections. Scale bar = 100 μm.

of the pathology (37). However, it should be noted that at least in one case in which there are no neuropathological abnormalities, reduction of mutant tau in the soluble fraction of cerebellum is already obvious (Fig. 3, lane cb1). This strongly suggests that significant decrease of mutant tau is attributed to its inherent properties, and its further decrease seen in frontotemporal regions may be caused by selective deposition of mutant tau. A similar diminution of all 6 isoforms of tau without reduced levels of tau mRNA was observed very recently in a sporadic FTD and a familial FTD linked to chromosome 17 with no identified tau gene mutation and no tau deposition (42). Although the neuronal death cannot be simply explained by loss of tau, considering little abnormality in tau-deficient mice (43), a decrease in the levels of tau may be associated with the neurodegeneration at least in a certain group of FTDs with or without tau mutations. Elaborate roles of tau on axonal growth may be taken into account for maintenance of neuronal processes when the neurons were impaired. This has recently been shown using another line of tau-deficient mice (44). Regarding potentially important degraded fragments (37), we were unable using our own antibodies to detect mutant tau-specific fragments in the soluble fraction (data not shown). Although we cannot completely exclude the possibility that this discrepancy comes from a different fractionation protocol, this particular finding may not be consistent among P301L brains.
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REFERENCES


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