βII-tubulin and phospho-tau aggregates in Alzheimer's disease and Pick's disease

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Abstract. The expression of βI-, βII- and βIII-tubulin isotypes was examined by immunohistochemistry in the entorhinal and transentorhinal cortices, hippocampus and dentate gyrus in normal human brains and in cases with Alzheimer's disease (AD), Pick's disease (PiD) and in argyrophilic grain disease (AGD). The results showed that βII-tubulin predominated in the upper layers (mainly layer II) and βIII-tubulin in the inner layers of the entorhinal and transentorhinal cortices in control brains. βII-tubulin immunoreactivity was higher than βIII-tubulin immunoreactivity in granular neurons of the dentate gyrus, whereas pyramidal neurons of the hippocampus proper were stained equally with anti-βII-tubulin and βIII-tubulin antibodies. No preferential layering distribution was observed for βI-tubulin. Polymerization assays with tubulin peptides following the method of microtubule-associated protein displacement demonstrated that the βI and βIII isotypes have a higher binding capacity for tau than does the βII isotype. Interestingly, about 60% of neurons with neurofibrillary tangles in layer II of the entorhinal and transentorhinal cortices in AD were selectively stained with anti-βII-tubulin antibodies. Moderate βII-tubulin immunoreactivity was also observed in Pick bodies in PiD. Taken together, these findings support the view that high βII-tubulin content is a contributing factor in the formation of abnormal hyper-phosphorylated tau aggregates.

Keywords: Alzheimer's disease, Pick's disease, tubulin, tau

1. Introduction

Tubulin constitutes the major component of microtubules; it is a dimeric protein composed of α and β subunits that. Both α and β exist as numerous isotypes. In mammals there are seven β-tubulin isotypes encoded by different genes [17,18]. All β-tubulin isotypes have the capacity to incorporate themselves into microtubules [16]. Differences among the isotypes are mainly centered on their C-terminal regions [28], which are involved in the binding of tubulin to distinct microtubule-associated proteins, including tau [24,25].

Tubulin is abundant mainly in the brain, and at least five β-tubulin isotypes are expressed in neurons. βI-tubulin, which is found in many tissues, accounts for about 3% of the total brain β-tubulin. The βII isotype accounts for about 58% of the brain β-tubulin, whereas βIII, which is present specifically in neurons, accounts for 25% of the total brain β-tubulin [1,22]. βIVa and βIVb are also present in the brain, together accounting for about 14% [1]. These β-tubulin isotypes can be identified by their reactions to specific antibodies recognizing their c-terminal regions [23].

Biochemical differences are probably related to special roles and functions of the tubulin isotypes [8]. For example, β-tubulin isotypes are differentially expressed in selected populations of cerebellar neurons, thus suggesting particular properties of individual cerebellar neurons under physiological condi-

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tions [6]. Moreover, modifications have also been observed in pathological scenarios; βII-tubulin expression correlates with axonal outgrowth during development, whereas the βII/βIII ratio is modulated in regenerating axons [14].

A complex cellular organization is encountered in the cerebral cortex [11,12]. Yet little is known about the distribution of β-tubulin isotypes in the various cortical layers. Furthermore, the probable relevance of β-tubulin isotyping on tau assembly to tubulin is not known. Finally, precise β-tubulin isotype expression may have consequences in those degenerative diseases of the nervous system in which there is an abnormal accumulation of phosphorylated tau in neurons, including cellular aggregates designed as neurofibrillary tangles and Pick bodies in Alzheimer's disease (AD) and Pick's disease (PiD), respectively [3,4,7,10,15,19,20, 27].

The present study examines the distribution of βI-, βII- and βIII-tubulin isotypes in the entorhinal cortex, subiculum and dentate gyrus in human brains of controls, and pathological cases of AD and PiD with special attention to neurofibrillary tangles and Pick bodies. Finally, polymerization assays with tubulin peptides following the method of microtubule-associated protein displacement have been used to increase understanding of the capacities of tau binding to βI-, βII- and βIII-tubulin isotypes.

2. Materials and methods

2.1. Human cases

The cases included were as follows. AD: four men and six women who were categorized as stage III (3 cases) and stage VI (7 cases) according to the nomenclature of Braak and Braak [3]. Their ages ranged from 68 to 82 years, and the mean age was 72.1 years. PiD: two men aged 73 and 69 years, and two women aged 71 and 82 years. Five age-matched controls (three men and two women) with no neurological disease and no abnormalities in the neuropathological examination were also used in this study. The delay between death and tissue processing was between 2 h and 6 h. All the samples were obtained following the recommendations of the local ethics committees of the University of Barcelona/Clinic Hospital and the Bellvitge Hospital (Barcelona and Bellvitge Brain banks). A complete neuropathological examination was carried out in every case in formalin-fixed tissue for no less than

three weeks; the tissue was then embedded in paraffin. For tubulin and *tau* immunohistochemical studies, fresh samples were fixed with 4% paraformaldehyde in phosphate buffer saline for 48 h and then embedded in paraffin.

2.2. Immunohistochemistry

Specific anti-βI-, βII- and βIII-tubulin immunohistochemistry was carried out following the avidin-biotin-peroxidase (ABC) method. De-waxed sections were first boiled in citrate buffer and then stored overnight at room temperature. After blocking endogenous peroxidase, the sections were incubated with normal serum and then incubated at 4 °C overnight with anti-βI, βII- and βIII-tubulin monoclonal antibodies used at a dilution of 1: 500. The monoclonal antibodies to βI-, βII- and βIII-tubulin have been previously characterized [1,22,23]. The antibodies to βI-, βII- and βIII-tubulin recognized a single band of 45 kDa in total cortical homogenates (data not shown). Some sections were processed with anti-*tau* phospho-specific antibody Thr[181] (Calbiochem), used at a dilution of 1:500. Then, the sections were incubated for 1 h with biotinylated anti-mouse IgG or anti-rabbit IgG diluted 1:100, followed by ABC at a dilution of 1:100 for 1 h at room temperature. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.05% hydrogen peroxide. Sections were counterstained with haematoxylin.

Double-labeling immunohistochemistry was conducted by incubating the sections following a two-step protocol. The sections were first incubated with anti-βI-, βII- and βIII-tubulin monoclonal antibodies, and the immunoreaction was visualized with diaminobenzidine and hydrogen peroxide as before. Subsequently, the sections were incubated with the rabbit polyclonal anti-*tau*, phospho-specific Thr[181]. The immunoreaction was visualized with $NH-4NiSO_4$ (0.05 M) in phosphate buffer (0.1 M), 0.05% diaminobenzidine, NH_4Cl, and 0.05% hydrogen peroxide. The first primary antibody was recognized as a brown homogeneous precipitate, whereas the second primary antibody was recognized as a dark blue precipitate.

Semi-quantitative studies were carried out in three non-consecutive serial sections of the entorhinal cortex per case. The number of neurons containing tau-immunoreactive and tubulin-immunoreactive tangles was expressed as a percentage of the total number of neurons with neurofibrillary tangles as revealed in double-immunostained sections.

2.3. Confocal microscopy

De-waxed sections of the entorhinal cortex in AD cases were stained with a saturated solution of Sudan black B (Merck) for 30 min to block the autofluorescence of lipofuscin granules present in nerve cell bodies, rinsed in 70% ethanol and washed in distilled water. The sections were incubated, at 4°C overnight, with the rabbit anti-tau Thr[181] antibody at a dilution of 1:500 and monoclonal mouse anti-βII-tubulin antibody at a dilution of 1:500 in a vehicle solution composed of Tris buffer, pH 7.2, containing 15 mmol/L NaN$_3$, and protein (Dako). After washing in PBS, the sections were incubated in the dark with the cocktail of secondary antibodies, diluted in the same vehicle solution as the primary antibodies, during 45 minutes at room temperature. Secondary antibodies were Alexa488 anti-mouse (green) and Alexa546 anti-rabbit (red) (both from Molecular Probes), and they were used at a dilution of 1:400. After washing in PBS, the sections were mounted in immuno-Fluore Mounting medium (ICN Biomedicals), sealed and dried overnight. Sections were examined in a Leica TCS-SL confocal microscope.

2.4. Protein purification

Microtubule protein was isolated from bovine brain by three temperature-dependent cycles of assembly-disassembly [26]. Microtubule pellets were frozen in liquid nitrogen after the second cycle and stored at −80°C. Before use, microtubule pellets were re-suspended in 0.1 M morpholinoethanesulfonic acid (Mes), pH 6.5, 0.5 mM MgCl$_2$, and 2 mM EGTA (buffer A), and then a third cycle was performed.

Tubulin peptides from different β isoforms, βI (431-444) EEEEDFGEEAEEEA, (β'I (408-426) FTEAES-NMNDLVSEYQQYQ, βII (434-440) GEFEEEG, βIII (435-450) EMYEDDEDESESQGPK, and βIII' (438-444) EDDEDES were synthesized on an automatic solid-phase peptide synthesizer (Type 430A, Applied Biosystems) and purified by reversed-phase HPLC on a NOVAPACK C-18 column, with a Waters 501 apparatus. The purified peptides were lyophilized and dissolved in buffer A, as previously described [9].

2.5. Polymerization assay

Microtubule protein from bovine brain (1 mg/mL) was incubated in buffer A, in the presence of 0.5 mM GTP and in the presence or absence of tubulin peptides.
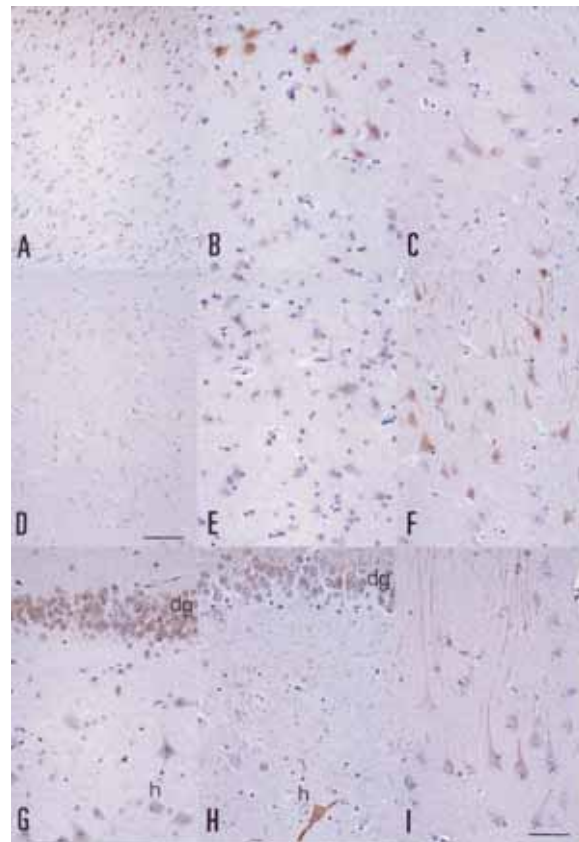

Fig. 1. βII-tubulin (A-C, G, I) and βIII-tubulin (D-F, H) immunoreactivity in the entorhinal cortex (A-F), dentate gyrus (G, H) and CA1 area of the hippocampus (I). βII-tubulin is more abundant in the upper layers (A, B), mainly layer II neurons, than in the inner layers (C) of the entorhinal cortex. In contrast, βIII-tubulin immunoreactivity is less abundant in the upper layers (D, E) than in the inner layers (F) of the entorhinal cortex in control brains. Granular neurons of the dentate gyrus (dg) are better stained with anti-βII-tubulin than with anti-βIII-tubulin antibodies, whereas the reverse is true for hilar (h) neurons (G, H). Pyramidal neurons of the CA1 region of the hippocampus are recognized by anti-βII-tubulin antibodies (I). Paraffin sections slightly counterstained with haematoxylin, bar = 25 μm.

Tubulin assembly was assayed for the sedimentation of polymers by using a Beckman air-driven ultracentrifuge for 15 min at 140,000 g [9]. The sediment protein was subjected to electrophoresis, and the tau content was determined by Western blot using the antibody 7.51 at a dilution of 1:100. The antibody 7.51 (kindly provided by Dr. C.M. Wischik, MRC, Cambridge, UK) is a well-characterized antibody raised against the binding region of tau to microtubules [21]. The amount of protein present in each sample was determined by densitometric analysis.

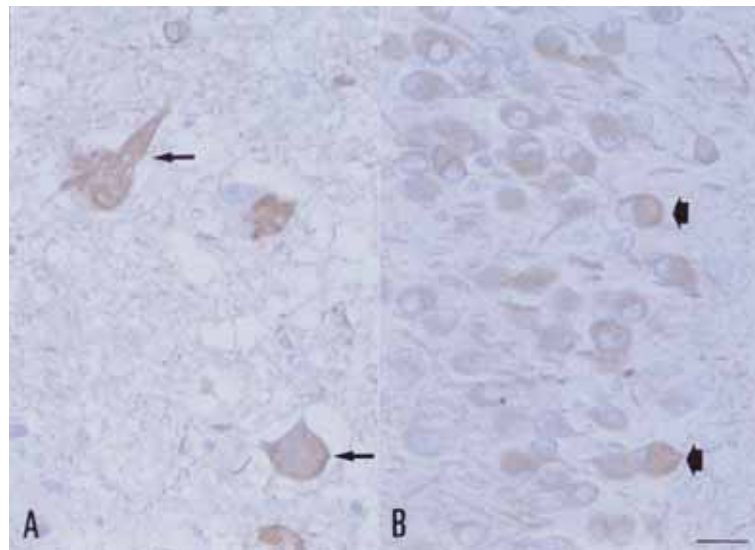The rationale for the use of the different β-tubulin peptides is the following: β-tubulin C-terminal re-

Fig. 2. βII-tubulin immunoreactivity is found in neurons with neurofibrillary tangles (long arrows) of layer II entorhinal neurons in AD cases (A) and in neurons with Pick bodies (short arrows) of granular neurons of the dentate gyrus (B) and entorhinal cortex in PiD. Paraffin sections slightly counterstained with haematoxylin, bar = 10 μm.

gion is involved in the binding of this tubulin subunit [25]. This C-terminal region can be categorized into two different parts: a) from residues 408 to 430, and b) from residues 430 to 450. The a) region is conserved in every tubulin isotype, being the most conserved part of the molecule, and it is not involved in the binding of β-tubulin to other microtubule associated proteins (MAPs) like MAP1 or MAP2 [9]. On the other hand, the b) region is the most variable part from one β-tubulin isotype to another, and it is involved in the binding of tubulin to MAPs [24]. Thus, we have studied the binding of these two tubulin binding regions, showing a similar length, to *tau* protein. The tested peptides have been peptide (from βI isotype) βI (EEEEDFGEEAEEEA) and peptide β'I (FTEAESNMNDLVSEYQQYQ), common to the different isotypes. These studies have been complemented with those testing the b) region of another β-tubulin isotype (βIII), specific for neurons [14,16,17], with a similar length βIII peptide (EMYEDDEDESESQGPK). To test for the minimum sequence of this βIII region involved in the binding to *tau* protein, we have analyzed a shorter region, β'III peptide (EDDEDES). The binding to this peptide has been compared to that, of similar length, present in the βII isotype (an isotype that is not specific for neurons). The βII isotype peptide was (GEFEEEG). A scheme indicating the localization of these peptides is shown in the inset of Fig. 4.

3. Results

Neurons in the distinct layers of the entorhinal and transentorhinal cortices were differentially stained with antibodies raised against βI-, βII- and βIII-tubulin isotypes in control brains. The βII isotype was mainly present in neurons of the upper layers, particularly neuronal islands of layer II, whereas the βIII isotype was preferentially located in neurons of the inner layers (Fig. 1A–F). The βI isotype appeared to be equally common in neurons of the upper and inner cortical layers of the entorhinal cortex. Granular neurons of the dentate gyrus were better stained with anti-βII-tubulin antibodies than with antibodies raised against βI- and βIII- tubulin isotypes (Fig. 1G, H). In contrast to the entorhinal cortex and dentate gyrus, no preferential staining was observed in the subiculum, in which all neurons were stained equally with antibodies to βII- and βIII-tubulin isotypes (Fig. 1).

βI-, βII- and βIII-tubulin immunoreactivity was present in pathological brains of the AD and PiD. Increased βII-tubulin immunoreactivity was found in about 60% of neurons containing neurofibrillary tangles of layer II of the entorhinal cortex in AD, as revealed by single (Fig. 2A) and double-labeling immunohistochemistry. Yet neuropil threads and dystrophic neurites of senile plaques in AD were not stained with anti-βI-, βII- and βIII-tubulin antibodies. Moderate βII-tubulin immunoreactivity was found in neurons with Pick bodies in the dentate gyrus (Fig. 2B)
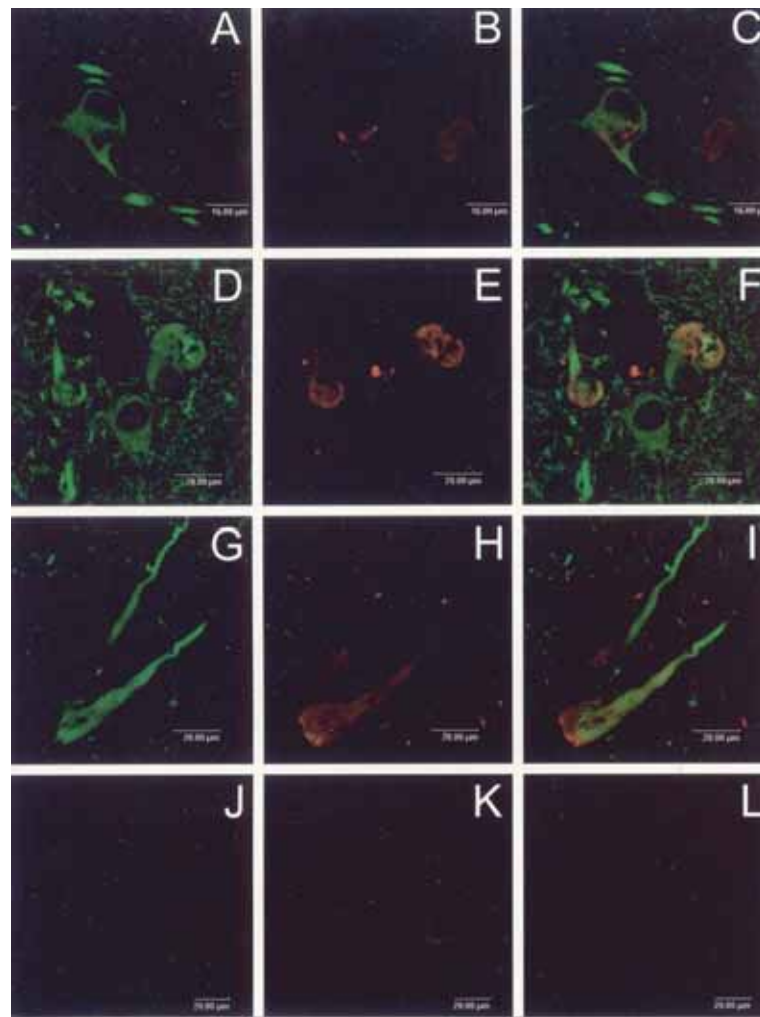
Fig. 3. Confocal microscopy showing co-localization of βII-tubulin (green) and phospho-tau Thr^{181} (red) in several neurons with neurofibrillar degeneration of layer II of the entorhinal cortex in AD (A, B; D, E; and G, H). Co-localization appears in the merge (yellow) column on the right (C, F and I, respectively). J, K, L shows incubation with only the secondary antibodies; no immunoreaction is seen.

and in layer II neurons of the entorhinal cortex in PiD, but Pick bodies were not stained with anti-βI- and anti-βIII-tubulin antibodies.

Confocal microscopy studies further demonstrated co-localization of βII-tubulin and phospho-tau aggregates (neurofibrillary tangles) in many neurons of the upper layers of the entorhinal cortex in AD (Fig. 3). It is worth noting that tubulin immunoreactivity often appears inside and outside the inclusion, thus indicating that not all tubulin is recruited in neurofibrillary tangles.

To learn whether the different β-tubulin isotypes might interact with the microtubule-associated protein *tau*, we tested *tau* binding to tubulin peptides following the method of microtubule-associated protein (MAP)

displacement [9]. As shown in Fig. 4(a), the peptide βI, but not the peptide β'I (with a similar length) interferes with the binding of *tau* with tubulin. It indicates that the βI, but not β'I peptide, contains a *tau* binding site. Also, in Fig. 4(a), it is shown that the βI peptide appears to have a higher binding capacity for *tau* than the βIII peptide (with a similar length). Figure 4(b) shows that a shorter peptide, β'III, is able to bind to *tau* protein, indicating that it probably contains the shorter tau binding region for that tubulin isotype. When that binding was compared to peptide βII, from the βII isotype with a similar length to peptide β'III, it was found that βII has much lower binding capacity for tau than the βIII peptide. We can thus infer that microtubules rich in βII would bind less tau than would microtubules
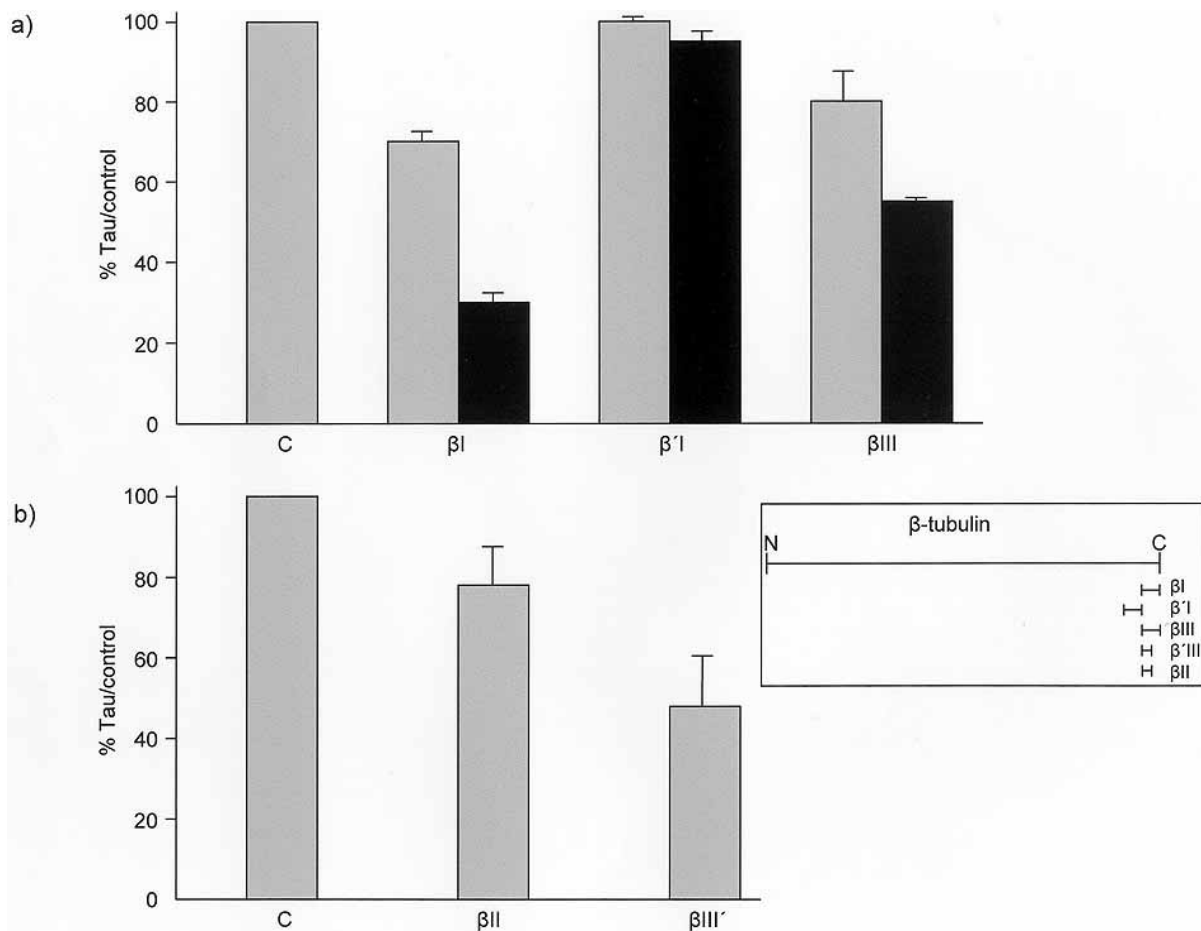
Fig. 4. a) Displacement of tau from microtubules assembled in the presence of C-terminal β-tubulin peptides. Microtubule protein (1 mg/ml) was assembled in the absence of any peptide (control) or in the presence of 10 μM (grey) or 25 μM (black) βI, β'I or βIII tubulin peptides for 30 minutes at 30°C in the presence of 1 mM GTP. b) As in a), but in this case the incubation was done in the presence of 25 μM βII or β'III peptides. The assembled polymers were sedimented in the Airfuge at 140,000 g, and the pellet fractions were subjected to gel electrophoresis. The proportion of tau remaining in the pellet fraction was quantified by densitometry in Western blots using the anti-tau antibody 7.51. The figure represents the percentage of tau remaining in the pellet fraction resulting from the average of thre experiments. The inset shows the localization in β-tubulin molecule of the different peptides used in the experiment. It indicates that peptides used in Fig. 4(a), βI, β'I, and βIII, have a similar length. Also, the length of β'III is similar to that of βII, tested in Fig. 4(b).

with low levels of βII. This result is similar to that previously found for MAP2 [9], a protein containing similar tubulin binding motifs to those of tau.

4. Discussion

The present study has shown selective expression of β-tubulin isotypes in neurons of the different layers of the entorhinal cortex, as revealed by specific antibodies to βI-, βII- and βIII-tubulin isotypes. βII-tubulin is particularly abundant in layer II of the entorhinal and transentorhinal cortices, whereas βIII predominates in the inner layers of the entorhinal cortex. Interestingly,

layer II neurons of the entorhinal and transentorhinal cortices are very vulnerable to neurofibrillary degeneration in AD, and neurofibrillary tangles first appear in these neurons at the early stages of the disease, and in the elderly [2,3,13].

Several factors may be involved in the selective vulnerability of cortical neurons to tangle formation. Neurofilament composition has been suggested as one such factor [13]. The present findings suggest that the concentration of βII-tubulin may also play a role. First, βII-tubulin is highly expressed in layer II neurons of the entorhinal and transentorhinal cortices. Second, βII-tubulin localizes in neurofibrillary tangles in about 60% of layer II entorhinal neurons in AD, as revealed

by immunohistochemistry. Third, tau shows a higher affinity for βI and βIII isotypes than for βII-tubulin, as determined by the MAP displacement method. Importantly, testing peptides with differential lengths have shown that it is the sequence and not the length of the peptide that has an effect in the competition experiments. Therefore, a higher proportion of βII may result in a smaller proportion of tau protein bound to microtubules. Consequently, the expected higher proportion of unbound tau in neurons rich in βII-tubulin may contribute to tau aggregation in aberrant structures such as the neurofibrillary tangles that occur in AD entorhinal cortex layer II neurons. Moreover, Pick bodies in the entorhinal cortex in PiD cases also predominate in layer II, thus further supporting βII-tubulin content as a component in the formation of certain phospho-tau aggregates.

βII-tubulin is also abundant in neurons of the dentate gyrus. Yet neurofibrillary tangles are rare in dentate gyrus neurons in AD. In contrast, Pick bodies in the dentate gyrus are mandatory in PiD [10]. It is clear that several factors may participate in the formation of abnormal tau aggregates in dentate gyrus neurons and tau isoforms appear to be crucial features that may explain the aggregation of particular tau isoforms into filaments in a given neurodegenerative disorder [5].

Taken together, the present findings support the concept that βII-tubulin participates in the formation of phospho-tau aggregates in neurofibrillary tangles and Pick bodies, but also that other factors are determining in disease-dependent particular inclusions. Interestingly, neuropil threads and dystrophic neurites surrounding senile plaques, which are located distally to the soma, are not stained with anti-βII-tubulin antibodies thus indicating sub-cellular differences in the distribution of βII-tubulin in neurons with phospho-tau aggregates.

Acknowledgements

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