Ubiquitin-negative mini-pick-like bodies in the dentate gyrus in p301l tauopathy

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Abstract. Neuropathological and biochemical findings are reported in a patient who had suffered from frontotemporal dementia associated with a P310L mutation in the \textit{tau} gene and included in the H1 haplotype. \textit{Tau} accumulation, as revealed with phospho-specific anti-\textit{tau} antibodies Thr181, Ser199, Ser202, Ser214, Ser262, Ser396, Ser422 and AT8 (Ser202 and Thr205), was found in neurons with pre-tangles, and astrocytes and oligodendrocytes through the brain. The most characteristic feature was \textit{tau} immunoreactivity decorating the perinuclear region and small cytoplasmic aggregates designed as mini-Pick-like bodies, mainly in the dentate gyrus. Inclusions were not stained with anti-ubiquitin antibodies and did not recruit tubulins. Tau accumulation in individual cells was associated with increased expression of kinases linked with \textit{tau} phosphorylation, mainly active (phosphorylated) stress kinases SAPK/JNK and p38 (SAPK/JNK-P and p38-P). Phosphorylated GSK-3\textit{\beta} at Ser9 (GSK-3\textit{\beta}-P), that inactivates the kinase, was particularly abundant in mini-Pick-like bodies, thus suggesting alternative roles of GSK-3 probably involved in cell survival. Western blots of sarkosyl-insoluble fractions revealed a double band pattern of phospho-\textit{tau} of 68/66 kDa and 64 kDa in the hippocampus and white matter in the P310L mutation. Sarkosyl-insoluble fractions of the hippocampus were enriched in p38-P and GSK-3\textit{\beta}-P in Alzheimer’s disease (AD) cases, processed in parallel for comparative purposes, but not in the P310L mutation. In addition, no bands of high molecular weight were found in P310L in contrast with AD in these fractions. These findings indicate that the major sites of \textit{tau} phosphorylation, and the expression of kinases involved in \textit{tau} phosphorylation are active in P310L mutation as in AD and other tauopathies. Yet the P310L mutation has particular phospho-\textit{tau} inclusions that are not tag with ubiquitin and appear to be rather soluble when compared with AD.

1. Introduction

Tauopathies are neurodegenerative diseases characterized by the abnormal hyper-phosphorylation and deposition of \textit{tau} in neurons and glial cells. Alzheimer’s disease (AD), Pick’s disease (PiD), progressive supranuclear palsy, corticobasal degeneration and argyrophilic grain disease (AGD) are common tauopathies \cite{7,27,38–40}. In addition, the term frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) designates inherited tauopathies associated with mutations in the \textit{tau} gene \cite{7,16,23,27}. Tauopathies have been reproduced in transgenic mice \cite{17,22,28}.

The P301L mutation is a common cause of familial tauopathy \cite{1,6,26,31,33,36,37}. Like other \textit{tau} mutations in the binding region encoded by exon 10, the P301L mutation greatly reduces the capacity to promote microtubule assembly but results in abnormal fibrillar aggregation \cite{3,20,42}. Frontotemporal atrophy with ballooned neurons in the frontal cortex, widespread \textit{tau}-immunoreactive neurons and glial cells, and a pattern of two bands of 66/68 kDa and...
64 kDa on Western blots have been observed in previous cases [1,31,37]. In addition, some structural aspects of tau phosphorylation in neurons and glial cells associated with the P301L mutation differ from one case to another. Thus, neurofibrillary tangles, and astrocytic plaques and tuft-like astrocytes appear to be common in some cases but are rare in others [1,31,16]. This indicates that, in addition to the common mutation, other factors, including the haplotype, may influence the phenotype of the mutation [33]. Previous studies have shown the P301L mutation in the H2 haplotype [41]. Interestingly, the presence of ubiquitin-negative, mutated tau-positive perinuclear reinforcements and small cytoplasmic aggregates reminiscent of mini-Pick bodies in neurons of selected areas, mainly granule cells of the dentate gyrus, has been recently emphasized in two patients with frontotemporal dementia bearing the P301L mutation [1]. The P301L mutation has been generated in two transgenic lines in mice [19,29,30].

The present study focuses on the tau deposits in neurons in a new case of P301L tauopathy with particular attention to tau phosphorylation and aggregation, and to the local expression of kinases involved in tau phosphorylation.

2. Material and methods

2.1. Case report

The proband was a man who had suffered from disturbances in social behavior starting at the age of 45 years, followed by intellectual impairment, language disorder, difficulty in calculation but with preservation of orientation and memory. The MRI carried out at the age of 49 showed frontotemporal atrophy. The course of the disease was progressive with apathy, irritability, apraxia and epilepsy, together with marked loss of language abilities. The patient died at the age of 52 years. His mother was affected by a similar disease; no neurological examination and post-mortem study was available.

The post-mortem delay between death and tissue processing was 3 hours.

2.2. Genetic study

Exons of the tau gene were amplified by using primers derived from 3′ and 5′ intronic sequences and polymerase chain reaction (PCR) conditions previously described [21] and analyzed through single strand conformation polymorphism (SSCP). After, electrophoresis, the gel was silver stained as described [4]. The SSCP analysis of exon 10 indicated the presence of an abnormal pattern in the proband when compared with controls. The polymerase chain reaction (PCR) product corresponding to the sample with the abnormal SSCP pattern was sequenced using the ABIPRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer, Foster City, CA, USA) following the manufacturer’s protocols and an automatic sequencer (ABI PRISM model 377). The exon 10 sequence revealed the presence of the P301L mutation in heterozygosis in both, the sense and the complementary strand. Tau haplotype H1/H1 was determined genotyping one of the polymorphism previously described [10].

2.3. Neuropathological study

The neuropathological examination was carried out in formalin-fixed tissue for no less than three weeks; the tissue was then embedded in paraffin. De-waxed sections, 5 µm thick, were stained with haematoxylin and eosin, and luxol fast blue-Klüver Barrera or processed for immunohistochemistry following the streptavidin LSAB method (Dako, Dakopats, Barcelona, Spain). After incubation with methanol and normal serum, the sections were incubated with one of the primary antibodies at 4°C overnight. Antibodies to phosphorylated neurofilaments of 170 kD or 200 kD (clones BF10 and RT97, Boehringer-Mannheim, Barcelona, Spain) were used at dilutions of 1:100 and 1:50, respectively. Antibodies to glial fibrillary acidic protein (GFAP, Dako), βA4-amyloid (Boehringer-Mannheim), and ubiquitin (Dako) were used at dilutions of 1:250, 1:5, and 1:200, respectively. Antibodies to α-synuclein (Dako) were used at a dilution of 1:100. Antibodies to pan-tau (Sigma, Madrid, Spain) were used at a dilution of 1:10. In addition, the following phospho-specific tau rabbit polyclonal antibodies were used: Thr181, Ser199, Ser202, Ser214, Ser231, Ser262, Ser396 and Ser422 (all of them from Calbiochem, VWR, Barcelona, Spain). The antibodies were used at a dilution of 1:100, excepting anti-phospho-tauThr181, which was used at a dilution of 1:250. The monoclonal antibody AT8 (Dr. J. Avila), that recognizes tau protein phosphorylated at both Ser202 and Thr205 [17] was used at a dilution of 1:200. Monoclonal antibodies to α-tubulin (Neomarkers, Molecular Probes, Leiden, The Netherlands) and rabbit polyclonal antibodies
to β2-tubulin (Dr. J. Avila) were used at dilutions of 1:100 and 1:500, respectively. Following incubation with the primary antibody, the sections were incubated with LSAB for 1 h at room temperature. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. Sections were counterstained with haematoxylin. Sections processed for phospho-tau immunohistochemistry were boiled in citrate buffer prior to the incubation with the primary antibody. Sections processed for βA4-amyloid and α-synuclein were pre-treated with 95% formic acid.

2.4. Kinase immunohistochemistry

Sections were processed with the streptavidin LSAB method, as previously. Sections were boiled in citrate buffer and then processed for immunohistochemistry. The anti-MAP kinase phospho-specific (MAPK/ERK-P) rabbit polyclonal antibody (Calbiochem) is raised against a synthetic phospho-tyrosine peptide corresponding to residues 196–209 of human p44 MAP kinase. The antibody detects phosphorylated Tyr204 of p44 and p42 MAP kinases (phospho-ERK1 and ERK2). The purified phospho-p38 MAP kinase (Thr180/Tyr182) (p38-P) rabbit polyclonal antibody (Cell Signaling, Izasa, Barcelona, Spain) detects p38 MAP kinase only when activated by dual phosphorylation at Thr180 and Tyr182. The purified rabbit polyclonal phospho-SAPK/JNK (Thr183/Tyr185) antibody (SAPK/JNK-P) (Cell Signaling) is produced against a synthetic phospho-Thr183/Tyr185 peptide corresponding to the residues of human SAPK/JNK. The antibody detects SAPK/JNK only when activated by phosphorylation at Thr183/Tyr185. The antibodies to MAPK/ERK-P and SAPK/JNK-P were used at a dilution of 1:100. The antibody to p38-P was used at a dilution of 1:200. The anti-GSK-3α/β monoclonal antibody (StressGen, Bionova, Madrid, Spain) reacts with 51 and 47 kDa proteins corresponding to the specific molecular weight of GSK-3α and GSK-3β. The antibody was used at a dilution of 1:100. The anti-phosphospecific GSK-3-β/Ser9 antibody (Oncogene, Bionova, Madrid, Spain) is a rabbit polyclonal IgG antibody specific for the Ser9 phosphorylated form of glycogen synthase kinase-3β. The antibody was used at a dilution of 1:100.

2.5. Biochemical study

For gel electrophoresis and Western blotting, fresh samples of the P301L mutation were immediately obtained at autopsy, frozen in liquid nitrogen, and stored at −80°C until use. For comparative purposes, fresh hippocampal samples of four patients with Alzheimer’s disease stage V of Braak and Braak, with similar post-mortem delays (between 2 and 5 h), were processed in parallel.

Fresh samples from the hippocampus and white matter (about 5 g) were homogenized in a glass tissue grinder in 10 vol (w/v) of cold suspension buffer consisting of 10 mM Tris-HCl (pH = 7.4), 0.8 M NaCl, 1 mM EGTA, 10% sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 10 μg/ml leupeptin and 5 μg/ml pepstatin. The homogenates were first centrifuged at 20,000 × g, and the supernatant (S1) was retained. The pellet (P1) was re-homogenized in 5 vol of homogenization buffer and re-centrifuged. The two supernatants (S1+S2) were then mixed and incubated with N-lauroylsarcosynate 1% for 1 h at room temperature while shaking. Samples were then centrifuged for 1 h at 100,000 × g in a Ti 70 Beckman rotor. Sarkosyl-insoluble pellets (P3) were re-suspended (0.2 ml per g of starting material) in 50 mM Tris-HCl (pH = 7.4). Protein concentrations were determined by the BCA method and 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was run using a maxi-protean system (Bio-Rad, Madrid, Spain). 100–200 μg of protein was loaded in each lane with loading buffer containing 0.125 M Tris (pH = 6.8), 20% glycerol, 10% mercaptoethanol, 4% SDS and 0.002% bromophenol blue. Samples were heated at 95°C for 5 min prior to gel loading. Total homogenates and fractions enriched with abnormal filaments were run in parallel. The proteins were then transferred to nitrocellulose membranes (Amersham) using an electrophoretic chamber system (Trans-Blot Electrophoretic Transfer Cell, Bio-Rad). Non-specific binding sites were blocked with Tris-buffered saline solution pH = 7.4 with 0.1% Tween-20 (TBST) containing 5% skimmed milk for 30 min, and incubated with one of the primary antibodies for 1 h at room temperature. Control of protein content in each lane was carried out by the staining of selected gels with Coomassie blue and of the membranes with Ponceau (Sigma). The rabbit polyclonal antibody to phospho-τThr181 was diluted 1:250. The rabbit polyclonal antibodies anti-phospho-τSer262 and Ser422 (all from Calbiochem) were used at a dilution of 1:1500. The p38-P antibody (Cell Signaling) was used at a dilution of 1:200. The anti-GSK-3-β/Ser9 antibody (Oncogene) was used at a dilution of 1:100. After washing, the membranes were incubated with the secondary antibody labeled with
horseradish peroxidase (Dako) diluted 1:1000 for 1 h at room temperature, washed again, and developed with the chemiluminescence ECL Western Blotting system (Amersham, Barcelona, Spain). Membranes were then exposed to autoradiographic films (Hyperfilm ECL, Amersham).

3. Results

3.1. General neuropathological findings

The macroscopical examination revealed moderate atrophy of the brain (1300 g) predominating in the frontal and temporal lobes, and striatum and slight atrophy of the thalamus. The cerebellum and brain stem were normal with the exception of slight pallor in the substantia nigra.

The microscopic study showed severe neuron loss and increased numbers of astrocytes in the cerebral cortex, and spongiosia in the upper layers predominating in the frontal and temporal lobes (Fig. 1 A). Ballooned neurons filled with phosphorylated neurofilaments and containing αB-crystallin were common in the frontal cortex (Fig. 1 B). The hippocampus and the dentate gyrus were best preserved in haematoxylin and eosin-stained sections, although pale cytoplasmic inclusions were seen in a few granule cells (Fig. 1 C). Slight neuron loss and astrocytic gliosis were present in the striatum, amygdala and substantia nigra. Slight myelin pallor, together with astrocytic gliosis, was found in the centrum semi-ovale. No α-synuclein inclusions and βA4-amyloid deposits were found.

Sections stained with anti-tau antibodies disclosed the presence of tau accumulation in neurons in the upper and inner layers of the cerebral neocortex (Fig. 2 A), entorhinal cortex (Fig. 2 B), subiculum and cellular layer of the hippocampus (Fig. 2 C), thalamus, subthalamus, striatum (Fig. 2 D), dentate gyrus (Fig. 2 E), amygdala and Meynert nucleus (Fig. 2 E), among other gray nuclei. Interestingly, tau deposition in the cerebral cortex displayed a peculiar pattern involving layer II/III and layer V neurons. The majority of neurons were pre-tangles, whereas neurofibrillary tangles were very rare. The most characteristic finding was tau-immunoreactivity decorating the perinuclear region and, particularly, small cytoplasmic aggregates resembling mini-Pick-like bodies. Mini-Pick-like bodies were most common in the dentate gyrus (Fig. 2 E) but were seldom encountered in the cerebral cortex (Fig. 2 B) and deep cerebral nuclei (Fig. 2 E). A few tau-immunoreactive substantia nigra, locus ceruleus, periaqueductal and periventricular nuclei, and reticular formation of the brain stem. In addition to neurons, tau-immunoreactive astrocytes were found in the cerebral cortex (Fig. 2 G and H). Astrocytic plaques were seen in the frontal cortex but not in the hippocampus and dentate gyrus. Tuft-like astrocytes were absent. Oligodendroglial inclusions, some of them reminiscent of coiled bodies, were abundant in the white matter (Fig. 2 I).

Tau-immunoreactive inclusions in neurons and glial cells were examined with antibodies to phospho-tau. Similar findings were found with the phospho-specific anti-tau antibodies AT8, and Thr181, Ser199, Ser202, Ser214, Ser262, Ser396 and Ser422. Pre-tangles, perinuclear halos and cytoplasmic neuronal aggregations designed as mini-Pick-like bodies were equally immunostained. Interestingly, the vast majority of granular neurons in the dentate gyrus were stained with anti-phospho-tau antibodies: more than a half contained mini-Pick-like bodies. Yet pre-tangles and tau-immunoreactive inclusions, including mini-Pick-like bodies, were not stained with anti-α-tubulin and anti-β-tubulin antibodies. Mini-Pick-like bodies, and the vast majority of neurons, excepting rare neurofibrillary tangles, were not stained with anti-ubiquitin antibodies (data not shown).

3.2. Phospho-kinase immunoreactivity

Neuronal immunostaining was rarely observed with anti-MAPK/ERK-P antibodies. Yet, small punctate SAPK/JNK-P-immunoreactive cytoplasmic granules were present in about 1/3 of granular neurons in the dentate gyrus (Fig. 3 A, C). Immunoreactivity to p-38-P was observed in more than a half of dentate gyrus granular neurons (Fig. 3 A, D), and in many cortical and CA1 pyramidal neurons with pre-tangles (Fig. 3 B, E). No differences in GSK-3α/β immunostaining was found between neurons with and without abnormal tau deposits (data not shown). Mini-Pick-like bodies were decorated with anti-GSK-3β-P antibodies (Fig. 3 F). However, GSK-3β-P was rarely encountered in neurons with no mini-Pick-like bodies, including those of the frontal and temporal neocortex.

3.3. Biochemical studies

Biochemical studies of total homogenates and sarkosyl-insoluble fractions disclosed a pattern of two bands of phospho-tau of 68/66 kDa and 64 kDa in the
Fig. 1. Marked loss of neurons and spongiosis of the upper layers in the frontal cortex (A). This is accompanied by ballooned neurons containing β-crystallin (B). No apparent cell loss occurs in the dentate gyrus although a few granule cells contain pale small cytoplasmic inclusions (D, arrow). Paraffin sections; CC: cerebral cortex; DG: dentate gyrus. A, bar = 25 μm. B and C, bar in C = 10 μm.

Fig. 2. Tau-immunoreactive neurons are seen in the frontal cortex (A), entorhinal cortex (B), CA1 area of the hippocampus (C), striatum (D), dentate gyrus (E) and Meynert nucleus (F). Most tau-immunoreactive neurons are pre-tangles showing perinuclear immunoreactive halos and cytoplasmic small inclusion roughly resembling mini-Pick bodies, which are particularly abundant in neurons of the dentate gyrus. In addition, tau-immunoreactive astrocytes are found in the cerebral cortex (G, H), and tau-immunoreactive oligodendrocytes in the white matter (I). Paraffin sections slightly counterstained with haematoxylin. CC: cerebral cortex; EC: entorhinal cortex; CA1: area of the hippocampus; str: striatum; DG: dentate gyrus; Mey: Meynert nucleus; As: astrocytes; WM: white matter. Bar = 10 μm.

hippocampus and white matter. The same results were obtained with the antibodies to phospho-tauThr181, phospho-tauSer262 and phospho-tauSer422 (Fig. 4).

Kinase expression in the hippocampus was examined in parallel in AD and P301L mutation for comparative purposes. Sarkosyl-insoluble fractions in AD, but not in P301L, were enriched in p38-P and GSK-3β-P. Moreover, p38-P-immunoreactive bands of high molecular weight were not found in the P301L mutation. Finally, the largest amount of GSK-3β-P was found in the sarkosyl-insoluble fraction in AD (Fig. 5).

4. Discussion

Tau accumulation in the patient with a P301L mutation in the tau gene and included in the H1 haplotype was found in neurons with pre-tangles, and astrocytes and oligodendrocytes through the brain. The most characteristic feature was tau immunoreactivity decorating the perinuclear region and small cytoplasmic aggregates designed as mini-Pick-like bodies, mainly in the dentate gyrus. Similar inclusions were reported in seminal cases [31] and have been recently examined by
Adamec et al. [1]. Perinuclear tau-positive immunoreactivity also occurs in FTDP-17 with a P301S mutation in the tau gene [8]. Pick-body-like neuronal inclusions have also been found in association with a G389R mutation in the tau gene in the context of FTDP-17 [36].

The sites of tau phosphorylation, as seen by using a panel of phospho-specific anti-tau antibodies, are similar in P301L tauopathy and other sporadic and familial tauopathies [11,15]. Phosphorylation sites include Thr181, Ser199, Ser202, Ser214, Ser262, Ser396 and Ser422, and those recognized by the antibody AT8 (Ser202 and Thr205). The present results have shown increased SAPK/JNK-P and p-38-P expression in association with tau deposits, whereas only a few neurons have been stained with the anti-MAPK/ERK-P antibodies. Phosphorylation of tau in the P301L mutation probably depends on the same kinases that phosphorylate tau in other tauopathies, including Alzheimer’s disease, progressive supranuclear palsy, corticobasal degeneration, Pick’s disease, argyrophilic grain disease and FTDP-17 [2,11–15,25,34–35,43–45]. Therefore, it is reasonable to conclude that mutated tau facilitates tau phosphorylation through a mechanism that is common to other tauopathies. In addition, GSK-3β-PSer9
is present in neurons and glial cells in sporadic and familial tauopathies [11,12,15,34]. It is interesting to note that GSK-3β-PSer9 decorates a few neurons in the cerebral cortex but the majority of neurons with mini-Pick-like bodies in the dentate gyrus in the P301L mutation. Phosphorylation of GSK-3β at Ser9 inactivates the kinase and then the capacity to phosphorylate substrates, but GSK-3β-PSer9 also prevents cell death by apoptosis in several paradigms [9]. It is feasible that multiple signals can be triggered by GSK-3 depending on the state and site of phosphorylation [5,24]. Granule cells in the dentate gyrus are largely preserved in number whereas neurons in the upper neocortical layers are devastated. It is tempting to speculate that neurons expressing GSK-3β-PSer9 are best equipped to cancel cell death programs in P301L tauopathy.

Although phosphorylation sites in tau and expression of kinases in target cells kinases are similar in the P301L mutation and other sporadic and familial tauopathies, it is important to note that tau deposits differ from those encountered in AD and PiD. A major point is the lack of staining with anti-ubiquitin antibodies in the vast majority of tau-containing neurons, excepting the few with neurofibrillary tangles, and the lack of ubiquitination of the perinuclear halo and mini-Pick-like bodies in P301L tauopathy, as previously stressed by Adamec.

Fig. 4. Western blots of sarkosyl-insoluble fractions of the white matter and hippocampus showing a pattern of two bands of 68/66 kDa and 64 kDa by using phospho-specific anti-tau antibodies Thr181, Ser262 and Ser422.
et al. [1]. Abnormal tau deposition in neurons is neither associated with abnormal accumulation of α-tubulin and β-tubulin, as occurs in neurofibrillary tangles in AD and Pick bodies in PiD. These findings further support the notion of structural differences between neuronal tau inclusions in AD and PiD, and P301L tauopathy. Pre-tangle neurons that are a characteristic feature in AGD are not ubiquitinated and do not recruit β-tubuline with this observation, p38-P and GSK-3β-P are enriched in sarkosyl-insoluble fractions in AD, but not in the P310L mutation. Moreover, p38-P bands of high molecular weight indicating either the formation of aggregates or abnormal cross-reaction with phospho-tau do occur in AD, but not in P310L mutation. Finally, higher GSK-3β-P expression is found in the sarkosyl-insoluble fraction in AD whereas the contrary occurs in P310L-tau mutation. Interestingly, the pattern of kinases in subcellular fractions in P310L mutation resembles that found in argyrophilic grain disease [11] in which, in addition to grains, pre-tangles rather than tangles are the major pathological abnormality. Together these results point to differences in solubility of tau and associated kinases in P310L mutation when compared with AD.

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