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Differential Incorporation of Tau Isoforms in Alzheimer's Disease

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Abstract

There are 6 different isoforms of tau expressed in the adult human brain, and little information is available on the cellular distribution of the isoforms. Tau inclusions are found in neurons and occasionally glia in a variety of diseases. Previous studies conducted on brain homogenates suggested that tau isoforms might be differentially incorporated into inclusions. To further elucidate the complex issue of tau isoform composition in Alzheimer's disease (AD) and other neurodegenerative diseases, monoclonal antibodies that differentiate between tau containing residues encoded by exon 10 (4R tau) and tau lacking exon 10 residues (3R tau) were used in single and double labeling immunohistochemistry as well as biochemical analyses of tau isolated from AD and other neurodegenerative diseases. Immunohistochemical analysis of the hippocampus in 34 AD cases performed with these antibodies showed both 3R and 4R tau isoforms in tangles. While biochemical studies showed that both isoforms were present in insoluble tau aggregates in AD hippocampus and cortex, not all tangles appear to be labeled with the 3R and 4R tau specific monoclonal antibodies. Similar studies in progressive supranuclear palsy and Pick's disease confirmed that these diseases were characterized by incorporation of specific isoforms in fibrillar lesions, but lesions in neither disease were exclusively composed of 3R tau or 4R tau isoforms.

Keywords

Alzheimer's disease; neurofibrillary pathology; Pick's disease; progressive supranuclear palsy; tau; 3R tau isoforms; 4R tau isoforms

INTRODUCTION

Tau is a microtubule-associated protein (MAP) predominantly expressed in neurons and preferentially localized to the axons [6,37,42]. Fibrillar aggregates of tau are characteristic hallmarks of several neurodegenerative diseases, collectively termed tauopathies. The primary tauopathies include Alzheimer's disease (AD), progressive supranuclear palsy (PSP), cortical basal degeneration (CBD), Pick's disease (PiD) and frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) [7,24,25,27,33]. Each of these

neurodegenerative disorders has a specific clinical and pathological phenotype, with tau pathology in distinct regions of the brain and in particular subsets of neurons and glial [10]. Biochemical data suggests that the tau pathology in these individual disorders are composed of distinct tau isoforms [15,16,21,22,38,39].

Alternative splicing of exons 2, 3 and 10 from the single tau gene yields six different isoform combinations in the adult human brain. Exon 10 belongs to the microtubule-binding domain (MTBD) of tau and is the second of four highly conserved repeat regions. There are three isoforms of tau that contain exon 10, referred to as four repeat (4R) tau, and three isoforms of tau that lack exon 10, referred to as three repeat (3R) tau [3,18,19]. Functional differences have been demonstrated between 4R and 3R tau. For example, it has been shown that 4R tau isoforms are more efficient at promoting microtubule assembly than 3R tau isoforms [20,23].

Whereas the mechanisms underlying neurofibrillary pathology are not well understood, the alterations affecting the inclusion or exclusion of exon 10 have been implicated in several tauopathies. For example, FTDP-17 is a disease caused by autosomal dominant mutations in the tau gene (*MAPT*), the majority of which are localized within or in proximity to the MTBD of tau [27,35]. To date more than 30 different mutations have been found; some mutations lie within the coding region of exon 10 and result in 4R tau with reduced ability to bind microtubules, other mutations affect the splicing of exon 10 and cause increased expression of the 4R tau isoforms resulting in altered ratios of 3R and 4R tau. Further, other mutations directly influence tau filament formation by augmenting the formation of beta-sheets around altered tau motifs (for a review see [29]). Variations in *MAPT* near or around the MTBD have been associated with PSP and CBD. Specifically, polymorphisms in an extended region that includes *MAPT* appear to be linked to higher frequency of PSP [11]. CBD has also been linked to this extended tau haplotype HI [4,26].

A number of diseases with tau pathology have been biochemically characterized based on whether or not 3R or 4R tau isoforms are found in the tau aggregates. Ultrastructural differences have also been found in the filaments that make up the neuronal aggregates [40]. Therefore, the diseases with tau pathology have been categorized into 3 groups; i) tauopathies in which the tau pathology is predominantly composed of 4R tau, ii) diseases where the tau pathology is predominantly composed of 3R tau and iii) diseases in which neurofibrillary tangles (NFT) contain a mixture of 3R and 4R tau [9].

Previous attempts to assess the isoform composition in the different tauopathies have only provided limited information, and in fact more recent studies have come to challenge the classification of the tauopathies as exclusively 3R or 4R tau diseases. Given the complexity of isoform profiles between the tauopathies and the different patterns of neurodegeneration observed within these diseases, we sought to expand on the results of previous studies and elaborate the distribution of 3R tau and 4R tau isoforms in AD, PSP and PiD. Towards this aim, we generated and characterized a 4R tau specific monoclonal antibody that was used in conjunction with a previously characterized 3R tau specific monoclonal antibody [13]. These monoclonal antibodies were used to examine the relative expression of 3R and 4R tau in sections from 34 AD cases by immunohistochemistry and comparative biochemical analysis of the PHF-tau. We also conducted immunohistochemical analysis of PSP and PiD cases for distribution of 3R and 4R tau in neurons and glia.

MATERIALS AND METHODS

Cases

Cases were obtained from the brain banks at Albert Einstein College of Medicine and Mayo Clinic Jacksonville. The clinical and pathologic features of each case are summarized in Table 1–Table 3.

Monoclonal antibodies

4R tau specific monoclonal antibodies were generated against a synthetic peptide corresponding to the amino acid sequence KVQIINKKLDLSNVQSK found in exon 10 of human tau. Antibodies were generated as described [12]. Briefly, tau deficient (–/–) mice, generated by a targeted disruption of tau exon one [41], were immunized using the synthetic peptide described above cross-linked with glutaraldehyde. Mice were injected intraperitoneally with solutions containing 1–2 mg/ml of the peptide (0.2 ml/injection). Blood samples were drawn and antibody serum titers were determined by ELISA using biotinylated peptides and by Western blot with bacterially expressed recombinant tau fusion proteins [8]. Spleen cells were collected from mice with the highest tau antibody serum titers and fused with myeloma cells (NSO cells) in the presence of polyethylene glycol (PEG). Fusion products were plated in 96-well plates in selection medium containing hypoxanthine-aminopterin-thymidine (HAT) (Invitrogen/Life Technologies, Carlsbad, CA). Positive clones were identified by assaying the culture media both by ELISA and by Western blot. Clones with high specificity for the peptide of exon 10 and for the recombinant tau isoforms containing exon 10 were selected and expanded. Three highly specific 4R tau monoclonal antibodies ET1, ET2 (both IgG1) and ET3 (IgG2b) were obtained. The 3R tau specific monoclonal antibody RD3 was generated against the peptide sequence KHQPGGGKVQIVYKPV incorporating amino acids from both exons 9 and 11 of tau and characterized as described [13]. Monoclonal antibody CP13 detects tau phosphorylation at serine 202. Human specific monoclonal antibody CP27 detects amino acids 140–160 of tau common to all tau isoforms.

Tissue culture and transient transfections

COS-7 cells and Neuro-2a (N2a) were grown in DMEM (Invitrogen/Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen/Life Technologies). Recombinant tau and MAP2B (a generous gift from Dr. B. Shafit-Zagardo) were expressed using pcDNA vectors. Cells were transfected with a mixture containing 2 µg of cDNA and lipofectamine reagent (Invitrogen/Life Technologies) for the COS-7 cells or with a mixture of 0.5 µg of cDNA and lipofectamine 2000 (Invitrogen/Life Technologies) for the N2a cells. Cells were transfected in serum free medium for 6 hr at 37°C and homogenized or fixed in 4% paraformaldehyde after 24–48 hr. Cells were homogenized in homogenization buffer (Tris-buffered saline (TBS), pH 7.4, containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM sodium orthovanadate, 2 mM EGTA, and 10mM sodium fluoride). Cell homogenates were frozen at –70°C over night, then thawed and spun at RT for 5 min at 15,000 rpm and used for either immunoblot or ELISA.

ELISA

ELISA using NeutrAvidin (Pierce) was performed as described [1]. Briefly, 96-well plates were coated with 5 µg/mL of NeutrAvidin in coating buffer and incubated at 37°C for 3 hr. Plates were then blocked with 2% bovine serum albumin (BSA) in TBS overnight at 4°C. Biotinylated peptides were both used at a final concentration of 0.08 µg/ml and left to incubate overnight at 4°C. Subsequently, wells were blocked with 5% milk in TBS for 1 hr at RT. For the screening of fusion clones a 1:10 dilution of conditioned medium was for the ELISA. To assess the sensitivity of antibodies ET1, ET2, ET3 and RD3 dilutions were started at 1:50,

followed by 3-fold serial dilutions. Conditioned media from the fusion clones and all primary antibodies were incubated for 1 hr at RT. Goat anti-mouse HRP-conjugated antibodies (Southern Biotechnology Associates Inc., Birmingham, AL) were used at a 1:500 dilution. Samples were analyzed using Horseradish Peroxidase Substrate Kit (Bio-Rad Laboratories, Hercules, CA). For the direct ELISA, plates were coated using a 1:25 dilution of cell lysates from COS-7 cells transiently transfected with tau in coating buffer and incubated at 4°C overnight. Wells were blocked with 5% milk in TBS. Primary antibodies (CP27, ET1, ET2, ET3 and RD3) were used starting at 1:5 dilutions, followed by 3-fold serial dilutions. Goat-anti mouse HRP-conjugated secondary antibodies and Horseradish Peroxidase Substrate Kit were used as described above.

Immunoblotting

Immunoblotting was performed as described [2]. Briefly, samples were separated using 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and these were incubated with the following primary antibodies: CP27 (1:1000), ET2 (1:500) and RD3 (1:10,000). Immunoblots were then incubated with HRP-linked goat anti-mouse IgG antibodies (1:500); (Southern Biotechnology Associates). Immunoreactive bands for the recombinant tau clones and for the fetal and normal brain samples were detected using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). For AD brain homogenates and sarkosyl preparations, immunoreactive bands on nitrocellulose membranes incubated with CP27 and RD3 were detected using 4-chloronaphtol and hydrogen peroxide in TBS. Immunoreactive bands on nitrocellulose membranes incubated with antibody ET2 were detected using SuperSignal West Pico chemiluminescent substrate (Pierce).

Immunocytochemistry

Paraformaldehyde fixed N2a cells transiently transfected with 3R and 4R tau cDNA were used for immunocytochemistry. Cells were permeabilized in 0.025% Triton-X and blocked in 5% milk in TBS for 1 hr at room temperature. Subsequently, cells were incubated with antibodies ET3 (1:100) and RD3 (1:100) overnight at 4°C and the next day were incubated in secondary antibodies goat anti-mouse IgG2B Alexafluor 488 and goat anti-mouse IgG1 Alexafluor 633 (Molecular Probes, Eugene, OR) for 1 hr at room temperature. Samples were dehydrated and mounted in Fluoromount (BDH Laboratory Supplies, Poole, England). Confocal images of fluorescent N2a cells were obtained using Leica ABOS confocal microscope using 20X, 40X and 63X oil immersion objectives. Lasers were set to detect at 488nm and at 633nm wavelengths to eliminate any bleed through between fluorescent probes. Z series of labeled cells were collected using the optimized step size and projected using the maximum pixel method.

Immunohistochemistry

Immunohistochemical analysis of 5–8 µm sections from paraffin embedded blocks of formalin fixed hippocampus from 34 AD cases, the basal ganglia of five PSP cases, and from the hippocampus, frontal cortex and superior temporal cortex of one PiD case was performed as follows: All sections were deparaffinized and rehydrated. For immunostaining with either RD3 or ET3 antibodies the sections were pretreated by microwaving for 10 min in TBS and then incubated for 30 min in 95% formic acid (Sigma Chemical Co., St. Louis, MO) for efficient antigen retrieval. Sections were blocked with 5% milk in TBS for 1 hr at RT and then incubated in primary antibodies at 4°C overnight. Antibodies dilutions were as follows: RD3 (1:100), ET3 (1:50), CP13 (1:25). The sections were then incubated with biotin-labeled secondary antibodies (Southern Biotechnology Associates) at a 1:500 dilution for 2 hr at RT and then with StreptAvidin conjugated Horseradish Peroxidase (Southern Biotechnology Associates) at a 1:500 dilution for one hr at RT. HRP-labeled areas were detected using a solution containing

0.3 mg/ml of 3,3'-diaminobenzidine (DAB) in 100 mM Tris, pH 7.4 and 0.02% hydrogen peroxide (H₂O₂). Sections were counterstained with toluidine blue.

For immunofluorescence, sections were incubated with antibody ET3 (1:50) dilution overnight at 4°C. The sections were then incubated with biotin-labeled goat anti-mouse IgG2B antibody (Southern Biotechnology Associates) at a 1:500 dilution for 2 hr at RT and then with Streptavidin AlexaFluor 633 conjugate (Molecular Probes, Eugene, OR) at a 1:500 dilution for 1 hr at RT, washed extensively with IX TBS and incubated with antibody RD3 at a 1:100 dilution overnight at 4°C. Subsequently, the sections were incubated in biotin labeled goat anti-mouse IgG1 (Southern Biotechnology Associates) at a 1:500 dilution for 2 hr at RT and then with Streptavidin AlexaFluor 488 conjugate (Molecular Probes) at a 1:500 dilution for 1 hr at RT. Control sections were stained with either antibody ET3 using goat anti-mouse IgG1 as a secondary antibody or antibody RD3 using goat anti-mouse IgG2b as a secondary antibody to rule out any cross-reactivity between the two isotypes. Sections were finally treated in 3% Sudan black (Sigma Chemical Co.) in 70% ethanol for 10 min at RT [36], and mounted with 80% Tris-buffered glycerol. Sections were imaged using the Leica AOBs confocal microscope (described above).

Sarkosyl extraction of insoluble material

Tissue samples were weighed and homogenized in 10 volumes of homogenization buffer (described above). The homogenized samples were spun at 15,000 rpm for 20 min; the supernatants were collected; and aliquots were resuspended in 5X sample buffer (32% 1M Tris, pH6.8, 5% SDS, 50% glycerol, and 1% β-mercaptoethanol) for analysis as the starting material. The rest of the sample was used for extraction of PHF-tau using the sarkosyl extraction of insoluble material [22]. Briefly, the supernatants of the brain homogenates were centrifuged at 35,000 rpm for 1 hr at 23°C; the resulting pellets were resuspended in 1% sarkosyl (Fisher Scientific) in TBS and incubated on shaker for 30 min at RT, and then centrifuged at 35,000 rpm for 1 hr at 23°C. The pellets from the ultracentrifugation were resuspended in IX reducing sample buffer and resolved by 10% SDS-PAGE, and analyzed by Western blot (for antibody dilutions, see above).

Thioflavine-S staining

Paraffin embedded sections of the AD cases were subjected to thioflavine-S staining. Briefly, sections were rehydrated, and then dipped in water. Sections were then incubated with 0.1% thioflavine-S (Sigma Chemical CO.) for 15 min. Sections were dipped two times in 70% ethanol for 15 seconds, and mounted with 80% Tris-buffered glycerol, pH 7.4. Sections were examined using the Leica AOBs confocal microscope and images were taken using a 405nm wavelength laser.

RESULTS

Characterization of monoclonal antibodies to 4R tau

We were successful in generating three highly specific 4R tau monoclonal antibodies - ET1, ET2 and ET3. Antibody ET2 showed high specificity for peptide 1157 spanning 17 amino acids exclusively found in exon 10 of tau (Fig. 1a). In contrast, ET 2 showed no reactivity with peptide 1176 spanning a sequence of 16 amino acids specific for the junction joining exons 9 and 11 found only on tau isoforms that contain 3 repeat regions (Fig. 1b). The other two ET antibodies, ET1 and ET3, also exhibited high specificity for peptide 1157 and no reactivity with peptide 1176 (not shown). A previously characterized 3R tau specific monoclonal antibody, RD3 [13], showed no reactivity with peptide 1157 (Fig. 1a), but was highly reactive towards peptide 1176 (Fig. 1b).

To determine if the 3R and 4R tau specific antibodies could detect full-length tau proteins, four recombinant tau isoforms containing either 3 repeat or 4 repeat regions were generated. CP27, a human specific monoclonal antibody that recognizes amino acids 140–160 (common to all tau isoforms), detected all 4 tau isoforms by Western blot (Fig. 1c). Tau protein loading was therefore normalized using CP27 antibody. Antibody RD3 displayed high specificity for 3R tau isoforms and did not detect any 4R tau isoform. ET2, on the other hand, was highly specific for the 4R tau isoforms and did not react with either of the 3R tau isoforms (Fig. 1c). Antibodies ET1 and ET3 also recognized only the 4R tau isoforms (data not shown). We found that RD3 and CP27 possessed approximately 10-fold higher sensitivity for tau isoforms than the ET2 antibody and therefore the working dilutions of RD3 and ET2 were adjusted accordingly to gain similar detection sensitivity for tau.

Considering that the ET monoclonal antibodies were highly reactive to the peptide amino acid sequence for exon 10, but were less reactive by Western blot on full-length tau containing exon 10 sequences, we examined the reactivity of ET2 and the other 4R tau specific antibodies by ELISA using the four full-length recombinant tau isoforms. By ELISA we found that the ET antibodies were minimally reactive with tau isoforms containing exon 10, while RD3 maintained its specificity for the 3R tau isoforms (not shown). This finding suggested that there may be conformational differences in the accessibility to the microtubule-binding domain of tau as a result of the presence of exon 10 and that treatments to unmask the 4R tau epitope are required to allow recognition with the ET antibodies.

The 3R and 4R tau specific monoclonal antibodies were then used to analyze homogenates from both fetal and normal adult human brains. RD3 showed strong reactivity with fetal brain that contains only the shortest tau isoform (Fig. 1d). ET2 appeared to detect a minimal 4R tau band in the fetal sample that could be dependent on the developmental stage of the fetus. Although, RD3 and ET2 antibodies were able to detect normal tau isoforms in adult brain we observed differences in the banding patterns from both antibodies (Fig. 1d).

To examine the potential use of ET3 in detecting 4R tau in cell culture, N2a cells were transiently transfected with equal amounts of both 3R tau and 4R tau and labeled using ET3 and RD3. We found that ET3 was highly specific in detecting only the 4R tau in cell culture and did not cross react with 3R tau (not shown). In cells double labeled with ET3 and RD3 both the 3R and 4R tau isoforms were detected equally (Figure 1e). Although not completely co-localized, all double transfected cells expressed both 3R and 4R tau isoforms at similar levels as confirmed by western blot (Fig. 1c).

The microtubule associated protein (MAP) 2b expressed in neurons has a microtubule-binding domain made up of 3 and 4 repeat regions. The repeat region encoded for by exon 16 of MAP2b shares significant sequence homology with exon 10 of tau [30]. Therefore, as part of our initial characterization of the 4R antibodies, we determined whether or not the ET antibodies could detect the 4R isoform of MAP2b. We found that none of the 4R monoclonal antibodies had any detectable reactivity with isoforms of MAP2b by Western blot (not shown).

Immunohistochemistry and comparative biochemistry in AD

Previous biochemical analysis of insoluble material isolated from bulk homogenates of AD brain indicated that the NFTs contain both 3R and 4R tau isoforms [21]. We analyzed the tau isoform composition of NFTs in a large pool of AD cases using the monoclonal antibodies RD3 and ET3 for immunohistochemistry. 34 different AD hippocampal sections were chosen that range in severity and, when possible, were assessed by thioflavine-S staining, a sensitive fluorescent method for detecting NFTs in AD. The number of NFTs detected with RD3 or ET3 immunohistochemistry was compared to those detected with thioflavine-S within different regions of the hippocampus. RD3 and ET3 staining patterns were also compared to CP13, a

monoclonal antibody that detects an AD specific phosphorylation of tau at serine 202. Previous studies have shown that CP13 detects early stages of neurofibrillary degeneration, labeling pre-tangles and intracellular NFTs but not extracellular NFTs. The results of the immunohistochemical study on the AD cases are summarized in Table 1, which shows NFT counts the CA1 sector of the hippocampus with CP13, RD3, ET3 and thioflavine-S. The entire hippocampus and entorhinal cortex were also examined for NFT density and tau isoform composition.

All sections incubated with RD3 and ET3 displayed reactivity to these antibodies, therefore indicating the presence of both 3R and 4R tau isoforms in NFTs of the hippocampus and cortex of AD. However, variability in staining patterns between individual cases was detected. The differences in staining appeared to follow a progression that was related to the severity of AD (Table 1). In particular, some advanced cases had large amounts of thioflavine-S positive NFTs, including many extracellular NFTs only detected by RD3, but not by ET3. Other cases had a more equal distribution of 3R and 4R tau immunoreactivity of NFTs comparable to that seen with thioflavine-S and CP13. Cases with milder neurofibrillary pathology, with mostly pretangles and early intracellular NFTs, had abundant CP13 staining, but limited amounts of both RD3 and ET3 immunoreactivity, indicating that these two isoform specific antibodies are not sensitive for recognizing pre-tangles. Consequently, they cannot be recommended as useful markers of early neurofibrillary pathology. In some AD cases, we were able to examine both anterior and posterior sections of the hippocampus and we found minimal differences between the two areas. However, in the anterior sections the pathology appeared more severe and displayed more abundant RD3 positive tangles as compared to the posterior hippocampal section from the same case.

Two severe AD cases (Table 1, cases 3 and 4) were selected to illustrate the variable pattern of staining seen with antibodies RD3 and ET3 in advanced AD cases. Case 3 (Fig. 2a–h) showed many thioflavine-S positive extracellular NFTs and an abundant number of RD3 positive NFTs. Adjacent sections from this case displayed negligible ET3 and CP13 staining. However, neuritic elements in the hippocampus were immunoreactive to both ET3 and CP13 (Fig. 2e and f). In contrast, in case 4 (Fig. 2i–p) NFTs reactive to both RD3 and ET3 as well as to CP13 and thioflavine-S appeared more evenly distributed throughout the sections. Especially noted is the increased number of 4R tau immunoreactive NFT in case 4 as compared to case 3. Higher magnification showed the fine details of immunolabeling of NFTs. While RD3 and ET3 antibodies appeared to stain only more advanced NFTs, ET3 also decorated a number of neuritic elements, and CP13 immunoreactivity was abundant in pretangles. RD3 was the only antibody in this study that reacted with extracellular NFTs (Fig. 2g and o).

Double-labeling analysis of AD hippocampal sections with both RD3 and ET3 monoclonal antibodies was used to determine whether individual NFTs in AD contain both 3R and 4R tau or if the staining segregates to different populations of NFTs. Based on the staining observations done with 3-3'-diaminobenzidine (DAB) (compiled in Table 1) two cases with predominantly RD3 stained tangles and minimal ET3 reactivity (cases 1 and 3) and one case with both RD3 and ET3 staining (case 6) were selected for this analysis. The CA1 hippocampal region of case 3 with predominantly RD3 stained NFTs (Fig. 3a–c) showed the distribution of 3R and 4R tau in hippocampus, with the majority of NFTs containing mostly the 3R tau isoform and only a few NFTs positive for 4R tau. The merged image showed a few double-labeled NFTs, but no NFTs that were only 4R tau positive. A similar finding was also observed in case 1 (not shown). Layers 2 and 3 of the entorhinal cortex of case 3, which had many extracellular NFTs, showed that the NFTs in this area were exclusively immunoreactive for 3R tau (Fig. 3d–f). Double labeling of case 6 (where DAB stained tissue seen in Fig. 4 showed a more even distribution of RD3 positive and ET3 positive NFTs) showed that the majority of the NFTs had immunoreactivity for both 3R and 4R tau (not shown). In the more posterior hippocampal

section of case 6 we could not detect regions with exclusive 3R or 4R tau staining (not shown). However, DAB stained sections of the anterior hippocampus of case 6 showed possible differences in staining patterns between RD3 and ET3. A few single 3R tau positive NFTs were observed in layers 2 and 3 of the entorhinal cortex (not shown).

High magnification images of double-labeled NFTs showed an overall similar staining with antibodies RD3 and ET3 throughout the NFT, although it was difficult to assess whether or not 3R and 4R tau isoforms completely co-localized throughout the NFT (Fig. 3g–i and j–l). Double labeling immunoelectron microscopy will be required to address this question.

To further investigate the variability of staining patterns observed among severe AD cases, twelve cases with advanced AD pathology, identified by thioflavine-S staining (Table 1), were chosen to compare the fidelity of the immunohistochemical analysis with 3R and 4R tau biochemical reactivity. Six of the cases (1, 2, 3, 7, 8 and 9) showed predominant RD3 and minimal ET3 immunohistochemical reactivity (Fig. 4a, left panels only cases 1, 2, and 3). Six other cases (4, 5, 6, 10, 11 and 12) showed both 3R and 4R tau isoform composition by immunohistochemical analysis (Fig. 4a, right panels only cases 4, 5 and 6). PHF-tau from hippocampal homogenates of the same 12 AD cases were analyzed using the sarkosyl extraction method. Brain homogenates (starting material) and the sarkosyl insoluble fraction were examined for total tau content by Western blot using CP27, and for isoform content using RD3 and ET2 (Fig. 4b, c, d and e). Working dilutions of the antibodies were based on the antibody reactivity with the full-length tau clones examined by Western blot (Fig. 1c). RD3 was diluted 10-fold more as compared to ET2. In each individual case total tau was assessed using CP27 analysis. Cases 1, 3, 7 and 8 presented much less total tau and proportionally much less 3R and 4R tau isoforms than the other cases. 3R and 4R tau were detected in the brain homogenates of all the cases; however, the banding patterns varied slightly between the cases.

Interestingly, even at a 10-fold higher dilution, RD3 could detect far more 3R tau using 4CN staining than antibody ET2 could detect 4R tau using chemiluminescence (shown in panels b, c and d) in the same brain homogenates. Comparison of case 2 with minimal 4R immunohistochemical reactivity to case 4 where 4R tau was detected in NFTs demonstrated no significant differences in the intensity of the 4R bands from the hippocampal brain homogenates.

Similar results were obtained from these cases with the sarkosyl preparations of insoluble PHF containing fraction (Fig. 4c,e). CP27 displayed the classic triple banding pattern of AD with tau proteins of apparent molecular weights of 68,65 and 62 kDa [21]. Cases 1, 3, 7 and 8 appeared to have proportionally less PHF-tau than other cases analyzed and, therefore, there appeared to be proportionally less 3R and 4R tau in these samples. Both 3R and 4R isoforms could be detected in the sarkosyl extracted material; however, RD3 detected the lower two bands with higher sensitivity than ET2 detected the upper two bands. The Western blot analysis also suggested that 3R tau isoforms are more abundant than 4R tau in the sarkosyl insoluble material. Western blot analysis did not show differences in the amount of 4R tau in case 2, in which immunohistochemistry displayed few or no NFTs with ET3 immunoreactivity when compared to cases 4 and 5 in which the 4R tau isoforms were readily detected in NFTs by immunohistochemistry. These results indicated that 4R tau was present in sarkosyl fractions even in cases where immunohistochemistry failed to detect these isoforms in tangles.

Immunohistochemistry of PSP and PiD

We used RD3 and ET3 to examine the tau isoform composition in PSP (a disorder with neuronal and glial lesions enriched in 4R tau) and in PiD (a disorder with neuronal lesions enriched in 3R). Paraffin embedded basal ganglia sections from 5 PSP cases and hippocampal and cortical sections from one case of PiD were studied. Immunohistochemistry with RD3 and ET3 was

compared to CP13, a good marker for tau pathology in these disorders. The density of neuronal and glial lesions that were tau positive in PSP and PiD is summarized in Table 2 and Table 3.

In the basal ganglia of PSP two main types of pathologies have been described: Tau positive NFTs in neurons and tau positive inclusions in glial cells referred to as tufted astrocytes [9]. CP13 detected these hallmarks in all of the cases examined and displayed a similar pattern in all cases. However, the estimated number of CP13 stained NFTs and tufted astrocytes differed from case to case (Table 2). Immunohistochemical analysis of these cases with monoclonal antibodies RD3 and ET3 confirmed previous findings that indicated that the 4R tau isoforms are the major component of tau inclusions in PSP basal ganglia. In all the cases examined we found that antibody ET3 detected similar numbers of neuronal NFTs as CP13 (Fig. 5a, b). We also found immunoreactivity to RD3 in NFTs, although sporadic and mostly visible using higher magnification (Fig. 5c,f). The RD3 staining was observed in only 3 out of the 5 cases examined. ET3 antibody also detected the tau positive tufted astrocytes (Fig. 5g,h). Only 2 cases displayed slightly less ET3 stained astrocytes in comparison to CP13 (Table 2). RD3 did not detect any tufted astrocytes in the basal ganglia of the cases examined (Fig. 5i).

Finally, we examined three sections from PiD, including hippocampus, temporal cortex and frontal cortex. In the hippocampal section an abundant number of neurons in the granule cell layer of the dentate fascia had CP13 immunoreactive Pick bodies (Fig. 6a). CP13 also labeled Pick bodies in pyramidal neurons in CA1 and CA2 of the hippocampus and neurons in the temporal cortex. As expected, 3R tau isoforms appeared to be the major component of Pick bodies. In the cortex a similar number of CP13 and RD3 positive Pick bodies were observed (Fig. 6a,c), whereas there was no ET3 staining (Fig. 6b). Similarly, comparable numbers of Pick bodies in the dentate fascia (Fig. 6d,f) and hippocampal pyramidal cells (not shown) were labeled with CP13 and RD3, while no ET3 staining was observed (Fig. 6e). PiD is not completely devoid of 4R tau [13,43]. In fact, several neuronal lesions in the temporal cortex of the PiD case reacted with ET3 (Fig. 6g–i), indicating that this PiD case contained some 4R tau isoform-positive lesions. Although, regional differences in the tau isoform composition of neuronal lesions was observed, 3R tau was the predominant isoform in both Pick bodies and neuronal lesions in PiD.

DISCUSSION

The classification of tauopathies into diseases containing lesions enriched in 3R tau isoforms, 4R tau isoforms or a mixture of 3R and 4R isoforms has been based largely on biochemical analysis of brain homogenates or PHF-tau extracts from different tauopathies [15,16,21,22, 38,39]. The majority of studies have been limited by the lack of monoclonal antibodies to conclusively differentiate 3R tau and 4R bands on Western blots and were dependent upon dephosphorylation of phosphorylated tau to match up bands with recombinant tau isoforms run in parallel [22,39]. The lack of highly specific monoclonal antibodies also limited our understanding of the cellular distribution of 3R and 4R tau isoforms in neuronal and glial lesions in neurodegenerative diseases. Here we report on the characterization of 4R tau specific monoclonal antibodies ET1, ET2 and ET3, used in conjunction with the previously characterized 3R tau specific monoclonal antibody RD3 [13]. In this study we show that the ET series of monoclonal antibodies are reliable tools for the analysis of 4R tau isoforms. They are highly specific for the exon 10 sequence of tau, and they can be used for ELISA, Western blot and routine immunocytochemistry on paraffin embedded tissue. None of the ET series are cross-reactive with 3R tau isoforms; they do not recognize fetal tau; and they do not display cross-reactivity with MAP2 isoforms. The specificity of the 4R tau antibodies for the amino acid sequence of exon 10 was equal to the specificity of the 3R tau antibody for the amino acid sequence encompassing the junction between exons 9 and 11; however, Western blot using recombinant full-length tau isoforms revealed that the sensitivity of the ET antibodies for 4R

tau was 10-fold lower than that of RD3 for 3R tau. Similarly, an ELISA using the recombinant full-length 3R and 4R tau isoforms showed a marked decrease in the sensitivity of the ET antibodies for 4R tau as compared to the 3R tau antibody. This indicates that the presence of exon 10 might mask the MTBD or create conformational differences between recombinant tau isoforms in solution.

Several recent studies have tried to elucidate the tau isoform content in the different tauopathies, and they have found that the lesions in these diseases may be predominantly but not exclusively composed of 3R or 4R tau isoforms. Our results confirm the findings of de Silva et al. [13] in that that majority of NFTs in PSP were immunoreactive with the 4R tau specific monoclonal antibody, while 3R tau appeared to be a minor component. In all of the five PSP cases examined we detected a predominant 4R tau staining. However, sporadic 3R tau staining was detected in some of the cases in the NFT found in the basal ganglia. This phenomenon is particularly common in neurons in the basal nucleus of Meynert, which are vulnerable to Alzheimer type pathology raising the possibility that 3R tau immunoreactive lesions may represent concurrent Alzheimer type pathology in these PSP cases. Tufted astrocytes did not display any immunoreactivity for 3R tau.

An important study, examining the tau isoform composition of the NFP in both sporadic and inherited frontotemporal lobar degeneration (FTLD), found pick bodies in cases with Pick-type histology were typically labeled with RD3 (3R tau) and not with ET3 (4R tau) antibodies [14]. We were limited in making firm conclusions about PiD given that we examined a single case. Specifically, we observed 3R immunoreactivity of Pick-bodies in the hippocampus in PiD, but found that a few Pick-type lesions in the temporal cortex had 4R tau immunoreactivity, indicating possible regional variability in tau isoform deposition or the presence of AD type neurofibrillary changes. Therefore, this study is in agreement with the other previous studies demonstrating the incorporation of 4R tau isoforms into some the PiD inclusions [14]. However, it is clear from this study and other studies using monoclonal antibodies that 3R tau isoforms appear to be predominant component in the Pick bodies found in PiD brain [14].

The ratio of 3R tau to 4R tau is altered in FTDP-17 by several different mutations in *MAPT*. In the normal adult brain this ratio is 1:1, indicating that that altering the tau ratio causes neurofibrillary pathology and neurodegeneration. Mice expressing exclusively the 6 human tau isoforms develop pathology perhaps because of an altered ratio of tau isoforms favoring the 3R tau isoform, with concomitant loss of mouse tau that is predominantly 4R [1]. In these mice, the insoluble material extracted with sarkosyl appears to be composed of only 3R tau isoforms, strongly suggesting that imbalances in composition of the tau isoforms leads to preferential incorporation and accumulation of tau into NFTs.

In sporadic AD there are no known mutations in the tau gene and the etiology is unknown. One recent study suggests that a misregulation of splicing machinery of tau leads to imbalances of tau ratios in sporadic AD [17]. Other studies have examined isoform imbalance in AD, and the results have been conflicting. Therefore, using our 4R monoclonal antibodies we wanted to examine the possibility that 3R and 4R tau isoforms could be altered in AD. AD may have a multi-factorial origin and there may be heterogeneity in isoform composition in the AD population that could only be assessed by examining a larger number of cases. Our examination of a large panel of AD cases by immunohistochemistry showed the isoform profile to be more complex. In the earlier stages of AD, the isoform specific antibodies detected only few lesions and were not effective at labeling pretangles. In these less severe cases the appearance of 3R to 4R tau labeled tangles appeared more equal. It is tempting to speculate that some of the tau is still bound via the MTBD to MT in the earliest lesions.

In severe endstage AD, there was more variability in immunoreactivity of NFTs. In regions with many extracellular NFTs, such as the subiculum, CA1 and the superficial layers of the entorhinal cortex, NFTs were immunostained only with the 3R antibody, RD3. In these cases the extracellular tangles were visualized by thioflavine-s, but there was minimal immunoreactivity with both the 4R tau antibody (ET3) and the M. phospho-tau (pSer202) CP13. Other advanced cases of AD had NFTs that were equally immunolabeled by three antibodies (RD3, ET3 and CP13) as well and with thioflavine-S.

The paucity of immunoreactivity of 4R tau observed in extracellular NFTs in our study is in agreement with previous studies conducted with another 4R tau monoclonal antibody [13]. The failure to detect the 4R tau epitope in extracellular NFTs has been suggested to be due to proteolysis of the 4R tau isoform [28]. Due to the apparent lack of ET3 reactivity in some advanced AD cases in our study and the excessive RD3 immunostaining in these cases, we decided to investigate the reason that the 4R tau epitope was not visualized in these cases. We questioned whether the 4R tau epitope had become degraded, buried by conformational changes, or if some AD cases had an imbalance in 3R and 4R isoforms that result in the eventual deposition of predominant 3R tau in NFTs. Comparative biochemistry of cases with 3R tau predominant immunostaining and other cases where there was immunoreactivity of NFTs with both 3R and 4R tau antibodies was used to further characterize the pathology in AD. Our results showed that in all cases an excess of the 3R tau isoforms was detected in brain homogenates and in the sarkosyl preparations of PHF-tau. This finding is in agreement with a previous report that showed a prevalence of 3R tau isoforms in the basal ganglia of AD brain [31]. Moreover, all cases, even those with predominantly 3R tau immunolabeled NFTs, displayed 4R tau immunoreactive bands in the homogenates. This was not unexpected since 4R tau is present in unaffected neurons in brain and most cases did show minor 4R tau presence by immunohistochemistry. Even in those cases where there were few 4R tau immunoreactive NFTs, there were nevertheless, 4R tau immunoreactive neuritic lesions, which also could contribute to the 4R tau in homogenates.

Unexpectedly we did not observe qualitative differences in the amount of insoluble 4R tau between cases with abundant or sparse 4R immunoreactive NFTs. These results indicate that although 3R tau is the major component of most NFTs, in some severe cases the presence of 4R tau is not lacking, but might merely be obscured or blocked. One possible explanation is with the greater abundance of 3R over 4R tau in most of cases, tau that accumulates in NFTs in later stages may preferentially be 3R tau. Minimal accumulation of 4R tau may be the seed and be present in early lesions, but eventually be obscured by co-deposition of 3R tau.

The differential immunoreactivity of NFTs in advanced cases of AD might also be due to differences related to disease severity. The most severe cases had developed abundant extracellular 3R tangles, while the less severe cases might have been earlier in the pathogenic cascade and in the process of developing this type of pathology, therefore showing more 4R expression. However, the nature of the lesions and the predisposition to develop extracellular tangles might differ among AD cases for unknown reasons. Therefore, the possibility that some AD cases have an intrinsic propensity to develop more 3R tau extracellular aggregates cannot be ruled out. Data on the genetic association between tau haplotype and AD is still evolving [5,34], and it would be interesting to further analyze the immunoreactivity of NFTs in AD with respect to *MAPT* haplotype. A tau haplotype designated H1c does appear to be associated with an increased risk of AD [32], and it will be important to examine tau lesion composition in cases with and without this haplotype.

3R and 4R tau do not differentially accumulate with subsets of NFTs in AD. The 3R predominant cases had many single labeled 3R NFTs in the hippocampus and entorhinal cortex. In these cases, 4R tau staining was minimal and it was not found in the absence of 3R tau

labeled NFTs. In cases where there was a more equal distribution of tau isoforms all NFTs appeared to be positive with both RD3 and ET3. Our analysis of double-labeled tangles at higher magnification shows that the tau isoforms often co-localize. It would be necessary to analyze more cases by double labeled immunohistochemistry and to examine the double labeled tangles by confocal microscopy to determine the ratios of 3R and 4R tau in individual tangles and draw further conclusions about the role of tau isoforms in neurofibrillary degeneration.

Characterization of the pathological hallmarks can give insight into the etiology of these diseases. In conclusion, we have generated 3 monoclonal antibodies highly specific for 4R tau isoforms. These antibodies appear to be interchangeable and suitable tools for a variety of techniques aimed at analyzing the expression of different tau isoforms within neurodegenerative lesions. 4R tau specific antibodies can be used in conjunction with the 3R tau specific monoclonal antibody RD3 for double labeling techniques that can be a very useful means to further characterize the tau isoform composition in many neurodegenerative diseases. Generation of additional highly specific monoclonal antibodies that could distinguish between tau isoforms containing the diverse N-terminal inserts would also provide additional insight into our understanding of tau pathology. Examination of a larger cohort of cases and additional brain regions with 3R and 4R tau specific monoclonal antibodies may help to elucidate tau pathology in neurodegenerative tauopathies.

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Fig. 1.

Characterization of tau isoform specific monoclonal antibodies. ELISA using peptides corresponding to exon 10 of tau and the junction between exons 9 and 11 of tau demonstrate the specificity of monoclonal antibodies ET2 (a) and RD3 (b). Western blots using 4 recombinant tau isoforms: A) 2+3+10+, B) 2+3+10-, C) 2-3-10+, D) 2-3-10-. CP27 a human specific monoclonal antibody that recognizes amino acids 140-160 common to all tau isoforms detects all 4 bands, ET2 reacts only with the tau isoforms containing exon 10; and RD3 only reacts with the tau isoforms lacking exon 10 (c). Western blots of samples from fetal (F) and normal adult (A) brain were stained using CP27, ET2 and RD3. ET2 reacts primarily with tau isoforms in the adult human brain while RD3 shows strong reactivity with tau in fetal

brain. Molecular weight bars correspond to $MW_r = 68$ kDa. (d). In N2a cells transiently transfected with both 3R (-2-3-10) and 4R (+2+3+10) tau isoforms RD3 (red) and ET3 (green) detect 3R tau and 4R tau equally well.

Fig. 2.

Demonstration of differences in staining patterns between two advanced AD cases. Paraffin embedded hippocampal sections from AD cases are stained with monoclonal antibodies CP13, ET3, RD3 and thioflavine-S. The CA1 region of the hippocampus of case 3 displays abundant RD3 (c and g) and thioflavine-S (d and h) positive tangles while minimal staining is observed with ET3 (b and f) or with CP13 (a and e). The CA1 region of the hippocampus of case 4 shows a more even distribution of tangles stained with all three antibodies and thioflavine-S (i-p). Magnification: Panels a-c and i-k, 125x; panels d and l, 250x; panels e-g and m-o, 500x; panels h and p, 800x.

Fig. 3. Double-label immunohistochemistry of AD cases using RD3 and ET3 monoclonal antibodies. Paraffin embedded sections for the CA1 region of the hippocampus of case 3 (a–c) confirms that in specific areas RD3 stains a more abundant number of tangles than ET3. Extracellular tangles in the cortex of case 3 and other cases are exclusively RD3 positive (d–f). A double-labeled tangle in the CA1 of case 3 (g–i) and a double-labeled tangle in the CA1 of case 6 (j–l) show that 3R and 4R tau are found in some of the same tangles but are not completely co-localized. Magnification: Panels a–c, 250x; panels d–e, scale bars = 40 μ m; panels g–i, scale bars = 16 μ m; panels j–l, scale bars = 8 μ m.

Fig. 4.

Comparative immunohistochemistry and biochemistry of the hippocampus of twelve severe AD cases. Immunohistochemical analysis of paraffin-embedded sections of cases 1, 2, 3, 7, 8 and 9 using monoclonal antibodies RD3 and ET3 show predominant RD3 staining and minimal ET3 staining (cases 7, 8 and 9 not shown). Cases 4, 5, 6, 10, 11 and 12 present a more even distribution of tangles stained with both isoform specific antibodies (a) (cases 10, 11 and 12 not shown). Western blot analysis of brain homogenates of cases 1–6 and cases numbered 7–12 using antibodies CP27 (1:1000) (4CN), RD3 (1:10,000)(4CN) and ET2 (1:500) (chemiluminescence) shows the presence of both 3R and 4R tau in the hippocampus (b and d). Western blot analysis of sarkosyl extracted insoluble material from cases 1–6 and cases

numbered 7–12 using antibodies CP27, RD3 and ET2 (same dilutions) shows that all cases appear to have PHF-tau containing 3R and 4R tau isoforms (c and e). ET2 blot in (e) is developed using 4CN. Molecular weight bars correspond to $MW_r = 68$ kDa.

Fig. 5.

PSP cases stained with monoclonal antibodies CP13, ET3 and RD3. Immunohistochemical analysis of paraffin embedded sections of the basal ganglia of PSP case 3 shows a similar number of tangles stained with CP13 and ET3 but no NFT staining with RD3 (a–c). PSP case 5, basal ganglia, shows NFT stained with CP13 and ET3 and, minimal RD3 staining in one or two NFTs (d–f). PSP case 4, basal ganglia shows staining of tau inclusions in glial cells (tufted astrocytes) with CP13 and ET3. RD3 did not stain tufted astrocytes in any of the PSP cases (g–i). Magnification: Panels a–c, 50x; panels d–f, 250x; panels g–i, 500x.

Fig. 6. Sections of different areas from a Pick's disease case stained with monoclonal antibodies CP13, ET3 and RD3. The temporal cortex of a Pick's disease case has abundant Pick bodies stained with both CP13 and RD3. No ET3 reactivity was observed in this area (a–c). Granule cell layer in the hippocampus had numerous Pick bodies that were positively stained with both CP13 and RD3 but no staining with ET3 was observed in these inclusions (d–f). Area of the cortex shows numerous Pick bodies stained with CP13 and RD3. This section shows some reactivity with ET3 (g–i). Magnification: Panels a–c, 125x; panels d–i, 250x.

Table 1

Immunohistochemical profile of AD brain with thioflavine-S and antibodies CP13, RD3 and ET3. Cases are listed in the table starting with most severe to less severe based on thioflavine-S staining (when available)

Age	Gender	Case#	Area	Thio-S	RD3	ET3	CP13
1	84	F	1	A.H.	****	*	**
2	73	F	3	H	****	**	*
3	94	F	7	H	****	*	*
4	91	F	31	H	****	*	**
5	73	M	32	H	****	*	**
6	73	F	8	H	****	****	****
7	87	F	2	H	****	*	**
8	66	M	33	H	****	0	****
9	82	F	4	H	****	****	****
10	89	F	6	H	****	****	****
11	83	M	9	H	****	****	****
12	85	M	10	H	****	****	****
13	75	M	11	A.H.	****	****	****
14	51	F	5	H	****	****	****
15	70	F	34	H	**	**	****
16	90	F	12	H	**	**	****
17	84	M	13	H	**	*	****
18	79	F	14	H	**	*	****
19	73	F	15	H	*	*	**
20	86	F	16	H	*	0	****
21	82	M	17	H	*	*	****
22	74	F	18	H	*	0	**
23	69	M	20	H	*	0	****
24	92	F	21	H	*	0	*
25	64	M	19	H	0	0	0
26	80	F	22	H	NA	****	*
27	78	M	23	H	NA	****	****
28	77	F	24	H	NA	**	*

	Age	Gender	Case#	Area	Thio-S	RD3	ET3	CPI3
29	82	F	25	H	NA	*	**	***
30	83	F	26	H	NA	**	***	***
31	83	F	27	H	NA	**	*	***
32	80	M	28	H	NA	0	0	*
33	85	F	29	H	NA	*	*	**
34	85	F	30	H	NA	0	0	0

Table 2

Immunohistochemical profile of PSP brain with antibodies CP13, RD3 and ET3

	Gender	Case #	Area	CP13/NFT	CP13/glia	ET3/NFT	ET3/glia	RD3/NFT	RD3/glia
1	M	1	BG	**	*	**	*	0	0
2	F	2	BG	**	**	**	**	0	0
3	F	3	BG	***	*	***	*	*	0
4	F	4	BG	**	***+	**	**	*	0
5	F	5	BG	***	***	***	**	*	0

Table 3
Immunohistochemical profile of PiD brain with antibodies CP13, RD3 and ET3

Case #	AREA	CP13	ET3	RD3
1	FC	***	0	**
2	TC	****	**	****
3	H	****	0	****