

Paradoxical phosphorylation of the serine 199 on tau proteins from young individuals

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The microtubule-associated tau proteins are abnormally aggregated in many tauopathies. Phosphorylation modulates the functions of tau. The serine 199 residue of tau is abnormally phosphorylated at early and late stages of Alzheimer's disease. The presence of the phosphorylated Ser199 was investigated in autopsy-derived and biopsy-derived brain tissue samples from non-demented individuals. A paradoxical expression was found in the hippocampus of the youngest ones, in granule cells of

the dentate gyrus and in pyramidal cells of the Ammon's horn, which are particularly prone to neurodegeneration in several tauopathies. The rate of positive cells decreased with age. These data emphasize the importance of the phosphorylation of the Ser199 residue of tau in ageing and susceptibility to neurodegeneration. *NeuroReport* 12:3177–3181 © 2001 Lippincott Williams & Wilkins.

Key words: Ageing; Alzheimer's disease; Pretangle; Pick's disease; Tau

INTRODUCTION

Tau proteins belong to the microtubule-associated protein (MAP) family, which promotes tubulin assembly and stabilizes the axonal cytoskeleton in developing and post-mitotic neurons [1]. In the human CNS, fibrillary aggregates of tau are formed in glial and/or neuronal pathological inclusions distinctive of the so-called tauopathies, such as Alzheimer's disease (AD) and many other neurodegenerative diseases [2]. A wide variety of tau protein variants are expressed in the human CNS. These combine six isoforms encoded by a single gene to a different status of tau phosphorylation [3]. The microtubule-binding domain includes 3 or 4 carboxy-terminal repeats, and the presence of a fourth repeat encoded by the alternatively spliced exon 10 increases the ability to promote microtubule assembly [3]. The ability to promote polymerisation of microtubules also depends on tau phosphorylation. Only six phosphorylation sites lie outside the microtubule-binding domain, including Ser199 [4]. The selective phosphorylation of the serine 199 (Ser199) residue of tau probably occurs *in vitro* in mitotic and in apoptotic neuron cells [5,6]. *In vivo*, phosphorylation of the Ser199 might occur early in pretangle neurons in AD as well as in the brains of aged individuals [7]. The aggregated tau are also hyperphosphorylated at Ser199 in many tauopathies, and in animal models of tauopathies as well [8,9].

Here we show by immunohistochemical techniques that the Ser199 is paradoxically hyperphosphorylated in the hippocampus of children and young adults, mostly in neuron subsets that are known to be vulnerable to neurofibrillary degeneration in the elderly.

MATERIALS AND METHODS

Patients: Formalin-fixed, paraffin-embedded brain tissue samples were obtained from 15 autopsy-derived cases and 14 surgical biopsies. Autopsy patients had neither neurological disease nor cognitive impairment. They ranged from 12 to 90 years old (mean age 44.8 years; eight male, seven female). Post-mortem delay at autopsy was <24 h. Conventional neuropathological procedures and immunohistochemistry (A β , ubiquitin, and α -synuclein) showed neither abnormal inclusion nor deposit.

The biopsy-derived samples were removed from patients who underwent surgical treatment of chronic epilepsy and gave their consent for the use of biopsy material. Patients ranged from 17 to 53 years old (mean age 33.8; 10 male, four female). To avoid extensive dephosphorylation of tau, all samples were immediately immersed in buffered formalin 4% in the Neurosurgery unit, and fixed for less than 24 h before embedding.

Immunohistochemistry: Immunohistochemistry was achieved with AD199, AD2, AT8, AT100 and tau-1. The polyclonal AD199 antibody is raised against a synthetic peptide that includes the phosphorylated Ser199 residue. It has been characterised previously [8,10]. Labelling with AD199 (diluted 1/500) was compared to the phospho-dependent anti-tau antibodies: AD2 (gift from Dr Mouton-Gilles, Montpellier, France; diluted 1/1000; Ser396/Ser404 epitope [11]), AT8 (Innogenetics, Gent, Belgium, diluted 1/1000; Ser202/Ser205 epitope), AT100 (Innogenetics, diluted 1/400; Ser212/Ser214), and to tau-1 (Boehringer Mannheim,

Germany, diluted 1/200, unphosphorylated Ser195/Ser198/Ser199/Ser202 epitope). Since tau-1 only labels non-phosphorylated tau epitopes, sections were incubated with or without purified alkaline phosphatase (type VIII, 400 UI/ml, Sigma, Temecula, CA) [12].

Deparaffinized sections (4 µm) were heated in citrate buffer pH 6.0 in a pressure cooker for 10 min, followed by incubation with either primary antibody or preimmune goat serum. The labelling was automatically achieved by a streptavidin-biotin complex method (Ventana Medical System, Tucson, AZ).

Labelling score: For each anti-tau antibody, the labelling intensity was scored as an average of the staining intensity in the pyramidal cell layer and the granule cell layer, taking in account the perikarya and the neuropile (0: no staining, +: weakly stained, ++: moderately stained, +++: strongly stained). To define the percentage of AD199-positive granule cells in the dentate gyrus, 400 perikarya were counted in the granule cell layer and considered as positive when conspicuously labelled.

RESULTS

Autopsy-derived brain tissue samples (Table 1): AD199 preferentially labelled the soma of the granule cells of the dentate gyrus and the pyramidal cells of Ammon's horn in the hippocampus. In the youngest patients (cases 1–9) AD199 staining was only observed in the hippocampus. The labelling pattern was cytoplasmic, diffuse and reminis-

cent of pretangle neurons seen in AD patients (Fig. 1). AD199 also labelled the pyramidal neurons of the subiculum and the uncus, as well as the pre-α neurons of the adjacent entorhinal cortex. AD199 labelled the soma of neurons and the axon hillock, whilst the neuropile was barely stained. No filamentous reinforcement was observed. AD2, AT8, and AT100 antibodies showed no immunoreactivities. A faint and diffuse tau-1 staining was present in the neuropile. Prior alkaline phosphatase treatment of the slides failed to enhance tau-1 labelling (tau-1/AP). In the oldest patients (cases 10–15), AD199 reactivity was no longer discernible in the granule cells in most cases (Fig. 2) but it was still present in the pyramidal cell layer. However, with increasing age, the pyramidal cell staining appeared of pretangle type and was intermingled with neurofibrillary tangles (NFT), as shown by AT8, AT100 and tau-1/AP.

Biopsy-derived brain tissue samples (Table 1): The granule cells of the dentate gyrus were immunoreactive with AD199. The percentage and staining decreased from childhood to adulthood (Fig. 2). The surrounding neuropile was faintly labelled, as was the soma of pyramidal neurons in the Ammon's horn. Conversely, tau-1 preferentially stained the neuropile but was weak in the soma. AD2 labelling was stronger than AD199 in the neuropile but absent in the soma, suggesting a difference between the localisation of tau phospho-epitopes.

Table 1. Comparison of Ser199P immunoreactivity to other anti-tau antibodies among autopsy and biopsy cases.

Autopsy cases	Age	Gender	Cause of death	AD199P	AD2	AT8	AT100	Tau1	Tau1+AP
1	12	F	Sjogren Larsson disease	++	0	0	0	+	0
2	14	M	Lymphoblastic acute leukaemia	+	0	0	0	+	0
3	18	F	AIDS	++	0	0	0	+	0
4	18	M	Burkitt's lymphoma	+++	0	0	0	+	0
5	19	M	Lymphoblastic acute leukaemia	++	0	0	0	+	0
6	20	M	AIDS	+	0	0	0	+	0
7	26	F	Obstetrical haemorrhage	+	0	0	0	+	0
8	31	M	AIDS	+	0	0	0	+	0
9	41	M	Coronary thrombosis	++	0	0	0	+	0
10	58	M	Control	+/-	0	0	0	+	0
11	77	M	Control	NFT	NFT	NFT	NFT	NFT	NFT
12	79	F	Control	NFT	NFT	NFT	NFT	NFT	NFT
13	83	F	Control	NFT	NFT	NFT	NFT	NFT	NFT
14	87	F	Control	NFT	NFT	NFT	NFT	NFT	NFT
15	90	F	Control	NFT	NFT	NFT	NFT	NFT	NFT
Biopsy cases									
16	17	M	Chronic epilepsy	+++	+	+/-	0	++	++
17	26	M	Chronic epilepsy	++	+	0	0	++	++
18	26	M	Chronic epilepsy	++	+	0	0	++	++
19	27	M	Chronic epilepsy	+	+	0	0	++	++
20	29	F	Chronic epilepsy	+	+	0	0	++	++
21	29	M	Chronic epilepsy	+/-	+	0	0	++	++
22	30	M	Chronic epilepsy	+	+	0	0	++	++
23	30	F	Chronic epilepsy	+	+	0	0	++	++
24	32	F	Chronic epilepsy	+	+	0	0	++	++
25	35	F	Chronic epilepsy	+/-	+	0	0	++	++
26	43	M	Chronic epilepsy	+	+	0	0	++	++
27	47	M	Chronic epilepsy	+/-	+	+/-	0	++	++
28	49	M	Chronic epilepsy	+/-	+	0	0	++	++
29	53	M	Chronic epilepsy	+	+	+/-	0	++	++

NFT, neurofibrillary tangles.

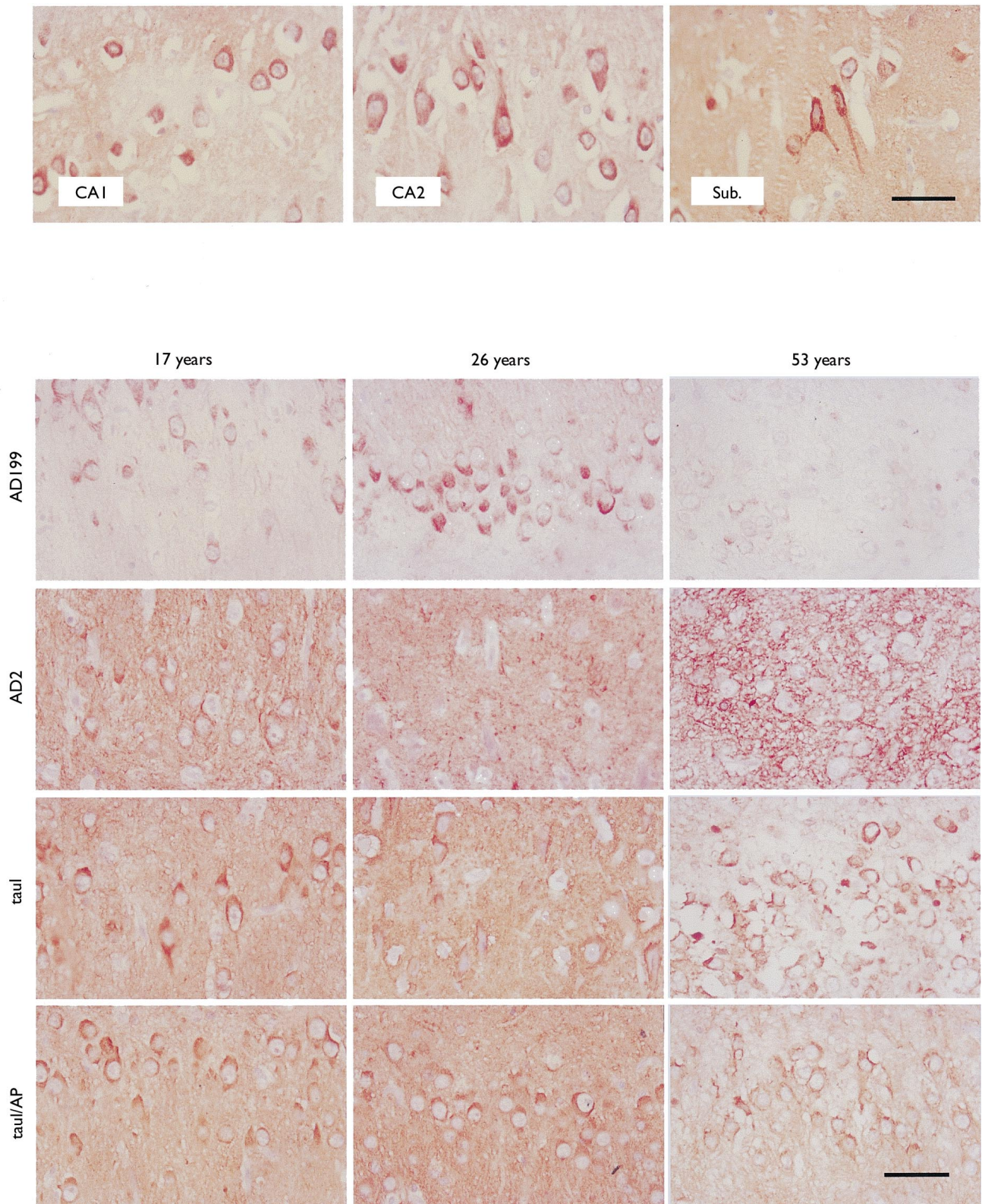


Fig. 1. Upper part: AD199 immunoreactivity in the Ammon's horn (CA1 and CA2) and in the subiculum (Sub) of a 12-year-old boy is strong, mainly localized in the soma and the axon hillock of the neurons. The phosphodependent antibodies AD2, AT8 and AT100 are negative (not shown). Bar = 50 μ m. Lower part: tau immunoreactivity in the granule cell layer of the biopsy-derived hippocampus samples in three patients, aged 17 (left vertical lane), 26 (middle) and 53 (right). AD199 (1st horizontal lane) is faintest in the oldest patient, whereas AD2 immunoreactivity (2nd lane) is strongest. Tau-1 labelling is similar (3rd and 4th lanes) from one case to another. Bar = 50 μ m.

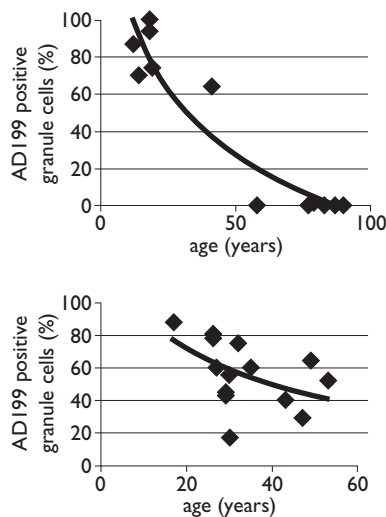


Fig. 2. Percentage of AD199-positive granule cells in the dentate gyrus of autopsy cases (upper part) and biopsy cases (lower part) showing a similar decrease in Ser199P-tau immunoreactivity.

DISCUSSION

Vulnerable neurons of tauopathies are preferentially labelled in children and young patients: AD199 showed immunoreactivity for the phosphorylated Ser199 epitope in pyramidal neurons of the hippocampus and of the entorhinal cortex in almost all patients. The granule cell layer of the dentate gyrus of the youngest patients was also labelled. These subsets of neurons are prone to neurodegeneration in tauopathies. The pyramidal neurons of the rhinal cortex and of the Ammon's horn undergo neurofibrillary changes early in AD [13]. In Pick's disease, Pick bodies concentrate in the granule cell layer of the dentate gyrus and in CA1 neurons [14]. Only 3 carboxy-terminal repeat (3R) isoforms are expressed in the granule cells of the dentate gyrus, because of the exclusion of exon 10 [15], and tau pathology in Pick's disease is mainly composed of 3R tau isoforms [10]. The Ser199 residue is located outside of the alternatively spliced sequences, and exon 10-positive tauopathies (progressive supranuclear palsy/corticobasal degeneration) as well as exon 10-negative tauopathy (Pick's disease) are immunoreactive with AD199 [10].

The staining pattern of the AD199-positive neurons is somatic and reminiscent of pretangle neurons: AD199 immunoreactivity in young patients was diffuse and/or finely granular. AD199-positive neurons were Gallyas negative, as described in pretangle neurons [16]. This is similar to the staining that was formerly described in non-demented controls with AT8, which includes Ser199 [17]. Few aberrant phosphorylation sites have been reported, i.e. serine 199, 202, 409 and 422, that are thought to occur in pretangle stage of AD [7]. Among phospho-dependent anti-tau antibodies, phosphorylated tau were only detected with AD199 in young adult cases. Similarly to AD199, tau-1 labelling was observed in the soma of the hippocampal neurons, but the staining intensity did not decrease with age, consistent with the presence of tau-1 immunoreactivity in post-mitotic neurons previously reported [18]. AT8 and

AT100 were negative in biopsy patients but labelled NFTs in the oldest autopsy cases. AT100 recognises a pathological epitope on tau and would not label pretangle neurons [19]. AT100 probably detected NFTs but pretangle neurons in infra-clinical AD patients.

AD199 immunoreactivity in the normal hippocampus might reflect mitotic mechanisms: Neurogenesis and gliogenesis have been demonstrated in the dentate gyrus and in Ammon's horn in the adult brain [20]. Microtubules support neurite outgrowth in interphasic neurons, and chromosome segregation during mitosis. In tauopathies, the hyperphosphorylation of tau may be an inappropriate mitotic answer of a neuron to an unknown signal that would lead to cell death [21,22]. In AD, tau phosphorylation is catalysed by cell cycle-associated, proline-directed kinases mitogen-activated protein kinase (MAPK), glycogen synthase kinase-3 β (GSK-3 β), cyclin-dependent kinase 5 (Cdk5), all of which being expressed in neurons. Hence our data suggest that one or more protein kinases are activated in subsets of neurons.

The equilibrium between phosphatases and kinases that modulates the AD199 immunoreactivity may be modified from childhood to elderly: On the one hand, kinases might be more active on the Ser199 residue of tau in children, on the other hand, phosphatase activity may increase in adulthood. Conclusive data support the hypothesis that the Ser199 site of tau would not be phosphorylated in normal adult brain [23,24]. In contrast, our study demonstrates that Ser199 is phosphorylated and suggests that the protein kinases GSK-3 β and Cdk5/cdc2 are active. Conversely, the preferential AD199 immunoreactivity in youth might also be the result of inactivation of phosphoserine/phosphothreonyl protein phosphatases and an imbalance of kinases/phosphatases activities [25]. According to several studies, the hyperphosphorylation of tau in AD would be mostly the result of a decrease in phosphatase activity [26], since the phosphorylation sites are almost the same in the fetus, in which the phosphatases are inactive, in biopsy-derived normal tau and in Alzheimer aggregated tau [25,27].

CONCLUSION

Our results demonstrate that phosphorylation of Ser199 is a major and specific event in the physiology of tau proteins. Indeed, we found that this phosphorylated epitope specifically and preferentially occurs in a few subsets of normal neurons, the pyramidal cells and the granule cells of the hippocampus, in young individuals. The phosphorylation of Ser199 decreases in ageing, almost totally in granule cells. In contrast, in pyramidal cells from aged normal controls, Ser199P is observed progressively in pretangle structures. Together, our results suggest that phosphorylation of Ser199 is a critical event for the imbalance of the most vulnerable neurons towards neurofibrillary degeneration in ageing and AD.

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