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Phosphorylated serine 199 of microtubule-associated protein tau is a neuronal epitope abundantly expressed in youth and an early marker of tau pathology

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Abstract Microtubule-associated protein tau is abnormally phosphorylated in many neurodegenerative disorders, and is the major component of neurofibrillary degeneration, a degenerating process with many biochemical phenotypes. The serine 199 (S199) residue of tau is phosphorylated at early and late stages of Alzheimer's disease (AD). We studied the immunohistochemical distribution of this phosphorylated epitope in AD and other neurodegenerative disorders, as well as in controls of different ages. The phosphorylated S199 (S199P) epitope was observed in tau lesions from numerous diseases with neurofibrillary degeneration. This epitope was found to be abundantly expressed in the hippocampus formation in childhood and in young adult brain samples, and more specifically in subsets of neurons vulnerable to neurodegeneration. Interestingly, our data suggests that S199P is particularly resistant to phosphatase activity occurring during post-mortem delays. We suggest a peculiar and important role of the S199 residue as a qualitative indicator of the normal and pathological phosphorylation status of tau proteins.

Keywords Tau · Pretangle · Neurofibrillary tangle · Neuropil threads · Alzheimer's disease

Introduction

Neurofibrillary degeneration is a pathological hallmark of Alzheimer's disease (AD) and other neurodegenerative disorders. Tau are microtubule-associated proteins (MAPs) [50] mainly found in neurons and, in some instances, in glial cells. A wide spectrum of tau proteins is expressed in the human nervous system, deriving from a single gene, made of 16 exons, and located on chromosome 17 [1]. Alternative splicing of the RNAs gives rise to six isoforms in the human central nervous system. The six resulting polypeptides have a molecular mass ranging from 45 to 65 kDa [9]. The isoforms differ by the presence of (1) either three or four repeats in the C-terminal part, resulting from the inclusion or exclusion of exon 10, and (2) no, one or two inserts of 29 amino acids in the N-terminal part, encoded by exons 2 and 3. Distinct regions support the functions of tau. The projection domain of tau encompasses the N-terminus and a proline-rich region; this domain is likely to interact with cytoskeleton and plasma membrane [8, 26]. The microtubule-binding domain includes the C-terminal repeats; the fourth C-terminal repeat increases the ability to promote microtubule assembly [23].

The phosphorylation status of tau is highly modulated during development and modifies its function. For instance, the rate of phosphorylation is developmentally regulated and the phosphorylation rate of tau, modulated by phosphatase activity, decreases from foetus to adulthood [36]. The increasing number of phosphorylated residues on tau decreases its ability to promote polymerisation of microtubules [32, 51]. There are 79 Ser or Thr putative phosphorylation sites on the longest brain tau isoform; 30 out of these sites were already demonstrated to be differentially and selectively phosphorylated [33].

Increasing number of phosphorylated sites and aberrant phosphorylation of tau are known to occur in neurofibrillary pathology [9]. The phosphorylation of tau differs from AD brain tissue to normal brain sample extracts, as shown by phosphorylation-dependent antibodies [11, 25, 27, 35]. However, only few residues have been reported, i.e. Ser199, Ser202, Ser409 and Ser422, that are thought to be abundantly or abnormally phosphorylated in pretangle stage of AD [31].

The phosphorylation status of these epitopes is likely to be critical in the pathophysiology of brain ageing, AD

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Table 1 Summary of clinical data (*AD* Alzheimer's disease, *CBD* cortico-basal degeneration, *DS* Down' syndrome/trisomy 21, *FLD* frontal lobe degeneration, *DLB* dementia with Lewy bodies, *MND* motor neuron disease, *DM* 1 myotonic dystrophy of type 1 (Steinert's disease, *PEP* post encephalitic Parkinsonism, *PiD* Pick's disease, *PSP* progressive supranuclear palsy)

Case	Diagnosis	Fixation (years)	Age (years)	Sex
1	AD	1 or less	65	F
2	AD	1 or less	67	M
3	AD	1 or less	68	F
4	AD	1 or less	71	F
5	AD	1 or less	74	F
6	AD	1 or less	76	F
7	AD	1 or less	80	M
8	AD	1 or less	81	F
9	AD	1 or less	83	M
10	AD	1 or less	83	F
11	AD	1 or less	85	F
12	AD	1 or less	89	M
13	AD	1 or less	92	F
14	AD	11	86	F
15	AD	18	72	M
16	AD	25	79	F
17	DS	6	48	F
18	DS	2	45	F
19	PEP	1 or less	67	F
20	PEP	1 or less	67	M
21	DLB	1 or less	76	M
22	CBD	1 or less	75	F
23	PSP	1 or less	69	M
24	PSP	1 or less	68	M
25	PSP	1 or less	67	M
26	DM 1	1 or less	65	M
27	DM 1	1 or less	61	M
28	DM 1	1 or less	64	M
29	FLD	1 or less	60	F
30	FLD	1 or less	65	F
31	FLD	1 or less	69	M
32	FLD	1 or less	34	F
33	FLD/MND	1 or less	69	F
34	PiD	1 or less	75	M
35	PiD	1 or less	70	M
36	Control	1 or less	12	F
37	Control	1 or less	14	M
38	Control	1 or less	18	F
39	Control	1 or less	18	M
40	Control	1 or less	19	M
41	Control	1 or less	20	M
42	Control	1 or less	26	F
43	Control	1 or less	31	M
44	Control	1 or less	41	M
45	Control	1 or less	58	M
46	Control	8	77	M
47	Control	7	79	F
48	Control	7	83	F
49	Control	7	87	F
50	Control	8	90	F

and "tauopathies". Since the Ser199 (S199) residue is already a candidate for phosphorylation in pretangle neurons in human as well as in animal models of neurodegeneration [21, 31], we have focused on this epitope of tau, and investigated its expression by immunohistochemistry and immunoblot performed on human brain tissue samples in AD, in other neurodegenerative diseases and in controls.

Material and methods

Patients

Brain tissue samples were removed from selected areas of patients with AD (*n*=16, ranging from 65 to 92 years), Down's syndrome (DS, *n*=2), post-encephalitic parkinsonism (PEP, *n*=2), dementia with Lewy bodies (DLB, *n*=1), cortico-basal degeneration (CBD, *n*=1), progressive supranuclear palsy (PSP, *n*=3), myotonic dystrophy of type 1 (DM 1, *n*=3), frontal lobe degeneration (FLD, *n*=5, 34 to 69 years) and Pick's disease (PiD, *n*=2). In each case, the diagnosis was clearly assessed by clinical data and conventional neuropathological procedures as well as immunohistochemistry (amyloid-β, tau, ubiquitin, and α-synuclein). When frozen brain tissue samples were available, the diagnosis was further confirmed by immunoblot analysis of pathological tau biochemical profile (performed with the AD2 antibody) [10].

Controls (*n*=15, 12–90 years) had neither neurological disease nor cognitive impairment. The death of the youngest patients was subsequent of Sjogren-Larsson's disease (case 36), lymphoblastic acute leukaemia (cases 37 and 40), acquired immunodeficiency syndrome (cases 38, 41 and 43), lymphoblastic lymphoma (case 39), obstetrical haemorrhage (case 42), and coronary thrombosis (case 44). Post-mortem delay at autopsy never exceeded 24 h. Clinical data are summarised in Table 1.

Immunohistochemistry

Immunohistochemical procedures were performed on formalin-fixed, paraffin-embedded brain tissue samples from the left hemisphere. Since tau pathology is restricted to a geographical pattern of distribution in most tau-linked neurodegenerative diseases [15], the samples were removed in Brodmann's areas 10, 21 and 39 (associative frontal, mid-temporal and parietal cortices), hippocampus and brain stem, mostly following a 1- to 3-months fixation period (except cases 14–18 and 45–50). Tau immunohistochemistry was achieved with AD199, AT8, AT100, AD2 and tau-1. AD199 is a recently developed polyclonal antibody, raised against a synthetic peptide of tau sequence that corresponds to the phosphorylated S199 epitope (S199P) [14, 41]. Labelling with AD199 (diluted 1:500) was compared to Gallyas staining [16], and to antibodies that overlap with the S199P in generating the epitope they were raised against.

These antibodies included the phospho-dependent anti-tau antibodies AT8 (Innogenetics, Gent, Belgium, diluted 1:1,000; phosphorylated Ser202/Ser205 epitope), AT100 (Innogenetics, diluted 1:400; phosphorylated Ser212/Ser214), AD2 (gift from Dr Mouton-Gilles, Montpellier, France; diluted 1:1,000; phosphorylated Ser396/Ser404 epitope) and tau-1 (Boehringer Mannheim, Germany, diluted 1:200). Tau-1 antibody is an antibody that only labels tau proteins among unphosphorylated Ser residues 195, 198, 199 and 202. It was used either directly or following dephosphorylation by purified alkaline phosphatase (type VIIL, from bovine intestinal mucosa, 400 UI/ml; Sigma, Temecula Calif.) [44]. Before labelling, 4-µm-thick sections were deparaffinized, and heated in citrate buffer pH 6.0, in a pressure cooker for 10 min. The sections were then incubated in diluting medium (Dako, Glostrup, Denmark) containing either primary antibody or pre-immune goat serum. For each primary antibody, the dilution was based upon the

optimal staining that could be obtained on brain samples after pressure cooker retrieval. The labelling was achieved by a streptavidin-biotin complex method and revealed by DAB or AEC chromogen in a Ventana ES automate. For confocal imaging, rehydrated sections were incubated with AD199 and AT8 for 24 h at room temperature, rinsed in phosphate-buffered saline-Tween 20 (0.05%), then incubated with a biotinylated goat anti-mouse IgG for 2 h (Dako; second step of the AT8 labelling). AD199 was then detected by a two-step labelling (cyanin3-conjugated goat anti-rabbit IgG, Jackson Laboratories, West Grove, Pa.) and AT8 by a three-step labelling (cyanin2-conjugated streptavidin, Jackson Laboratories). The endogenous non-specific autofluorescence of lipofuscin was reduced by Sudan black dye before the final rinsing procedures [40]. Double-labelled sections were examined under a Leica laser scanning fluorescence microscope.

Specificity of AD199

Sections from controls and demented patients were incubated with purified AD199, and adsorbed AD199. AD199 was purified by affinity chromatography as previously described [43]. Briefly, the phosphorylated peptide was covalently coupled to NHS-fast flow Sepharose (Pharmacia Biotech, France), following the manufacturer's instructions. AD199 antiserum (500 µl) was diluted in 9 vol TBS-T (50 mM TRIS pH 8.0, 100 mM NaCl, 0.1% Tween 20) and gently agitated overnight at 4°C with an orbital shaker. The media was rinsed with 20 vol TBS-T and the polyclonal antibody AD199 was eluted in 2 vol acetate buffer pH 2.0 and neutralised with TBS [43]. Additional sections were incubated with AD199 following dephosphorylation by purified alkaline phosphatase pre-treatment.

Immunoblot

Brain tissue sample homogenisation in SDS buffer, proteins resolution by SDS-PAGE and Western blots were performed as previously described [41].

Results

Labelling pattern of tau pathology in demented patients

AD199 stained neurofibrillary tangles (NFTs), neuropil threads and neuritic plaques in AD and DS. The labelled NFTs were either of intra-neuronal type (Fig. 1A), or of extra-neuronal type (Fig. 1B). Well-preserved neurons displayed a diffuse or finely granular cytoplasmic staining of pretangle appearance (Fig. 1C). Neuritic plaques were strongly stained (Fig. 1D). NFT-bearing neurons were stained by AD199 in the brain stem of PEP (Fig. 1E). Neuronal and glial inclusions in PSP/CBD, mainly coiled bodies and rare tufted astrocytes, were also labelled (Fig. 1F). Large neurons of the motor areas, and neurons of the brain stem were positive in PSP, as well as glial processes and astrocytes in the same areas. In PiD, Pick bodies were immunoreactive in the dentate gyrus, pyramidal cell layer and temporal cortex (Fig. 1G). In DLB patient, NFTs and neuritic plaques were labelled, but Lewy bodies and Lewy neurites were negative in the cortex, the hippocampus and the brain stem samples. In FLD patients, the somas of the pyramidal cells of the Ammon's horn and granule cells of the dentate gyrus were reactive, as well as large neurons of the dorsal nucleus of the vagus nerve in brain stem samples. The labelling remained, although Western blot disclosed a dramatic decrease in all six isoforms of tau (Delacourte et al., unpublished data), as recently reported [53]. In DM 1 patients, AD199 stained the soma of cortical neurons (Fig. 1H) and the neuropil. Rare nerve fibres were also stained in the white matter.

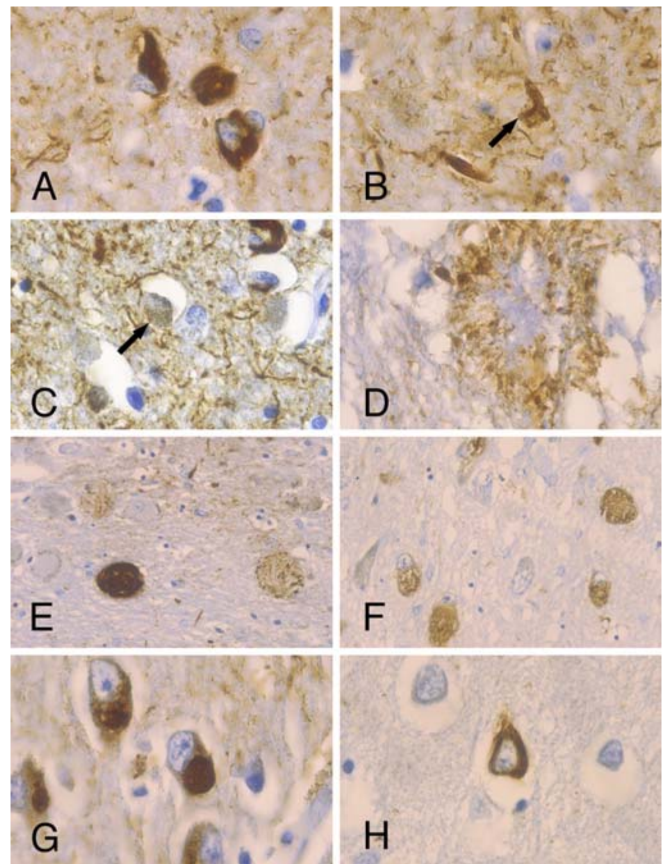

Fig. 1A–H AD199 immunolabelling of formalin-fixed, brain tissue samples. **A–C** Brodmann's area 21 in an AD patient (no. 10) showing intra-neuronal NFTs (**A**), extra neuronal NFTs (**B**, *arrow*) and pretangle stage neurons (**C**, *arrow*) in the third and fifth cortical layers. **D** Immunolabelling of neurofibrillary degeneration remains in an 18-year fixed brain tissue sample (BA 21, case 15). **E** Strong labelling of intra-neuronal NFTs in the mesencephalon of a PEP case (no. 20). **F** Intra-neuronal NFTs are also labelled in the locus coeruleus in a PSP case (no. 25). **G** Pick bodies are detected by AD199 (dentate gyrus, case no. 34). **H** NFTs are labelled in the motor cortex in a DM 1 patient (no. 26) (*AD* Alzheimer's disease, *NFT* neurofibrillary tangle, *PEP* post encephalitic parkinsonism, *PSP* progressive supranuclear palsy, *DM 1* myotonic dystrophy of type 1). Original magnification **A–D**, **G**, **H** ×1,000; **E**, **F** ×400

Sensitivity of immunohistochemistry using AD199

The pattern of AD199 staining was similar to that of Gallyas staining when brain tissue was fixed in formalin for less than 1 year. Longer fixation time before sampling resulted in a loss of immunoreactivity for all anti-tau antibodies. In contrast, AD199 immunoreactivity still remained after 11- and 18-year fixation in 4% formalin (cases 14, 15; Fig. 1D), but was no longer observed after a

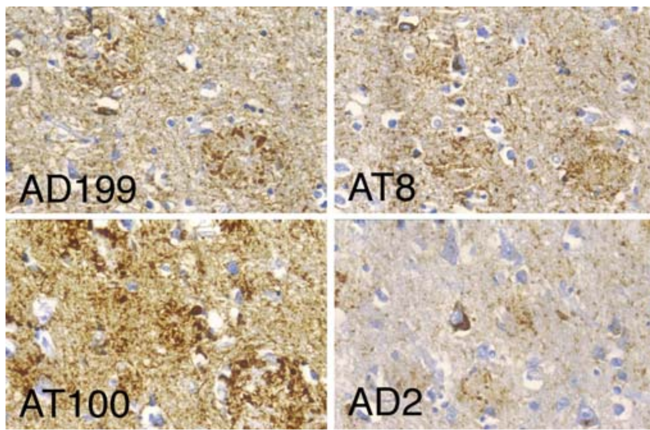

Fig. 2 Serial sections of Brodmann's area 10, cortical layer 3, in a 92-year-old AD patient (case 13). AD199, AT8 and AD2 show similarly NFTs, neuropil threads and plaques. Alzheimer tau pathology is strongly immunoreactive with AT100. Original magnification ×400

25-year fixation period (case 16). In AD, DS and PEP patients, AD199 showed a staining pattern similar to that of AT8 and AT100 (Figs. 2 and 4, first column); it was more sensitive than AD2 and tau-1 following alkaline phosphatase treatment. All patients whose the brain tissue samples were AD2 and AT8 positive were also AD199 positive.

In PSP patients, tau-positive cortical and brain stem neurons were evenly stained, whatever the anti-tau antibody used. In PiD patients, Pick bodies as well as Alzheimer-type changes (i.e. NFTs, neuropil threads and neuritic plaques) were strongly reactive for AD199, AT8 and AD2, while only Pick bodies and rare NFTs were labelled by AT100. In DM 1 patients, the somas of very few neurons were reactive for AT100 in the cortex, whereas AD199, AT8 and AD2 stained more tau-positive neurons.

AD199 pretangle-like immunoreactivity in young controls

In all young controls (cases 36–44), the hippocampal region was immunoreactive for AD199 (Fig. 3). AD199 mainly labelled the large pyramidal neurons of the entorhinal cortex, the pyramidal neurons of the CA3 and CA2 sectors of the hippocampus, the granule neurons of the dentate gyrus and, to a lesser extent, the pyramidal neurons of the CA1 sector, the subiculum and the temporal cortex. There was no staining in the frontal and parietal associative cortices. The cytoplasm of neurons was diffusely stained without tangle appearance, more or less granular, without any relationship with age. Such pre-tangle type immunostaining was absent with the antibodies AT8, AT100 and AD2.

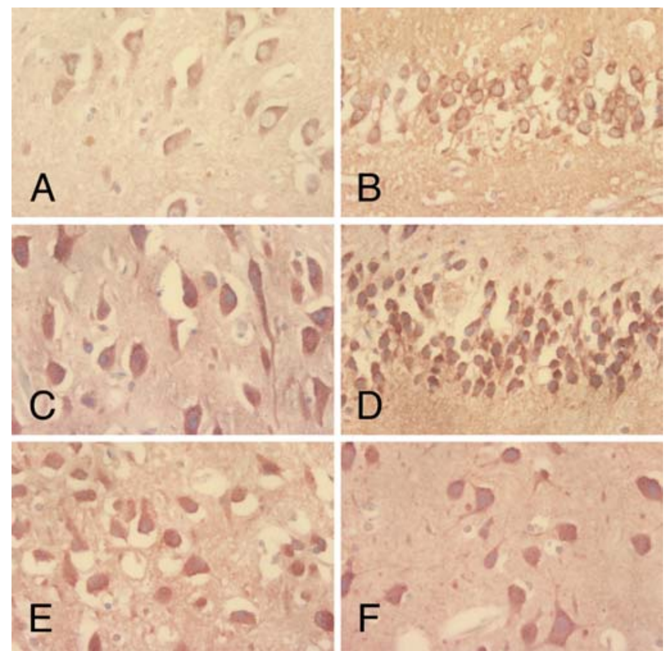

Fig. 3 AD199 immunolabelling of the hippocampus formation of young controls, showing neuronal, cytoplasmic immunoreactivity in the pyramidal neurons of the CA3 sector of the Ammon's horn (**A**) and in the granule neurons of the gyrus dentatus (**B**) in a 12-year-old boy (case 36). The same pattern of staining is demonstrated in the 12- to 41-year-old patients, particularly in the Ammon's horn pyramidal neurons, the granule neurons of the gyrus dentatus, the subiculum, the uncus and the entorhinal cortex. **C–F** An 18-year-old patient; **C** CA1 sector, **D** dentate gyrus, **E** uncus, **F** entorhinal cortex. Original magnification ×400

AD199 immunoreactivity in the oldest controls

AD199 staining was rare and weak in controls over age of 50 years (Figs. 4, 5). Of particular interest, granule neurons of the dentate gyrus were weakly and diffusely stained in the youngest patients (Fig. 5), whereas a subset of labelled NFT-bearing pyramidal cells and neuropil threads appeared in the oldest patients. In the oldest patient (no. 50, 90 years), AD199 disclosed conspicuous neurofibrillary tau pathology in the entorhinal cortex and hippocampus, without any pretangle neurons. In the same patient, rare NFTs and threads were labelled in the frontal cortex. The Western blot analysis confirmed the presence of phosphorylated S199 in the youngest patients and its decrease in the oldest patients (Fig. 6).

Phospho-dependence of the AD199 labelling

The phospho-dependence of AD199 was assessed following its purification with the phospho-peptide. The resulting purified fraction of AD199 provided a weaker staining than the neat serum. However, the labelling pattern of the brain tissue from demented patients and controls was similar to that obtained with the non-purified one (Fig. 7). The

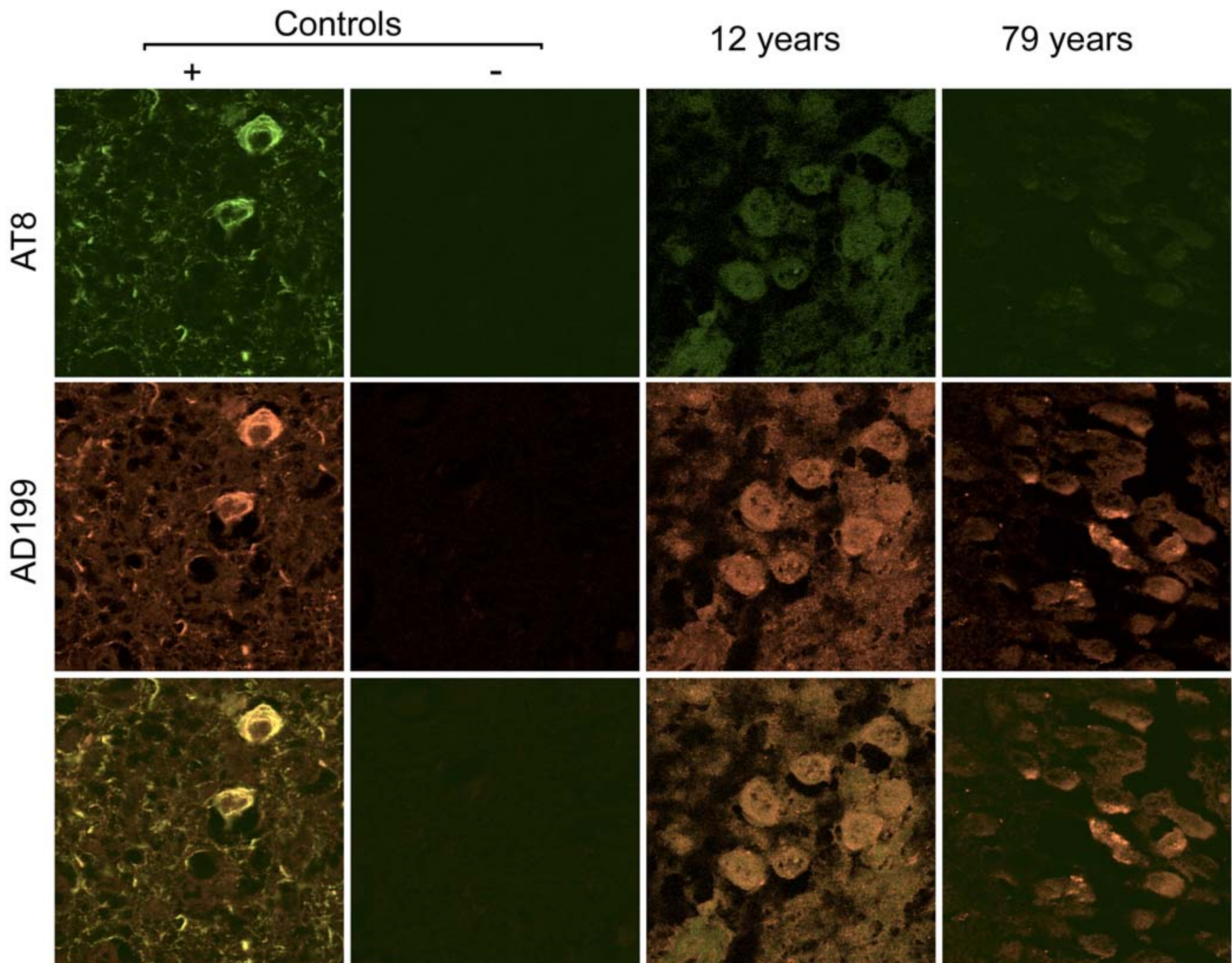

Fig. 4 Confocal imaging of AT8 immunoreactivity (*1st lane*, *green*) vs AD199 (*2nd lane*, *red*); the signals are summarised in the *third lane* (colocalisation is demonstrated by a *yellow* colouration). In the first column, showing the 3rd cortical layer of the frontal cortex in an AD patient, NFTs and neuropil threads are similarly immunoreactive for both antibodies. As a negative control, the primary antibodies were omitted when processing the same brain tissue sample (*2nd column*). The *3rd column* demonstrates the gyrus dentatus of the hippocampus of a 12-year-old boy, where AD199 immunoreactivity pattern is reminiscent of pretangle neurons and stronger than AT8 immunoreactivity. In a 79-year-old woman (*4th column*), AD199 reactivity is weaker than in the 12-year-patient. Original magnification ×1,000

pre-incubation of the sections with alkaline phosphatase abolished most of the staining.

Discussion

The early modulation of phosphorylation of tau might be a key event in normal ageing and degenerative diseases. We have shown immunoreactivity for the phospho-dependent, anti-S199P, AD199 antibody in pathological lesions of AD/DS/PEP, FLD, PSP/CBD, PiD, and DM 1 as well as in pretangle and/or normal neurons in control cases. Phosphorylation of the S199 residue may be a very early marker of tau pathology, and a marker of neuronal vulnerability.

S199P is an informative site on tau proteins

The S199 of tau belongs to a subset of phosphorylation sites supposed to highly modulate the cell sorting in neuron [12], and the tau region surrounding this epitope is critical in the pathophysiology of tauopathies. Dephosphorylation of tau proteins at several sites, including S199, displaces tau from axons to dendrites, and the combined phosphorylation of the Ser199/Ser202 residues of tau is highly indicative of Alzheimer neurofibrillary pathology, as shown by AT8. Antibodies tau1, AT8 and AT100 only provide indirect data about S199 phosphorylation status. Tau 1 epitope includes the non-phosphorylated S199 [5, 6, 22, 44]. Although AT8 is very sensitive in AD, it shows minimal cross-reactivity with “normal” tau [37], and it labels tau when phosphorylated at Ser202 and Thr205 [17, 44].

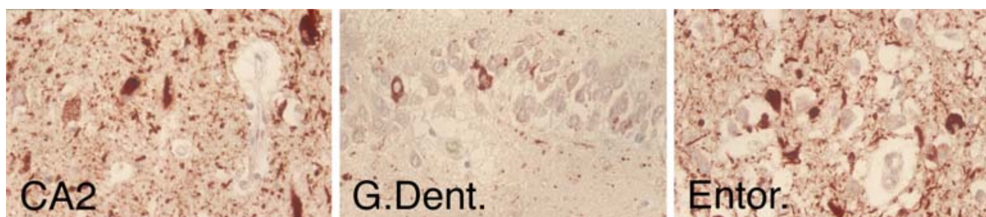

Fig. 5 AD199 staining of the hippocampus formation in the oldest controls, showing NFTs and neuropil threads in pyramidal cell layer of the Ammon's horn (CA2). Labelled neurons are rare in the granule cell layer of the gyrus dentatus when compared to young controls (*G. Dent.*). Labelling of intra- and extra-neuronal tangles in the entorhinal cortex is consistent with low stage Alzheimer pathology (*Entor.*). Original magnification ×400

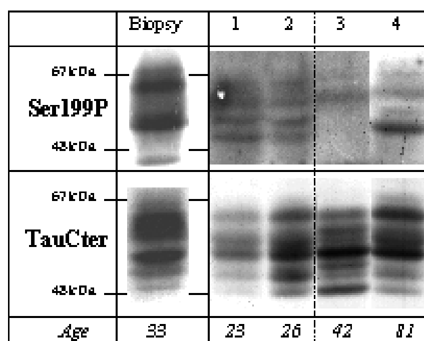

Fig. 6 Western blot detection of tau proteins in frontal brain homogenates from controls of different ages. *Upper part*: immunodetection of tau proteins phosphorylated on S199, with AD199 (numbering according to longest tau isoform). *Lower part*: immunodetection of all tau variants with anti-Tau Cter. Note the strong detection of S199P epitope in brain homogenates from a biopsy specimen (as a triplet of 60-, 64- and 69-kDa), and in autopsic samples (45 and 62 kDa after post-mortem delay) from young patients (*lanes 2*, *3*), but decreasing frequently in older controls (*lane 3*, 42 years), but not in all aged controls (*lane 4*, age 81 years) (*S199* Ser199, *S199P* phosphorylated S199)

AT100 is very specific of Alzheimer tau pathology. Although AT100 reacts with the abnormal Thr212/Ser214 epitope, it gives information on S199 because it is suggested that AT100 epitope in vitro requires a prior phosphorylation of S199 [52]. Our data show that AT100 staining is always associated with AD199 staining.

Little is known about the isolated S199 site. A polyclonal S199P that was raised against a synthetic peptide phosphorylated at S199 residue has been previously reported [29, 45], but the expression of this epitope has not been extensively investigated in different neurodegenerative diseases. Since AD199 immunoreactivity was shown in the brain tissue from all the demented patients in our series, our results suggest that antisera directed against S199P are highly informative in tauopathies.

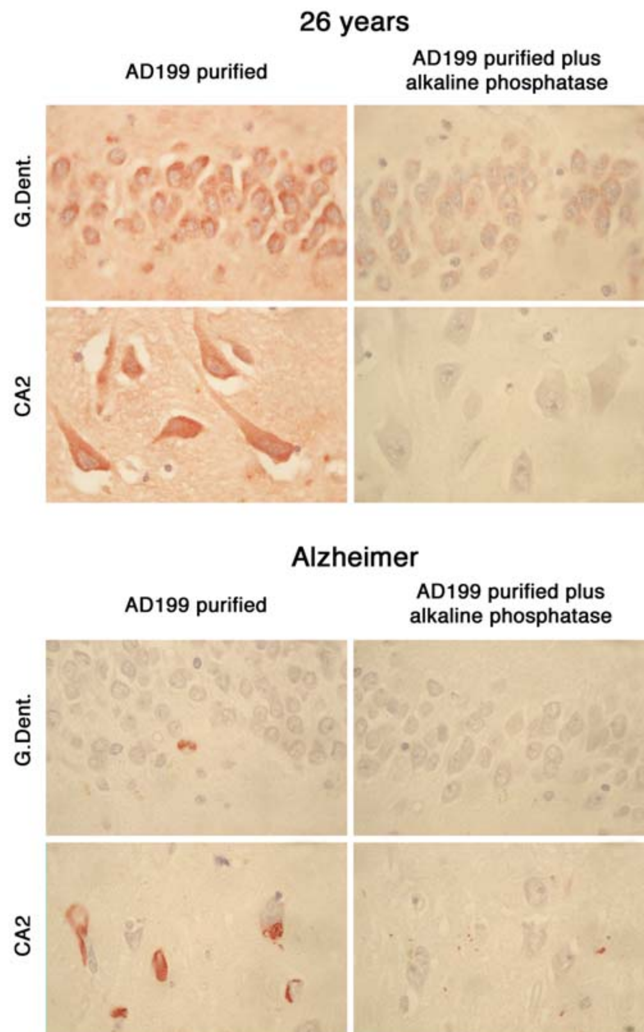

Fig. 7 AD199 staining with the chromatography affinity-purified antibody. In the *upper part*, AD199 remains in the granule neurons of the gyrus dentatus and the pyramidal neurons of the CA2 sector of the Ammon's horn in a 26-year-old patient (on the *left*). Most of the signal has disappeared after alkaline phosphatase treatment (on the *right*) showing that AD199 is a phospho-dependent antibody. In the *lower part*, only NFTs are labelled by the purified AD199, in the CA2 sector of an AD patient, most of the staining is abolished by alkaline phosphatase. Original magnification ×400

AD199 is a valuable tool for the histochemical diagnosis of tauopathies

S199P may be a major epitope of early and late aggregates in many tauopathies. In autopsy-derived formalin-fixed brain tissue samples, AD199 may be a valuable and

sensitive tool in detecting tau pathology, such as intra- and extra-neuronal tangles, neuropil threads, and Pick bodies. Four different profiles of tauopathies are distinguished by their biochemical properties on Western blot analysis, i.e. class I (mainly AD, DS and PEP), class II (PSP, CBD and argyrophilic grain dementia), class III (PiD) and class IV (DM 1); FTDP17 demonstrates tauopathy of class I, II or III type [13]. AD199 does not differentiate any of these subclasses. Previously, immunoblot analysis using AD199 have already shown the involvement of S199P in AD (class I), PSP/CBD (class II), in Pick disease (class III) [14, 15], and in an experimental model of transgenic mice bearing the P301L tau mutation (one of the mutations known to induce FTDP-17 in man, Dutch family 1-type, tauopathy class II) [21]. Here we have also confirmed S199P immunoreactivity in DM 1 patients by immunohistochemistry. Immunoreactivity was mainly restricted to NFT-bearing neurons of motor areas. It must be recorded that tau pathology has already been described in DM 1 by immunohistochemistry, and AD199 immunoreactivity as previously shown by Western blot analysis [42, 47].

AD199 stains pretangle-like neurons in FLD

In contrast to tau-linked neurodegenerative diseases, we did not show any pathological tau-positive inclusion in FLD. All six isoforms of tau protein are under-expressed in the brain of FLD patients [53]. By histochemistry, the cytoplasm of groups of hippocampus and brain stem neurons was diffusely immunoreactive, but there were no NFTs, as expected. Indeed, FLD is known to lack specific histological features such as NFTs [34].

AD199 labels pretangle-like neurons in adult controls

A major feature in control cases was that AD199 stained pretangle neurons and/or normal neurons in autopsy-derived, formalin-fixed brain tissue samples. Data are available on the 199/202 complex phosphorylated epitope of tau but there are very few data on the S199P residue. A polyclonal antibody raised against the combined phosphorylated Ser199/Ser202 has been shown to label neuropil threads in middle-aged (62–65 years) non-demented brains (pretangle stage) and NFTs in aged non-demented patient brains (66–85 years) [31]. In middle-aged brains, the labelling was stronger with anti-Ser199/Ser202 antibody than with anti-Ser231, Ser396, Ser409 and Ser422 antibodies (phosphorylation sites known to be implicated in neurofibrillary pathology in AD). The Ser199/Ser202 epitope is expressed in granulovacuolar degeneration in non-demented subjects older than 74 as well as in AD patients [28]. In the ageing human brain, although AT8 detects mainly PHF-tau, pretangle neurons are somehow immunoreactive [3]. Focusing on entorhinal/transentorhinal pre-α neurons, Braak et al. [7] have shown that AT8 immunoreactivity is demonstrable in pretangle stage. AT8 immunoreactivity has also been found in the hippocampus and entorhinal cortex of aged animals (brown lemur, Rhesus monkey, Hamadryads baboon, rabbit, guanaco, reindeer and American bison), mainly in the somas and processes of hippocampus/entorhinal neurons, in animals the behaviour of which was normal [24]. Recently, it has been proposed that the susceptibility of a neuron to neurodegeneration would parallel a sequential phosphorylation of tau residues [2]. Here, the phosphorylated Ser262 residue is suggested to be expressed early in the pre-tangle stage, whereas the S199P epitope would appear later, in the intracellular NFT stage. S199P was investigated by the mean of AT8 and AT100, and we speculate that S199P is abundantly expressed in pre-tangle stage as shown by AD199.

AD199 shows that S199 epitope of tau is abundantly phosphorylated in brain regions in young adults and children

The S199 site of human tau was initially supposed to be not phosphorylated in normal adult brain [5, 22]. Here, we show that tau proteins in biopsy-derived brain tissue samples are phosphorylated at the S199 residue and suggest that the S199P is a constitutive and normal site of phosphorylation. The S199 residue is phosphorylated in vitro by proline-directed protein kinases, including glycogen synthase kinase 3β (GSK-3β) and cdk5/cdc2 [38, 48]. Interestingly, cdk5/cdc2 and hyperphosphorylated tau colocalise in early stage NFT-bearing neurons and pretangle neurons in AD [39]. Cdk5/cdc2 promotes the expression of GSK-3β in vitro and may interfere with the phosphorylation of S199 at the pretangle stage and early stage of NFT [39]. There are high similarity in the sites of phosphorylation of PHF-tau, normal adult and foetal human tau. However, tau in adult brain have a lower number of phosphorylated residues than tau in foetus and PHF-tau [35]. Compared with the adult, the foetal central nervous system express higher levels of kinases and lower levels of phosphatases [35].

Immunoreactivity for S199P in non-diseased neurons in adulthood and childhood are supported by in vitro data [20], suggesting that tau is phosphorylated at Ser199/Ser202 (and Ser202/Thr205) before polymerisation into PHF-tau. Non-filamentous tau would therefore remain accessible to phosphatases and the S199P epitope might disappear in older adults.

It is suggested that hyperphosphorylation of tau might be due to inactivation either of phosphoseryl/phosphothreonyl protein phosphatases [18, 19] or imbalance of kinases/phosphatases activities [35]. When protein phosphatase 2A (PP2A) activity is inhibited in vitro by okadaic acid treatment, it mimics the hyperphosphorylation of tau and the 198/199/202 sites are hyperphosphorylated. As in AD, the hyperphosphorylation of S199 in youth might be mostly the result of a decrease in PP2A activity, or reduce specificity of phosphatases to this site [4].

Since the S199P epitope is exposed to phosphatases in post-mortem brain, persistence of immunoreactivity is

probably related to the abundance of phosphorylation of the S199 epitope in young cases.

A similar S199P-like immunoreactivity has already been shown in rat [30, 45, 49]. This site (Ser190 in rat) is phosphorylated in the foetus and neonate as well as in the adult rat brain. In young rat brain, the S199P epitope of tau and GSK-3β colocalize in the soma of pyramidal neurons. Expression of GSK-3β is broad before birth and reaches a climax around days 8–11 [46].

Altogether, our histochemical data on autopsy-derived brain tissue samples and Western blot analysis of biopsy-derived tissue may indicate that (1) the phosphorylation activity is increased at the specific S199, and/or (2) phosphatase activity is low at this site, and/or (3) the phosphorylated S199 is more or less resistant to phosphatases after death.

Conclusion

S199P is a conserved phosphorylated site observed in numerous neurodegenerative disorders and enables the visualisation of early stages of neurodegeneration, even when aggregates of tau are not detectable by biochemical approach. Antisera specific to S199P of tau protein show that (1) S199P is a major epitope of pathological aggregates in tauopathies and may be a highly sensitive tool for detecting the early and late tau pathology in formalin-fixed, paraffin-embedded, brain tissue samples, (2) S199P is an early epitope in the aggregates of neurofibrillary pathology, and (3) S199P is abundantly expressed in large pyramidal neurons in children and young adults, reflecting a vulnerability of the neuronal populations to neurofibrillary degeneration.

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