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Different distribution of phosphorylated tau protein isoforms in Alzheimer's and Pick's diseases

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Abstract Tau proteins aggregate into different neuronal inclusions in several neurodegenerative disorders. In Alzheimer's disease (AD), hyperphosphorylated Tau from paired helical filaments (PHF) of neurofibrillary tangles, named PHF-Tau, have an electrophoretic profile with four main bands (Tau 55, 64, 69, 74 kDa). In Pick's disease, phosphorylated Tau from Pick bodies are made of two major components (Tau 55, 64 kDa) and a minor 69 kDa. Here we show, using specific antibodies against translated exon 2, 3 or 10 of Tau isoforms, that the set of Tau isoforms engaged in the most insoluble part of PHF in AD is made of Tau isoforms with exon 10 while they are lacking in phosphorylated Tau from Pick's disease. Our results suggest that specific sets of Tau isoforms distinguish between typical neuronal inclusions.

Key words: Neurodegeneration; Tau protein; Alzheimer's disease; Pick's disease; Antibody

1. Introduction

Paired helical filaments (PHF) are the main structural components found in degenerating neurons of Alzheimer's disease (AD). These pathological filaments are composed of abnormally hyperphosphorylated Tau proteins, named PHF-Tau or pathological Tau proteins [1–4]. In many other neurodegenerative diseases, Tau-positive inclusions are also observed (for review see [4]), including corticobasal degeneration [5], progressive supranuclear palsy [6,7], amyotrophic lateral sclerosis/parkisonism-dementia complex of Guam [8,9], myotonic dystrophy [10] and Pick's disease (PiD) [5,11]. Tau protein aggregation is associated with neurofibrillary degeneration (NFD) and the presence of degenerating neurons in neocortical association areas is well correlated with cognitive impairment [12]. Interestingly, the Tau profile is disease specific [4]. For example, pathological Tau are composed of four main components (Tau 74, 69, 64 and 55 kDa) in AD whereas they are made of two major components (Tau 64, 55 kDa) and a minor Tau 69 kDa in PiD [4,11].

Tau are phosphoproteins which belong to the family of microtubule-associated protein. In human brain, alternative mRNA splicing of a single gene transcript generates six Tau isoforms [13]. These isoforms differ by the insertion of 29 or 58 amino acids, translated from exon 2 or exons 2 and 3, near the amino-terminal part, and/or by the insertion of a fourth microtubule-binding motif translated from exon 10, at the half carboxy-terminal part of Tau.

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In vitro studies have recently shown that Tau isoforms with three microtubule-binding motifs aggregate more efficiently into PHF-like structure, in the presence of heparin, sulfated glycosaminoglycans or RNAs [14–16]. In addition, both sulfated glycosaminoglycans and RNAs have been detected in degenerating neurons of AD [15,17], suggesting a putative role in NFD. Nevertheless, it remains to determine if in AD, PHF are preferentially made up of Tau isoforms with three and/or four microtubule-binding motifs, and if Tau proteins with four microtubule-binding motifs play a role among the types of different Tau neuronal inclusions.

We have addressed this issue using newly developed isoform-specific anti-Tau antibodies, such as antibody Tau-E10 [18] which distinguishes Tau isoforms with four microtubule-binding motifs from the other Tau isoforms. Moreover, AD PHF-Tau from brain tissue homogenates were compared to phosphorylated Tau proteins from PiD, a neurodegenerative disease where Tau proteins essentially aggregate into straight and random filaments in Pick's bodies and therefore typically different from PHF of neurofibrillary tangles of AD [11,19,20].

2. Materials and methods

2.1. Patients

All of the AD patients met the NINCDS-ADRDA criteria and were histopathologically confirmed for the definite diagnosis of AD, as already reported [21]. The five PiD cases were the same as those described by Delacourte et al. [11]. Control brain tissues, obtained from non-demented patients, did not display neurofibrillary pathology in the neocortex. AD, PiD and control brain tissue samples consisted of gray matter from the frontal lobe to avoid the possibility of contaminating aggregated Tau proteins with aggregated Tau proteins which are generated in the hippocampal formation during aging [22].

2.2. Monoclonal and polyclonal antibodies

AD2 is a protein A-purified monoclonal antibody raised against a crude PHF preparation obtained from AD brain [23]. It detects NFTs by light microscopy [5], PHFs by electron microscopy [24] and PHF-Tau on immunoblots [23]. It specifically recognizes phosphorylated serines 396 and 404 (numbering according to the longest human brain Tau isoform) in the carboxy-terminal part of Tau [23]. AD2 was used at 0.2 μg/ml in Tris-buffered saline containing 0.05% Tween-20 (TBS-T). Tau-1 is a phospho-dependent monoclonal antibody (Boehringer Mannheim GmbH, Germany) recognizing Tau proteins at the unphosphorylated sequence 192–204 [25]. It was used at 1:5000 in TBS-T.

M19G is a polyclonal antibody raised against a synthetic peptide corresponding to the first 19 amino acids of Tau [5,23]. Antibodies 304, Tau-E3 and Tau-E10 are Tau isoform-specific. Polyclonal antibody 304, raised against the 29 amino-acid insert (exon 2) [26], was kindly provided by Dr. M. Goedert. Tau-E3 and Tau-E10 were both obtained by immunizing New Zealand rabbits with synthetic peptides (Neosystem, France) corresponding to the first 16 amino acids encoded by exon 3 and the first 10 amino acids encoded by exon 10 of Tau, respectively. The specificity of Tau-E10 for Tau proteins with the translated sequence of exon 10 was already established using re-

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combinant Tau isoforms [18]. Tau-E3 specificity was also verified using the same approach (data not shown).

These phospho-independent polyclonal antibodies recognize both normal Tau and PHF-Tau. M19G, 304, Tau-E3 and Tau-E10 were used in TBS-T at a final dilution of 1:5000, 1:1000, 1:5000 and 1:2000, respectively.

2.3. Monodimensional (1-D) and two-dimensional (2-D) gel electrophoresis

For 1-D and 2-D gel electrophoresis, frontal brain tissue samples were freshly homogenized in Laemmli sample buffer (1:10) containing 5% of SDS. Brain homogenates were aliquoted and frozen at −80°C until used. Each aliquot was used not more than twice. 1-D and 2-D were performed as already described [18,27]. Tau isoforms distribution was investigated using 7.5–15% polyacrylamide gradient gels. On 2-D gels, isoelectric points (p*I*) were determined using the carbamylated CPK (MW=45 kDa; p*I* range 4.9–7.1) of the Carbamylyte™ calibration kit (Pharmacia) [27].

2.4. Western blots

Proteins resolved on 1-D and 2-D gels were transferred onto nitrocellulose membranes (Hybond™ ECL™, 0.45 μm pore size, Amersham) for 90 min at 0.8 mA/cm^2 using an LKB Multiphor II Nova Blot (Pharmacia). Proteins were stained with Ponceau Red, in order to check the quality of the resolution for the 1-D and 2-D and to visualize the carbamylated CPK on 2-D blots. Blocking was carried out in TBS-T containing 5% dry milk and blots were incubated with the antibodies. Monoclonal and polyclonal antibodies were detected with horseradish peroxidase-labeled sheep anti-mouse and anti-rabbit immunoglobulins both adsorbed with human serum proteins (Sigma Immuno Chemicals), respectively. Blots were revealed by chemiluminescence using the ECL™ Western blotting system (Amersham).

3. Results

3.1. Tau in AD and PiD: monodimensional analysis

The typical AD PHF-Tau components of 55, 64 and 69 kDa and an additional band of 74 kDa were immunolabeled by antibody AD2 (Fig. 1: AD, AD2). A vertical smear was always associated with the detection of PHF-Tau in AD brain tissue homogenates, as already described [18,23]. Using M19G antibody, both PHF-Tau and normal Tau proteins were observed. The smear was moderatly immunostained and the 74 kDa band was well visible (Fig. 1: AD, M19G).

Tau isoform distribution was further investigated using isoform-specific anti-Tau antibodies. Antibody 304, specific for Tau proteins with the translated exon 2, immunolabeled PHF-Tau 74, 69 and 64 kDa (Fig. 1: AD, 304). Lower MW were also detected with antibody 304, corresponding to normal Tau proteins (see 2-D Western blot analysis chapter), and catabolic products. Antibody Tau-E3, raised against the exon 3 translated sequence, detected the PHF-Tau bands 74 and 69 (Fig. 1: AD, Tau-E3). Interestingly no smear was observed with both antibodies 304 and Tau-E3. Conversely, antibody Tau-E10, specific for Tau proteins with exon 10 translated sequence [18], immunostained the 74, 69 and 64 PHF-Tau bands and intensively the vertical smear (Fig. 1: AD, Tau-E10). The Tau-E10 immunoreactive smear was very similar to the one observed with antibody AD2. In control brain tissue homogenates, normal Tau proteins are detected by Tau-1 (Fig. 1: Ctrl; Tau-1), an antibody recognizing unphosphorylated Tau proteins at the epitope ranging from amino acid 192 to 204 (numbering according to the longest Tau isoform) [25]. Antibody Tau-E10 labeled Tau proteins with four microtubule-binding motifs and their specific carboxy-terminal catabolic products. The vertical smear was totally absent (Fig. 1: Ctrl, Tau-E10). Isoform-specific anti-Tau antibodies

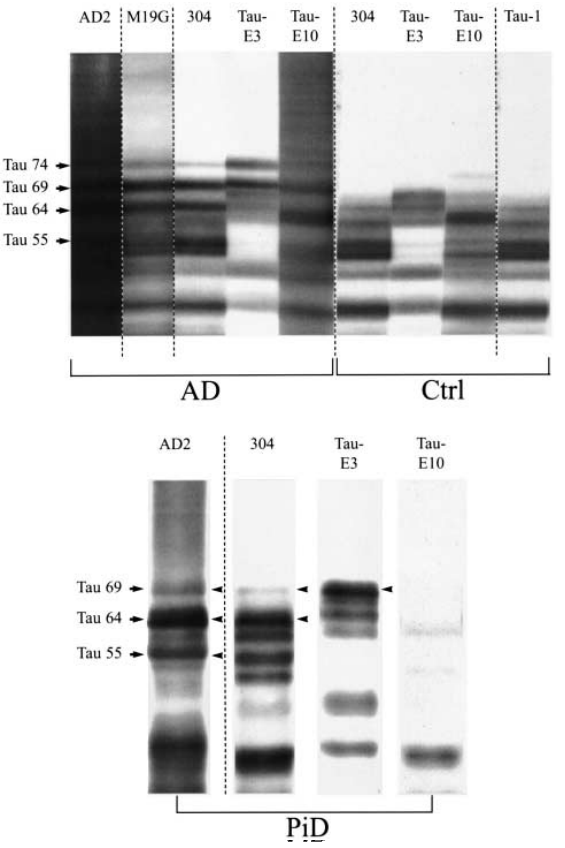

Fig. 1. Immunoblotting of Tau proteins isoforms and their distribution in AD, control brain and PiD. Brain tissue homogenates of AD, control (Ctrl) and PiD were compared. Phosphorylated Tau proteins at serines 396 and 404 (numbering according to the longest Tau isoform) were immunolabeled with AD2. In AD they correspond to the typical four PHF-Tau bands indicated by arrows (AD: AD2 lane; Tau 74, 69, 64, 55). In PiD they are resolved as two main phosphorylated Tau components (Tau 64, 55) and a less immunoreactive 69 kDa Tau band (PiD: AD2 lane, indicated by arrowheads). Unphosphorylated Tau proteins were detected with Tau-1 (Ctrl Tau-1). Both phosphorylated and dephosphorylated Tau proteins were immunostained with the antibody M19G (AD, lane M19G). Tau proteins with the translated sequence of exon 2, 3 and 10 were selectively detected with antibodies 304, Tau-E3 and Tau-E10, respectively (AD, Ctrl, PiD: lanes 304, Tau-E3 and Tau-E10). Note that Tau-E10 immunostains both PHF-Tau and a vertical smear in AD, while it did not stain phosphorylated Tau components of PiD brain tissue homogenates (indicated by arrowheads). As well, immunostaining of normal Tau proteins with the translated exon 10 sequence in PiD was very light in comparison to AD and Ctrl brain tissue homogenates.

detected normal Tau proteins (Fig. 1: Ctrl, 304, Tau-E3, Tau-E10), but none of the antibodies immunoreacted with a smear in control brain tissue homogenates.

Using the same approach, phosphorylated Tau proteins from PiD were investigated. AD2 immunolabeling showed the typical Tau doublet (Tau 55 and 64) and a faintly reactive 69 kDa band (Fig. 1: PiD, AD2), as already described [5,11]. A vertical smear, less intensively labeled than in AD, was also visible. Using the isoform-specific anti-Tau antibodies, the 69 kDa band was detected by antibodies 304 and Tau-E3 and the 64 kDa band was strongly labeled with antibody 304 (Fig. 1: PiD, 304 and Tau-E3). Antibody Tau-E10 failed to react with any of the phosphorylated Tau proteins and no smear was visualized (Fig. 1: PiD, Tau-E10). A weak signal was ob-

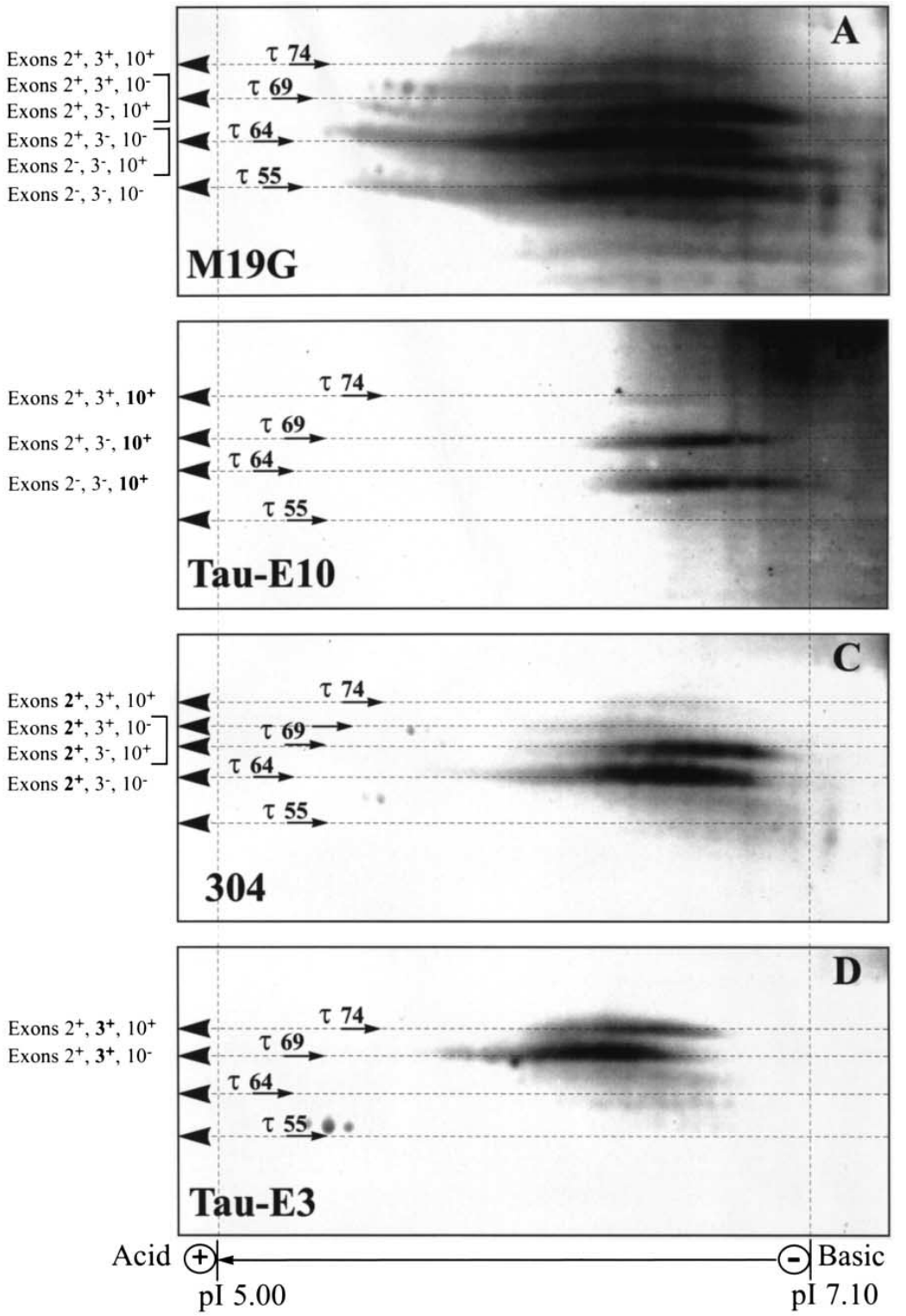

Fig. 2. Two-dimensional characterization of the Tau isoforms distribution in AD PHF-Tau. PHF-Tau proteins from an AD brain tissue homogenate are resolved by 2-D gel electrophoresis and immunoblot with either the antibody M19G (A), Tau-E10 (B), 304 (C) or antibody Tau-E3. All the PHF-Tau components are observed with antibody M19G (A: τ74, τ69, τ64 and τ55). For each of the PHF-Tau components the Tau isoforms content is determined using isoform-specific anti-Tau antibodies Tau-E10 (B), 304 (C) and Tau-E3 (D), respectively. The corresponding translated exons are indicated laterally by arrowheads. Note that antibody Tau-E10 (B) detect a basic vertical smear and that PHF-Tau with the translated sequence of exon 10 are the less acidic Tau isoforms among AD PHF-Tau proteins. Two-dimensional immunoblots are orientated with the basic origin on the right and the acidic end on the left. p*I* 5.00 and 7.10 are indicated on the *x*-axis.

served for normal Tau proteins and carboxy-terminal Tau catabolic products with antibody Tau-E10. It needed a longer time of exposure than for the other antibodies (AD2, 304, Tau-E3) and for obtaining Tau-E10 signal in both AD and

control cases. The same results were obtained for the five PiD cases analysed.

3.2. Two-dimensional Western blot analysis of AD PHF-TAU

AD PHF-Tau are known to be hyperphosphorylated [27] while normal Tau proteins are dephosphorylated during post-mortem delays [28]. Both species of Tau proteins are present in AD brain tissue homogenates. Therefore 2-D gel analysis was used to separate normal Tau from PHF-Tau, since PHF-Tau are acid and normal Tau are basic [18]. This approach enabled us to define the precise Tau isoform distribution among AD PHF-Tau components. M19G detected six PHF-Tau spots in the pH gradient, ranging from p*I* 5.5 to 7.1. Normal Tau proteins were not observed in this pH gradient since their p*I* was over 7.1 (Fig. 2A). The 74 kDa spot was detected by antibodies Tau-E10, 304 and Tau-E3 (Fig. 2B–D). The 69 kDa PHF-Tau component was resolved into two spots. The highest and most acidic one, was immunolabeled by antibodies 304 and Tau-E3 (Fig. 2C,D). The lowest and basic one, was detected by antibodies 304 and Tau-E10 (Fig. 2B,C). The 64 kDa PHF-Tau component was also made of two spots (Fig. 2A). The more intensively labeled spot, characterized by the highest MW and the most acidic p*I*, was only detected by antibody 304 (Fig. 2C). The other spot was less acidic and was immunostained by Tau-E10 (Fig. 2B). Interestingly, Tau-E10 immunoreactive Tau isoforms were the less acidic spots resolved by 2-D (Fig. 2B). Furthermore, the vertical smear was also visualized on 2-D blots, slightly detected by M19G, strongly by antibodies Tau-E10 but not detected by antibodies 304 and Tau-E3 (Fig. 2A–D).

4. Discussion

2D-Gel electrophoresis coupled with Western blotting is a powerful approach to resolve and analyse PHF-Tau proteins, the structural components of neuronal inclusions in AD [18]. Using specific antibodies against Tau isoforms with exon 2, 3 and 10, we were able to determine the precise isoform contents of PHF-Tau of AD and the phosphorylated Tau components of PiD. In AD brain homogenates, Tau isoforms with exon 10 were both observed in the soluble and the most insoluble bulk of PHF-Tau. Conversely, phosphorylated Tau in PiD were lacking of Tau isoforms with the translated exon 10 sequence, showing a dramatic difference between these two types of neurofibrillary degeneration.

In AD brain tissue homogenates, Tau proteins variants were distributed in numerous fractions including normal and PHF-Tau proteins, each group being heterogeneous because of their phosphorylation state [3], or resulting from other post-translational modifications [4], or to partial truncation during the catabolic process. As a consequence, Tau protein fractions have different immunochemical and physical properties [29,30].

On 2D gels, normal Tau proteins were distinguished from PHF-Tau, since they are dephosphorylated during post-mortem delay and thus have a basidic profile [23,27,28] while PHF-Tau are more resistant to dephosphorylation [27] and remain acidic [18]. PHF-Tau could be subdivided into two groups: hyperphosphorylated Tau which are SDS-soluble (PHF sol-Tau) and composed of the whole molecule, as shown by their immunoreactivity with both amino- and carboxy-terminal specific anti-Tau antibodies. The second group corresponds to amino-truncated phosphorylated Tau aggregates (PHF agg-Tau) not solubilized by SDS and heat treatment. Therefore, they are resolved as a smear on both 1-D and 2-D blots while they are totally absent from control brain tissue homogenates, reflecting the typical neurofibrillary aggregation process which occurs in AD. In addition to these two major groups, a third one is represented by soluble Tau catabolic products derived from normal and PHF agg-Tau [18].

These subgroups of Tau proteins from AD brain tissue likely correspond to those described in the literature [29,30]. However, in the present study, we addressed the question of the distribution of Tau isoforms among these different bulks of Tau.

PHF sol-Tau were resolved as four main acidic components (Tau 74, 69, 64 and 55 kDa). The six isoforms expressed in the normal adult CNS tissue were found in the composition of PHF sol-Tau components [18,26], in good agreement with the results of Goedert et al. [26]. The 55 and 74 kDa components were both made of an unique Tau isoform. Tau 55 was not detected by antibodies against exons 2, 3 and 10 and corresponds to the shortest Tau isoforms, also referred to as fetal Tau isoform. The 74 PHF-Tau component is immunolabeled by all of the isoform-specific anti-Tau antibodies and corresponds to the longest Tau isoforms with amino-acid sequences translated from exons 2, 3 and 10. Tau 69 and 64 components are both made of two Tau isoforms. Tau 69 is composed of the Tau isoform including the two amino-terminal inserts (exons 2, 3) and the Tau isoforms with the amino-terminal insert translated from exon 2 and four microtubule-binding motifs (exon 10). Tau 64 is composed of isoform without translated sequence of exon 3 but with one amino-terminal translated insert (exon 2) and the isoform made of four microtubule-binding motifs. The composition of PHF-Tau we described is similar to the one resulting from in vitro phosphorylation of recombinant Tau protein isoforms by the protein kinase GSK3β [31]. However, it is interesting to note that Tau isoforms lacking exon 10 are the most acidic isoforms on 2-D blots, likely because the addition of the translated exon 10 sequence, which correspond to a fourth microtubule-binding motif, is responsible for a less acidic character of Tau proteins [32].

Moreover, we show that Tau with four microtubule-binding motifs are largely integrated in the PHF agg-Tau fraction. At the opposite of 304 and Tau-E3 antibodies, Tau-E10 antibody strongly reacted with the PHF agg-Tau smear of AD brain tissue homogenates while immunostaining was totally absent in control brain tissue homogenates. Our results corroborate the study of Jakes et al. [33] which have shown with a different approach that both three and four microtubule-binding motifs Tau isoforms are present in the PHF-core [33].

Furthermore, we have tested Tau-E10 antibody on brain tissue homogenates from five PiD cases [11]. PiD is a neurodegenerative disease of particular interest since intraneuronal aggregation of Tau is almost totally made of Pick bodies [8,11], a neuropathological feature easily distinguishable from the typical NFT observed in AD [11,19,20]. While AD2 shows the typical doublet of phosphorylated Tau components (Tau 55, 64), the minor 69 kDa Tau component and a vertical smear [11], Tau-E10 did not stain any of the phosphorylated Tau doublet components, but a light immunolabeling was obtained for normal Tau as well as low MW

catabolic Tau products, after a longer time of exposure. Our results strongly suggest that aggregated Tau protein of Pick bodies are devoid of Tau isoforms made of four microtubule-binding motifs. Those results strengthen the idea that Tau isoforms with exon 10 are directly implicated in the formation of PHF in AD for two reasons: first, because they are associated to the most insoluble PHF-Tau fraction, which mainly contains the PHF-core; second, because they are lacking in the composition of Pick bodies, composed of straight and random filaments, but not composed of PHF [34].

In conclusion, we show that Alzheimer's and Pick's disease are two neurodegenerative disorders with different types of neuronal inclusions that comprise different types of Tau isoforms. Tau isoforms with the exon 10 translated sequence are likely to play an important role in the formation of abnormal Tau filaments that constitute the neuronal inclusions. The fact that Tau isoforms with exon 10 are a marker of Alzheimer-type neurofibrillary degeneration, but are not in Pick's disease, is of particular interest since the use of such isoform-specific anti-Tau antibodies could help for an ante-mortem diagnosis of AD [35].

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