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Levels of normal and abnormally phosphorylated tau in different cellular and regional compartments of Alzheimer disease and control brains

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Abstract

Microtubule associated protein tau is abnormally phosphorylated in Alzheimer disease (AD) brain. In the present study we investigated (i) whether tau is axonal or both axonal and somatodendritic, (ii) whether tau is a marker of Alzheimer neurofibrillary pathology, and (iii) whether the levels of tau in the cytosol (100,000 × *g* supernate) from AD brain are altered. Frozen autopsied tissue from 20 AD, 17 normal aged and 15 neurological control cases obtained 3–8 h postmortem were analyzed. Levels of normal, total, and abnormally phosphorylated tau were determined by a radioimmunoslot-blot assay using mAb Tau-1 as the primary antibody. Both frontal gray matter homogenate and cytosol from normal brains had 30–45% higher levels of normal tau than the corresponding fractions from the white matter. In AD frontal and temporal cortices, the total tau levels were 6- to 7-fold higher than in cerebellar cortex ($P < 0.01$ and $P < 0.02$). Furthermore, tau levels of cerebellar cortex, an area of the brain unaffected with Alzheimer neurofibrillary changes, were indistinguishable between AD and control groups. The levels of normal tau in cytosol from both frontal gray and white matters in AD were reduced by approximately 40% ($P < 0.05$). The levels of total tau in AD frontal and temporal cortex were 4- to 5-fold higher than in the corresponding tissue from control cases ($P < 0.01$) and this increase was in the form of abnormally phosphorylated tau. These studies suggest (i) that there is probably at least as much tau in the somatodendritic compartment as in the axonal compartment, (ii) that the abnormally phosphorylated tau is a biochemical marker of the neurofibrillary pathology in AD, and (iii) that the levels of normal tau are significantly reduced in the 100,000 × *g* brain supernate from AD cases.

Key words: Alzheimer disease; Microtubule associated protein tau; Abnormally phosphorylated tau; Neurofibrillary degeneration; Radioimmunoslot-blot assay

1. Introduction

Tau is one of the major microtubule associated proteins of a normal mature neuron in the brain. In human brain six isoforms of tau have been identified and these isoforms are the products of alternate splicing of a single tau gene [1]. Tau promotes the microtubule assembly from the tubulin subunits and stabilizes the formed microtubules [2,3]. Tau is a phosphoprotein and the degree of phosphorylation determines its microtubule polymerizing activity. The in vitro phosphorylated tau does not promote the microtubule assembly [4].

Normal tau contains 2–3 mol of phosphate per mol of the protein. In Alzheimer disease (AD) brain tau is abnormally hyperphosphorylated [5–8]. The abnormal tau from AD brain, which contains 5–9 mol of phosphates per mol of the protein [8], does not promote the microtubule assembly [6,9,10]. One of the histopathological hallmarks of Alzheimer disease (AD) is the presence of paired helical filaments (PHF) in selected neurons in the neocortex, especially the hippocampus, and the degree of these neurofibrillary changes correlates with the severity of dementia in the afflicted patients [11,12]. Accumulations of the PHF in the affected neurons are seen in the cell body as neurofibrillary tangles, in dystrophic neurites in the neuropil as neuropil threads [13], and in dystrophic neurites surrounding a core or wisp of extracellular β-amyloid in neuritic (senile) plaques. The microtubule associated protein tau in abnormally hyperphosphorylated form is the major protein subunit of PHF [5,7,14,15]. In addition to PHF, there is a significant pool of soluble abnormally phosphorylated tau in AD brain [8]. We have previously reported that the levels of total tau in frontal cortex homogenates were several-fold higher in AD than in control cases and this increase was in the form of the abnormally phosphorylated tau [16].

The present study was undertaken to examine: (i) whether tau is only axonal or also somatodendritic in distribution; (ii) whether the abnormally phosphorylated tau is a biochemical marker of neurofibrillary pathology in AD brain; and (iii) whether the levels of tau are altered in AD brain cytosol.

2. Materials and methods

Monoclonal antibody Tau-1 [17] was generously supplied by Dr. L.I. Binder, Molecular Geriatrics, Lake Bluff, IL, USA. Tau-1 is a phosphate-dependent antibody to a non-phosphorylated epitope of tau involving Ser^{199}/Ser^{202} (of human tau_{441}). The abnormally phosphorylated tau from AD brain which is phosphorylated at Ser^{199} and/or Ser^{202} is recognized by Tau-1 only after these sites are dephosphorylated; alka-

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Abbreviations: AD, Alzheimer disease; PHF, paired helical filaments; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline; BSA, bovine serum albumin.

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line phosphatase is one of the phosphatases that can dephosphorylate the abnormal tau at the Tau-1 site [5]. ^{125}I-labeled antibody to mouse IgG was purchased from Amersham (Arlington Heights, IL, USA). Alkaline phosphatase was obtained from Boehringer Mannheim (Indianapolis, IN, USA).

2.1. Tissue used, preparation of brain homogenates and cytosols, and protein assays

AD and control brains obtained between 2 and 8 h postmortem and stored frozen at −75°C were used for this study. The AD cases were histopathologically confirmed, and the controls were age-matched. Frontal cortex from fourteen AD, eleven neurological controls, and nine normal controls, and temporal cortex from ten cases each of AD and neurological controls, and seven normal controls cases were assayed for the levels of normal and abnormally phosphorylated tau. Cerebellar cortex from five AD and four cases each from normal and neurological control groups were also analyzed for the tau levels in the homogenates. Approximately 1–2 g was chiselled off from the frozen tissue, thawed and cleaned off the meninges. Gray matter was separated from the white matter at 4°C, and each tissue was homogenized (20% w/v homogenate) by 20 strokes in a glass-Teflon homogenizer at 4°C in 10 mM Tris, pH 7.2, containing 1 mM each of EDTA and EGTA and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). An aliquot was saved and the rest was centrifuged at 100,000 × *g* using Beckman TLA-45 rotor for 10 min at 4°C. Homogenates and supernatants were kept frozen at -75°C in aliquots until used. The protein concentrations of the tissue fractions were determined by the method of Lowry et al. [18] as modified by Bensadoun and Weinstein [19].

2.2. Radioimmunoslot-blot assay for normal and abnormally phosphorylated tau

Levels of normal and abnormally phosphorylated tau were determined by a radioimmunoslot blot assay described previously [16]. Briefly, 1 μg total protein/250 μl of 50 mM Tris, pH 7.6, and 200 mM NaCl (TBS) and a cocktail of protease inhibitors (0.15 μM aprotonin, 1 μM leupeptin, and 1 μM pepstatin A) and phosphatase inhibitors (1 mM Na_3VO_4 and 5 mM NaF) was applied in two sets of triplicates to a nitrocellulose membrane containing seventy-two slots and adsorbed by employing a moderate vacuum. After blocking with 5% bovine serum albumin (BSA)-TBS, the membrane was cut into two sets, one was treated at 37°C for 18 h with alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN, USA) in 0.1 M Tris pH 8.0, containing the protease inhibitors aprotonin (0.3 μM), leupeptin (10 μM), pepstatin A (1.43 μM) and phenylmethylsulfonyl fluoride (1 μM), while the other set was incubated in the same cocktail without alkaline phosphatase. These blots were then blocked with 5% BSA in phosphate-buffered saline, incubated with 1:25,000 diluted anti-tau mAb, Tau-1 [17] for 16 h at room temperature, washed to remove excess antibody and incubated with 15 ml of 1% BSA, 0.05% Tween-20 in TBS containing ^{125}I-labeled antibody to mouse IgG (Amersham, Arlington Heights, IL, USA) at 0.05 μg/ml (13–20 μCi/μg), for 2 h at room temperature. After washing with PBS-Tween-20, the slots were cut from the membrane and counted with 3 ml of Gammascint (National Diagnostics, Somerville, NJ, USA) per strip.

Fresh bovine brains were used to isolate tau [14] which was used as the standard in concentrations between 0 and 10 ng/μg BSA. For homogenates 0.35 μg and 1 μg and for cytosolic fractions 1 μg protein per slot were applied.

3. Results

3.1. Distribution of normal tau in frontal white and gray matter

The normal tau levels in both frontal and temporal gray matter homogenates were highest in AD and lowest in the neurological controls (Fig. 1, F/GH and T/GH). The differences in the tau levels between the AD and the neurological controls were statistically significant ($P < 0.05$), whereas those between AD and the normal aged groups were insignificant. In frontal white matter similar but insignificant differences between the three groups were observed (Fig. 1, F/WH). In cerebellar cortex homogenate there were no significant differences in the levels of normal tau among the three groups (Fig. 1, Cb/GH). A comparison of the tau levels in each group between the gray and the white matter homogenates, representing somatodendritic and axonal compartments, respectively, revealed no significant differences in the number of cases studied, although all the tau values were 20–60% higher in the gray matter than the white matter (Fig. 1, compare F/GH with F/WH).

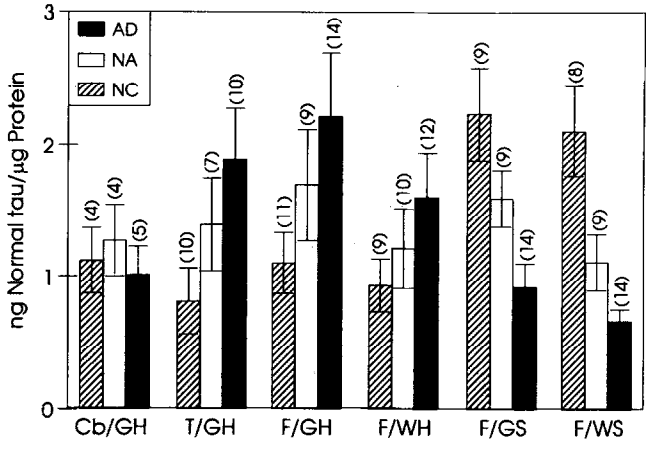

Fig. 1. Normal tau levels in brain white and gray matters. Tau levels were determined in homogenates of gray and white matter and the 100,000 × *g* supernate fractions from AD and control brains by radioimmunoslot blot assay using the monoclonal antibody Tau-1 as the primary antibody as described in section 2. The bar diagram shows mean ± S.E.M. of each group and the number of cases analyzed are in parenthesis. AD: Alzheimer disease; NA: Normal aged controls; NC: Neurological controls; F, Frontal; T, Temporal; Cb, Cerebellum; G, Gray matter; W, White matter; H, Homogenate; and S, 100,000 × *g* Supernate.

Unlike tau levels in the whole tissue homogenate, the cytosolic normal tau levels in frontal gray matter were approximately 40% lower in AD as compared to normal ($P < 0.05$) and neurological controls ($P < 0.01$). Similarly, the normal tau levels in white matter cytosol from AD cases were also found to be approximately 40% lower than the identically treated normal ($P \sim 0.05$) and the neurological control cases ($P < 0.001$) . The differences in tau levels between gray and white matter cytosols were found to be insignificant in all the three groups of cases.

3.2. Distribution of abnormally phosphorylated tau in affected and unaffected regions of AD brain

Histopathologically neurofibrillary degeneration is extensive in the neocortex whereas the cerebellar cortex is practically uninvolved in AD brain. In order to learn the association of the abnormal phosphorylation of tau with the neurofibrillary changes, the levels of abnormally

phosphorylated tau and as an internal control of normal tau were determined in frontal and temporal cortices representing the affected areas and the cerebellar cortex as the unaffected area of the AD brain. As a control, the levels of the normal and the abnormally phosphorylated tau were also determined in neurological and normal aged control brains. The normal tau levels in the frontal and temporal gray matter homogenates were similar to that of the cerebellar cortex in the two control groups, whereas in AD cases the levels of normal tau in frontal and temporal cortices were significantly greater ($P < 0.05$) than the cerebellar cortex (Fig. 1, compare F/GH and T/GH with Cb/GH). The abnormally phosphorylated tau levels both in AD temporal and frontal cortex were several-fold higher than in cerebellar cortex (Fig. 2; T/GH vs. Cb/GH: $P < 0.05$; F/GH vs. Cb/GH: $P < 0.01$); the cerebellar cortex which is unaffected with neurofibrillary pathology in AD had undetectable levels of the abnormal tau (Fig. 2; Cb/GH). Furthermore, the levels of total tau in the frontal and temporal cortex homogenates were several-fold higher in AD than the control cases and this increase was largely in the form of the abnormally phosphorylated tau (see Figs. 1 and 2). These data suggested that the levels of abnormally phosphorylated tau are associated to the histopathology of the disease.

A comparison of abnormally phosphorylated tau levels between the frontal gray matter and the white matter supernates demonstrated consistently higher average values in the white matter in all the groups (Fig. 3). The abnormally phosphorylated tau levels were 0.07–0.17 ng/μg cytosolic protein in gray matter and 0.11–0.49 ng/μg protein in white matter cytosol. However, the differences were not statistically significant in the number of cases studied. Similarly among the three groups, apparently higher values of abnormally phosphorylated tau were observed in AD cases, though without any significant difference from the control groups.

4. Discussion

To understand the role of tau in Alzheimer neurofibrillary degeneration, it is critical to learn the cellular distribution of this protein quantitatively in normal brain and in affected and non-affected areas of AD brain. Based on immunocytochemical studies tau was previously reported to be mainly an axonal protein [17] and was suggested to be misdirected from the axonal to the somatodendritic compartment in neurons with neurofibrillary pathology in AD [20]. Employing a quantitative immunoassay the present study demonstrates (i) that there is at least as much tau in the somatodendritic compartment as in the axonal compartment in normal human brain; (ii) that abnormally phosphorylated tau is a biochemical marker of Alzheimer neurofibrillary pathology and the neurofibrillary changes are several-fold greater in the somatodendritic compartment than the axonal compartment; and (iii) that in 100,000 × g supernates of AD neocortex there is an approximately 40% decrease in the levels of normal tau.

Immunocytochemistry of normal tau is problematic because it is very sensitive to fixatives and the conditions of fixation (see [17,21]). This problem has lead to earlier studies reporting that tau is axonal [17,20,22]. Subsequent immunocytochemical and in situ hybridization studies also showed the presence of tau in the somatodendritic compartment of normal mature neurons [23,24]. Since the axonal volume in grey matter is a very small fraction of that in the white matter, and the white matter contains very few neuronal cell bodies, the grey matter and the white matter are representative of the somatodendritic and the axonal pools, respectively. In the present study, employing a quantitative radio-immunoassay we have found that there is 30–45% more tau in normal brain gray matter than in the white matter, although because of the biological scatter the differences

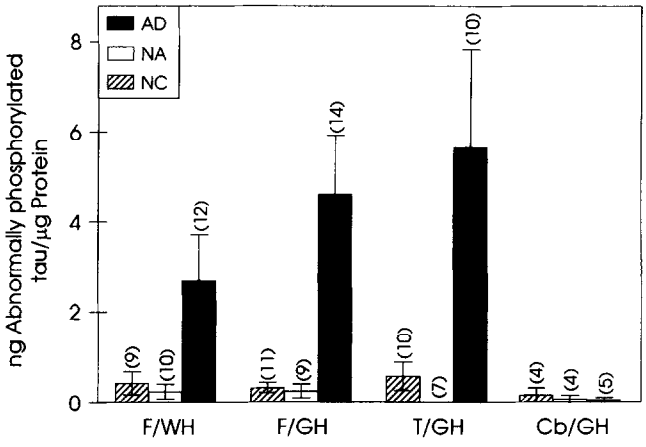

Fig. 2. Levels of abnormally phosphorylated tau in AD and control brains. As described in section 2 the abnormally phosphorylated tau levels were determined as a difference between total tau and normal tau levels. The total tau and normal tau levels were determined by Tau-1 antibody by radioimmunoslot-blot assay with and without pretreatment of the blot with alkaline phosphatase respectively. Rest of the details are the same as in Fig. 1.

Table 1
Summary of cases used

Diagnosis	Age (years) mean ± S.E.M.	Sex M/F	PMI (hours) mean ± S.E.M.
AD (20)[a]	73 ± 3	8/12	4.2 ± 0.3
NA (17)	72 ± 6	9/8	5.7 ± 1.1
NC[b] (15)	81 ± 3	11/4	3.8 ± 0.3

AD: Alzheimer disease; NA: normal aged controls; NC: neurological controls; PMI: postmortem interval. [a]Number of cases. [b]5 cases of Parkinson's disease, 2 cases of Lewy body disease, 3 cases of Multi-infarct dementia, 1 case of Huntington disease and 4 cases of unknown diagnosis.

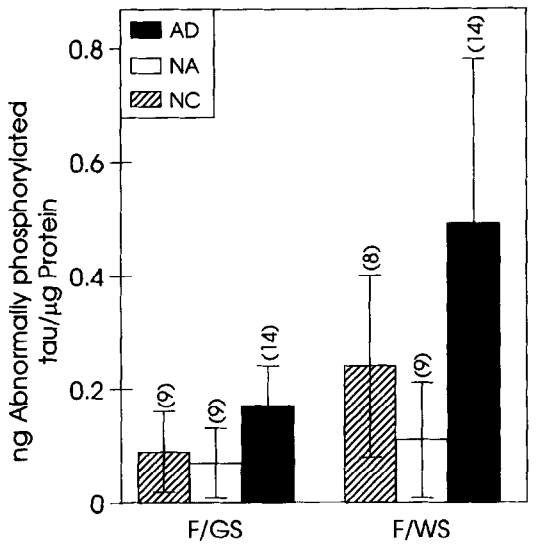

Fig. 3. Levels of abnormally phosphorylated tau in frontal gray and white cytosols from AD and control cases. Abnormally phosphorylated tau levels were determined in 100,000 × *g* supernatants of frontal gray and white matter homogenates from AD, and control groups. Rest of the details are the same as in Figs. 1 and 2.

in the number of brains examined were not statistically different. The statistically insignificant increase in normal tau in gray matter over white matter was observed both in tissue homogenates as well as in the cytosols. These findings suggest that there is at least as much tau in the somatodendritic compartment as in the axonal compartment. Some of the tau measured in total tissue homogenate can also be of glial origin, since tau has been observed immunocytochemically in oligodendrocytes and astroglia [23,25]. However, given the larger proportions of gila in white matter than gray matter, it is unlikely that there is a significantly greater contribution of glial tau in gray matter than white matter.

The levels of abnormally phosphorylated tau corresponded to the known topography of neurofibrillary pathology in AD. In AD brains the levels of abnormally phosphorylated tau were several-fold greater in frontal and temporal cortices than the cerebellar cortex; cerebellar cortex is known to be minimally affected with neurofibrillary changes in AD [26]. Most of the abnormally phosphorylated tau in AD brain was sedimentable. Less than 1% of the abnormal tau in homogenate from AD frontal cortex was recovered in the cytosol (taking cytosol as 20% of total tissue protein) from the same. In contrast, the levels of the abnormal tau in cytosol from frontal white matter were several-fold greater than in the corresponding gray matter and approximately 4% (taking cytosol as 20% of total tissue protein) of the white matter abnormal tau was not sedimentable. Taken together, these studies suggest (i) that the abnormally phosphorylated tau is a biochemical marker of Alzheimer neurofibrillary pathology and (ii) that in AD the abnormal tau in the cerebral white matter might be different from that in the corresponding gray matter. The nature of these differences is, at present, not known. It is possible that the white matter abnormal tau is less hyperphosphorylated than that in the gray matter.

The levels of normal tau in the frontal gray matter and white matter homogenates from AD were approximately two-fold greater than the neurological controls and only slightly higher than the normal aged cases. The levels of normal tau in cerebellar cortex from AD and both normal and neurological control groups were similar. These studies suggest that there is no decrease in the levels of normal tau in AD brains, and that there might be a slight increase in the areas of AD brain affected with neurofibrillary pathology.

Unlike brain homogenate, the cytosol (i.e. 100,000 × *g* supernate) prepared from the same had approximately 40% lesser normal tau in AD than normal controls in both frontal gray matter and white matter. This pattern of decrease in normal tau in the cytosol was specific to AD because in the neurological control group the pattern was opposite. A recent study has shown that in AD brain, the normal tau binds to the abnormally phosphorylated tau co-sedimenting with the latter [10]. The decrease in the normal tau in cytosol from AD brain observed in the present study, is probably due to the binding of normal tau to the soluble abnormally phosphorylated tau and sedimenting at 100,000 × *g* during the isolation of the cytosol.

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