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Modified tau is present in younger nondemented persons: a study of subcortical nuclei in Alzheimer's disease and progressive supranuclear palsy*

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Summary. Tau-positive neurons relating to neurofibrillary tangles and diffuse cytoplasmic stainings were quantitatively examined in the brains of 61 nondemented persons including 24 age-matched controls, 10 patients with Alzheimer's disease (AD) and 5 with progressive supranuclear palsy (PSP). In nondemented persons, the locus ceruleus (LC) was found to contain tau-positive neurons initially in persons in their 30s, whereas the hippocampus contained such neurons initially in persons in their 40s. The LC had a higher incidence and density than the hippocampus in almost all age classes. As neuronal tau accumulation is considered a histological change occurring with normal aging, the LC might be involved in the earliest aging in the normal brain. In AD there was conspicuous tau accumulation in the same sites which were vulnerable to tau accumulation in the age-matched controls. In PSP tau accumulated heavily in a set of sites different from the age-matched controls and AD. Thus, subcortical tau accumulation in AD is increased far more than that under normal aging process, while that in PSP is not simply in an increased state of the normal aging process.

Key words: Modified tau – Subcortical nuclei – Alzheimer's disease – Progressive supranuclear palsy – Normal brain

In Alzheimer's disease (AD) and several other diseases such as Guam parkinsonism-dementia complex, postencephalitic parkinsonism and Down's syndrome, one of the most prominent morphological changes is the presence of numerous neurofibrillary tangles (NFTs) in the cerebral cortex. These tangles are also found to a smaller degree in nondemented aged humans. There have been many studies reporting these histological changes observed in the cerebral cortex of patients with AD and of normal, elderly nondemented subjects [1, 7, 8, 27, 40, 41, 45].

NFTs are also one of the pathological hallmarks of progressive supranuclear palsy (PSP). NFTs in PSP, however, differ from those in AD by their topographical distribution; subcortical nuclei are most severely involved by the formation of NFTs, whereas the cerebral cortex appears unaffected [16, 17]. There have been several studies [9, 12, 15] on the occurrence of NFTs in the subcortical nuclei of normal nondemented subjects to compare them with PSP patients.

Recent advances in immunological approaches to AD NFTs have revealed that an abnormally phosphorylated form of microtubule-associated protein (MAP) tau, i.e., modified tau, was a major antigenic constituent of NFTs in AD [10, 14, 22, 46, 47]. In addition, NFTs in PSP were shown to share antigenic determinants with those in AD: tau antigenicity is prominent in both types of tangles [2, 35]. NFTs in AD were also reported to share antigenic determinants with other cytoskeletal proteins such as high- and middle-molecular weight neurofilaments (NFH and NFM) [23, 30, 49] and MAP2 [21, 48] and non-cytoskeletal protein such as ubiquitin [29]. On the contrary, NFTs in PSP have been variously reported to have either positive or negative reactivity to NFH and NFM antibodies [35, 36] and to ubiquitin antibodies [24–26], but reactions to antibodies to the epitopes in MAP2 have not yet been well described.

The purpose of the present study is to evaluate the prevalence and severity of tangle formation in the subcortical nuclei and the hippocampus of normal nondemented persons, and to compare the results with AD and PSP patients. For this, we evaluated tau immunoreactivity as a major antigenic constituent of NFTs. Modified tau accumulates not only in the form of tangles but also as a cytoplasmic diffuse immunostaining of some neurons lacking NFT. These diffuse cytoplasmic stainings are reported to lead to their accumulation in

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the form of NFTs [3, 18, 33]. Thus, we examined and evaluated NFT-bearing neurons and diffuse tau stainings, and supplemented them with silver impregnation using a modified method of Gallyas [5] and of Bielschowsky [13].

Materials and methods

Materials

For nondemented persons, we investigated the brains of 61 subjects (ranging in age from 23 to 91 years) with no evidence of dementia as judged from retrospective examinations of the detailed clinical records available and also with no evidence of clinicopathologically proven neurological diseases, severe head trauma, systemic diseases affecting the central nervous system or respirator brain. These 61 brains were examined for the presence of beta-amyloid deposits (senile plaques and amyloid angiopathy) with beta-protein immunolabeling coupled with formic acid pretreatment [20]. Amyloid deposits were not found in the brains from persons in their 20s to 40s, but were identified in normal limits of distribution and density in the brains from persons over 50 years of age, as described [31]. We also examined 10 patients with AD (ranging from 52 to 67 years, mean 61.1 ± 5.9) and 5 patients with PSP (54 to 74, mean 66.2 ± 7.9), and compared them with 24 age-matched controls (50 to 79 years, mean 64.6 ± 8.4) selected from the 61 nondemented persons. The AD cases fulfilled the established criteria for the diagnosis of the disease [19]. The diagnosis of PSP was confirmed by a characteristic clinical history and histological examination [39]. The interval between death and autopsy was less than 24 h. Brain tissues from most cases were fixed in 10% buffered formalin for 2 to 3 weeks, and those from the remaining ones for 1 month to 3 years. The variation in fixation time had no appreciable effect on the pattern of tau immunostaining. Blocks from the hippocampus, basal ganglia, midbrain and pons were embedded in paraffin, and cut into 5-μm-thick sections. Five serial sections prepared from each block were stained with hematoxylin-eosin, Klüver-Barrera, modified Bielschowsky and Gallyas impregnation, and tau immunohistochemistry, respectively. We examined the following nine sites: the hippocampus (CA1 and subiculum), globus pallidus, putamen, subthalamic nucleus, nucleus basalis of Meynert, substantia nigra, nucleus raphe dorsalis, locus ceruleus and pontine nucleus.

Immunostaining

Affinity-purified antibody against the modified tau protein integrated into paired helical filaments (PHFs), anti-tau, used in this work has been characterized and established as described [38]. On immunoblots of total homogenate from rat brain, the anti-tau recognized only tau polypeptide bands. The anti-tau was further confirmed not to cross-react with NFH and NFM, MAP2 or ubiquitin using immunoblot membranes containing Triton X-100-insoluble cytoskeletal proteins from rat spinal cord [6], heat-stable MAPs purified from rat cerebrum [11, 37] or pure ubiquitin [Sigma, St. Louis, Mo.] (data not shown). The unlabeled antibodies biotin-streptavidin method (Stravigen kit, BioGenix Laboratories, Dublin, Calif.) was used to immunostain the sections. After deparaffinization, the endogenous peroxidase was blocked. To enhance the immunoreactivity of tau, formic acid pretreatment was performed for 3 min [38]. After washing with tap water and 50 mM Tris-HCl pH 7.6, the tissue sections were incubated overnight with the anti-tau (0.5 μg/ml) at 4 °C. The following steps were carried out using a Stravigen kit: diaminobenzidine tetrahydrochloride was used to develop color. To readily differentiate the diaminobenzidine product from melanin pigment, the sections including the substantia nigra and locus ceruleus were bleached with a 0.25% aqueous solution of potassium permanganate for 4 min, followed by treatment with 1% oxalic acid solution, prior to incubation with the anti-tau. To confirm that the bleaching had no influence on the sensitivity and intensity of tau immunostaining, some sections of the hippocampus, pons or midbrain were examined. In the sections with or without potassium permanganate bleaching, there was no appreciable difference in quantity and staining intensity of modified tau immunoreactivity. The sections were counterstained with hematoxylin to distinguish the nuclei of neuronal cells.

Silver staining

For comparison with the immunohistochemical method, silver staining was carried out on the serial sections according to a modified method of Gallyas [5] and of Bielschowsky [13]. To differentiate NFTs clearly from the silver-impregnated melanin pigments, deparaffinized sections including substantia nigra and locus ceruleus were bleached in an aqueous solution of 0.6% KOH and 0.6% H_2O_2 for 15 to 30 min before silver impregnation. This method of bleaching did not inhibit silver impregnation. Use of KOH and H_2O_2 for 15 to 30 min allowed only partial bleaching, but was sensitive enough for differentiation of NFTs and impregnated melanin.

Quantitative analysis

The number of tau-positive neurons in the immunostained sections, the number of silver-impregnated tangles in the Gallyas-stained sections, and the total number of nucleated neuronal cells in the Klüver-Barrera-stained sections were counted within each site under a 200-fold magnification (Olympus light microscope BH2). In the putamen, however, small and large neuronal cells were counted under a 400-fold magnification to differentiate them from glia cells. The regions for quantification were as follows: an entire area on the sections was investigated for the CA1 and subiculum of the hippocampus, globus pallidus, subthalamic nucleus, nucleus basalis of Meynert and nucleus raphe dorsalis. For the substantia nigra and locus ceruleus, the left or right side was arbitrarily chosen, and investigated entirely. For the putamen and pontine nucleus, a region was selected randomly and the investigation finished when the total counted neurons reached over 500 and 1000, respectively.

The incidence of cases showing tau-positive neurons designated as the incidence of tau-positive neurons in each site was expressed as the number of cases with tau-positive neurons divided by the total number of cases examined (in %). The incidence of impregnated tangles was similarly defined. To compare the severity of tau accumulation or tangle formation among the different sites, the density of tau-positive neurons or impregnated tangles in each site was calculated as the number of tau-positive neurons or impregnated tangles divided by the total number of neuronal cells within an investigated area (in %). The mean density of tau-positive neurons or impregnated tangles in each site was obtained by averaging the density of tau-positive neurons or impregnated tangles in all positive cases.

Statistical analyses of the mean density of tau-positive neurons in each site were performed as follows: comparisons among AD, PSP and the age-matched controls were analyzed initially by a one-way analysis of variance (ANOVA) to test the equality of group means and then by the Bonferroni correction to compare all the subgroups. In the putamen, subthalamic nucleus and pontine nucleus, however, the aged controls were excluded from the statistical analyses since only one or no positive cases were found, and the comparisons between AD and PSP were performed by Student's *t*-test.

Results

Morphology of modified tau accumulation

On paraffin sections of formalin-fixed brain tissue, immunohistochemistry with the anti-tau revealed NFTs, cytoplasmic staining of some neurons lacking NFTs, degenerating neurites of senile plaques and neuropil threads.

Immunostained NFTs adopted either a flame-shaped appearance as typically seen in pyramidal cells of the hippocampus and neocortex, or a globose form as seen in the subcortical nuclei (Figs. 1, 2). NFTs were intensely stained, regardless of cortical or subcortical localization. Extracellular tangles appeared weaker in the staining intensity and less compact than intracellular tangles. A similar picture of tangle staining was seen using the modified Gallyas (Fig. 2B) and Bielschowsky methods. In the putamen of patients with PSP, a tau-positive and Gallyas-positive component was found in the short dendrites of innumerable small stellate neurons, as reported by Probst et al. [35], in addition to large globose tangles in the large neurons. This dendritic staining was not recognized by the modified Bielschowsky method. Tau immunoreactivity was also seen in the cytoplasm of a small number of morphologically normal neurons lacking NFT (Fig. 2A). This cytoplasmic staining appeared diffuse or fine granular. Such diffuse tau stainings were not recognizable using the silver methods (Fig. 2B). For convenience, we used the term "tau-positive neurons" to designate collectively NFT-bearing neurons, diffuse cytoplasmic stainings and tau-positive small stellate neurons in the putamen.

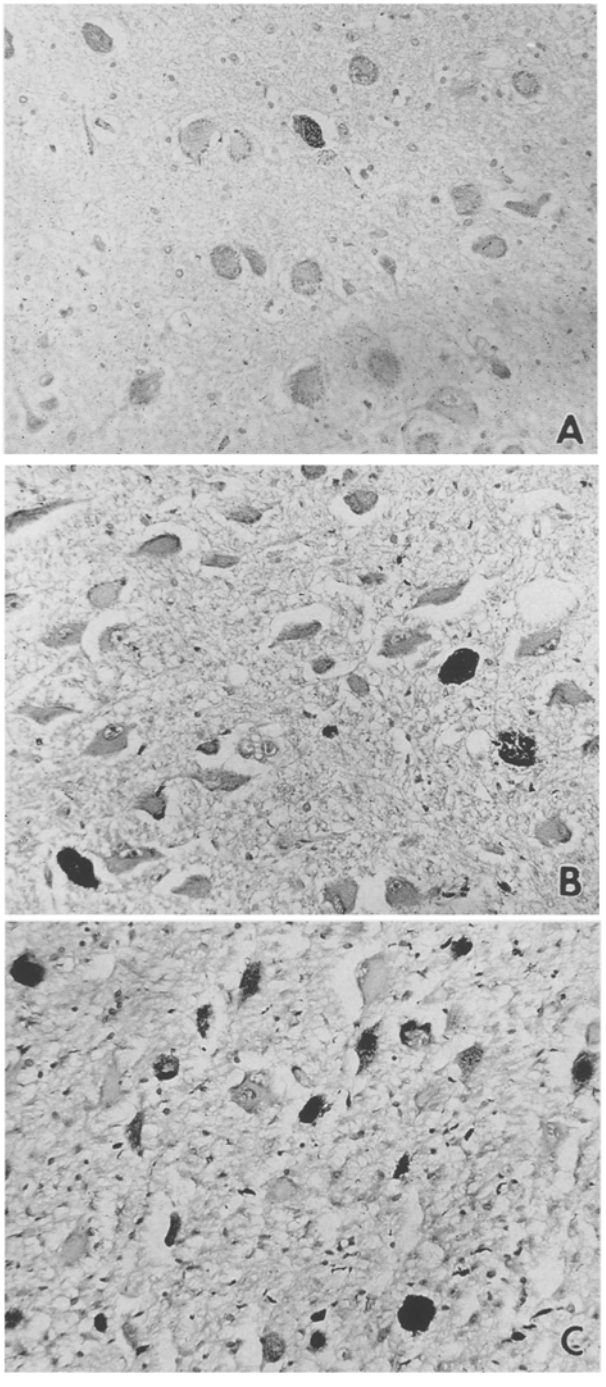

Fig. 1. The locus ceruleus neurons from a 35-year-old nondemented subject (**A**), an 80-year-old nondemented subject (**B**) and a 72-year-old patient with progressive supranuclear palsy (PSP; C). The immunostaining with anti-tau revealed neurofibrillary tangle (NFT)-bearing neurons in different proportions. Neuronal melanin was fully bleached with potassium permanganate. **A–C** × 180

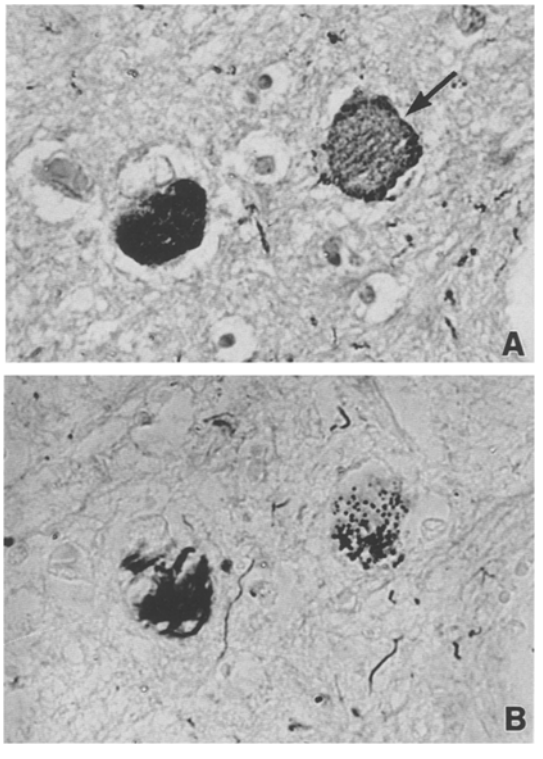

Fig. 2. The locus ceruleus neurons from a 90-year-old nondemented subject. **A** NFT and diffuse cytoplasmic staining without evident NFT (*arrow*) was immunolabeled with anti-tau. Neuronal melanin was bleached as in Fig. 1. **B** NFT was impregnated but the neuron which had diffuse cytoplasmic tau staining in **A** was not with Gallyas stain. Neuronal melanin was incompletely bleached with KOH and H_2O_2. **A, B** × 520

Table 1. Incidence of tau-positive neurons and Gallyas-impregnated tangles, and mean density of tau-positive neurons in each age class of nondemented persons

Age	Hip	GP	Put	Sth	NBM	SN	NRD	LC	PN
20–29	0/0(5)	0/0(5)	0/0(5)	0/0(5)	0/0(4)	0/0(5)	0/0(5)	0/0(5)	0/0(5)
(n = 5)	–	–	–	–	–	–	–	–	–
30–39	0/0(10)	0/0(10)	0/0(10)	0/0(9)	0/0(9)	0/0(10)	20/0(10)	56/0(9)	0/0(9)
(n = 10)	–	–	–	–	–	–	1.3 ± 0.2	3.2 ± 3.7	–
40–49	8/8(12)	0/0(12)	0/0(12)	0/0(12)	8/0(12)	18/0(11)	8/8(12)	45/0(11)	0/0(11)
(n = 12)	0.6	–	–	–	0.7	0.2 ± 0.1	1.4	2.2 ± 1.0	–
50–59	25/13(8)	0/0(8)	14/0(7)	0/0(7)	38/13(8)	25/0(8)	38/25(8)	75/25(8)	0/0(8)
(n = 8)	0.8 ± 0.1	–	0.2	–	9.6 ± 12.0	0.2 ± 0	8.1 ± 3.4	4.5 ± 6.3	–
60–69	29/14(7)	13/0(8)	0/0(8)	0/0(6)	75/13(8)	29/0(7)	25/13(8)	100/13(8)	0/0(8)
(n = 8)	2.6 ± 2.5	0.4	–	–	8.4 ± 4.8	0.2 ± 0.1	5.6 ± 2.0	3.7 ± 2.7	–
70–79	100/100(8)	25/13(8)	0/0(8)	20/0(5)	57/14(7)	14/0(7)	25/25(8)	100/75(8)	0/0(8)
(n = 8)	6.6 ± 5.0	0.5 ± 0.4	–	0.3	10.9 ± 4.5	0.2	7.1 ± 4.5	7.7 ± 4.3	–
80–	80/80(10)	10/0(10)	30/0(10)	0/0(4)	70/50(10)	44/11(9)	80/70(10)	89/78(9)	11/0(9)
(n = 10)	10.9 ± 6.3	0.4	0.2 ± 0	–	8.5 ± 4.6	0.7 ± 0.4	9.2 ± 7.8	11.4 ± 6.1	0.1

In the upper lines in each age class values are the incidence (%) of tau-positive neurons/Gallyas-impregnated tangles, and values in parentheses are total number of cases examined in each site. The lower lines give values of mean ± SEM density of tau-positive neurons averaged for positive cases

–: Density was not given because of the absence of any positive case

Hip: Hippocampus; GP: globus pallidus; Put: putamen; Sth: subthalamic nucleus; NBM: nucleus basalis of Meynert; SN: substantia nigra; NRD: nucleus raphe dorsalis; LC: locus ceruleus; PN: pontine nucleus

Neuronal tau accumulation in nondemented persons

The quantitative data of tau-positive neurons and silver-impregnated tangles in each site of each age class of nondemented persons are given in Table 1. Tau-positive neurons occurred initially in younger persons and showed a higher density than silver-impregnated NFTs in all sites examined (data not shown for the density of silver-impregnated NFTs).

Tau-positive neurons were found in the locus ceruleus at a younger age than those found in the hippocampus, and their number in the locus ceruleus exceeded that in the hippocampus in almost all age classes (Fig. 3). Regarding the density of tau-positive neurons, the locus ceruleus had a higher density than the hippocampus. On the other hand, the evaluation of silver-impregnated tangles revealed that the hippocampus involved initial tangle formation and had the highest incidence of NFTs in almost all age classes, a finding consistent with reported data [27, 43, 44].

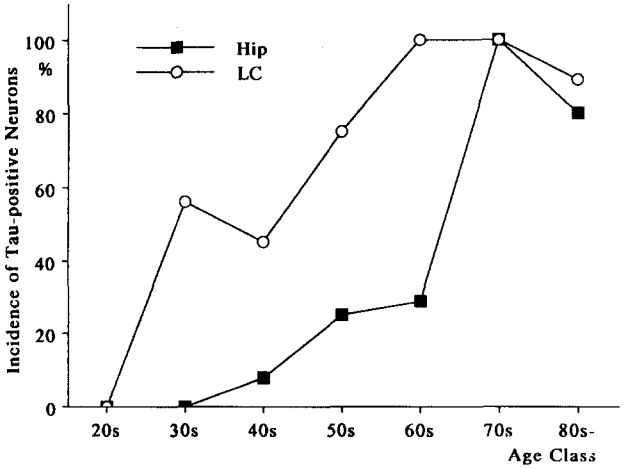

Fig. 3. Incidence (%) of tau-positive neurons in the hippocampus and locus ceruleus in each age class of nondemented persons (see Table 1). Abbreviations are the same for Table 1

Neuronal tau accumulation in AD, PSP and the age-matched controls

The results of quantification of the incidence and the mean density of tau-positive neurons in each site of patients with AD and PSP, together with those of the age-matched controls, are given in Table 2. In the controls, tau-positive neurons were often noted in the nucleus basalis of Meynert, substantia nigra, nucleus raphe dorsalis and locus ceruleus, together with the hippocampus, but less often in the globus pallidus, putamen and subthalamic nucleus, and practically not in the pontine nucleus. In AD and PSP, tau-positive neurons were almost consistently detected in all subcortical sites examined of all cases.

A comparison was made of the mean density of tau-positive neurons among AD, PSP and the age-matched controls (Table 2). Statistical analyses revealed that AD showed a significantly higher density of tau-positive neurons in the hippocampus and nucleus basalis of Meynert as compared with the controls and PSP, while PSP did so in the globus pallidus, putamen, subthalamic nucleus and pontine nucleus as compared with the controls and AD. The globus pallidus in AD and the hippocampus and nucleus basalis of Meynert in PSP showed no difference in the density from the controls. In the locus ceruleus, nucleus raphe dorsalis and substantia nigra, AD and PSP had a significantly higher density as compared with the controls, and there was no difference between the two diseases.

Table 2. Incidence of tau-positive neurons and mean density of tau-positive neurons in the aged controls, Alzheimer's disease (AD) and progressive supranuclear palsy (PSP) cases, and results of statistical analyses of the mean density

	Control (n = 24)	AD (n = 10)	PSP (n = 5)	AD vs Control	PSP vs Control	AD vs PSP
Hip	52(4.9 ± 4.7)	100(80.6 ± 12.4)	100(2.8 ± 4.1)	***	NS	***
GP	13(0.5 ± 0.3)	90(3.2 ± 2.7)	100(58.1 ± 29.8)	NS	*	*
Put	4(0.2)[a]	90(1.0 ± 0.8)	100(10.4 ± 5.3)	a	a	*[b]
Sth	5(0.3)[a]	100(5.5 ± 8.8)	100(62.6 ± 14.8)	a	a	***[b]
NBM	57(9.5 ± 6.3)	100(83.5 ± 10.8)	100(12.5 ± 7.7)	***	NS	***
SN	23(0.2 ± 0.0)	100(15.9 ± 9.1)	100(33.3 ± 16.1)	**	*	NS
NRD	29(7.1 ± 3.0)	100(43.1 ± 25.6)	100(38.4 ± 12.7)	**	*	NS
LC	92(5.4 ± 4.6)	100(58.5 ± 18.6)	100(57.7 ± 16.4)	***	**	NS
PN	0(–)	40(0.3 ± 0.5)	100(14.6 ± 8.0)	a	a	*[b]

Values are incidence (%) of tau-positive neurons in each site, and values in parentheses are mean±SEM density (%) of tau-positive neurons averaged for positive cases. One-way analysis of variance (ANOVA) was performed to show the significant difference in the group means at the significance level 1%, followed by Bonferroni correction for significance

[a]In the putamen, subthalamic nucleus and pontine nucleus, statistical analyses for the aged controls were not done since only one or no positive case was found

[b]Statistical analyses only between AD and PSP using Student's t-test

***, ** and *: Significantly different at the significance level 0.1%, 1% and 5%, respectively

Control: the age-matched controls; NS: not significant; other abbreviations are the same as for Table 1

Discussion

A modified form of tau accumulating in NFTs, senile plaque neurites and neuropil threads is relatively resistant to formalin fixation, whereas normal (unmodified) tau is more sensitive [34]. Application of tau antibodies to formalin-fixed human tissue sections led to the detection of the well-preserved form of tau, i.e., modified tau protein, but practically little normal tau [10, 22, 34]. Several studies [28, 32], which used optimized fixatives that effectively preserved normal tau immunoreactivity, revealed that normal tau is expressed in neuronal cell bodies and glia cells in addition to the initially reported localization in axons [4]. Neuronal tau accumulation observed in this present study is, however, demonstrated by applying anti-tau to formalin-fixed brain tissues and is associated solely with modified tau.

One of the most important observation of this study is that the brain stem nuclei, especially the locus ceruleus, were found to involve neuronal tau accumulation in younger nondemented subjects and at a higher density than the hippocampus. This earlier detection of tau accumulation in the locus ceruleus is due to the combination of tau immunostaining enhanced with formic acid pretreatment and the clear visualization of diaminobenzidine product by melanin bleaching. Our immunohistochemical method is more sensitive for the detection of NFTs than the modified Gallyas and Bielschowsky methods. In addition, our method detected a few neurons which had no evident NFTs but did have diffuse cytoplasmic tau immunoreactivity. Thus, the overall higher sensitivity of the tau immunohistochemistry used accounted for the differences observed between the silver stains and tau immunostaining.

The histological and immunohistochemical examination of the brains from nondemented persons in their 30s revealed no additional abnormalities typical of aging such as neuronal loss and senile plaques other than tau accumulation largely in the locus ceruleus and less often in the nucleus raphe dorsalis. As neuronal tau accumulation relating to tangle formation is considered one of the major histological changes of normal aging found in nondemented brains, it is likely that normal physiological aging in the central nervous system begins in the locus ceruleus in earlier life. However, no neurophysiological counterpart is known at present regarding the earliest involvement of the locus ceruleus in tau accumulation within the brains of younger nondemented persons.

The distribution of NFTs differs between AD and PSP. In AD, the involvement of brain stem neurons in tangle formation is also a pathological marker as is tangle formation in the cerebral cortex, but the involvement of the basal ganglia are relatively uncommon in tangle formation [42]. In PSP, on the other hand, the widespread and massive presence of NFTs is observed in the brain stem, basal ganglia and cerebellum, but not in the cerebral cortex [16, 17]. This study confirmed the above findings by an overall quantitative evaluation of tau-positive neurons in various subcortical nuclei and the hippocampus of AD and PSP, and comparing them with those in the age-matched controls. In the controls, the nucleus raphe dorsalis, locus ceruleus, nucleus basalis of Meynert and hippocampus were considered to be vulnerable to tau accumulations (Fig. 4). In AD the distribution patterns of tau-positive neurons were identical to those in the controls, with one exception being that in the substantia nigra. The vulnerable sites in AD, however, had a much higher density of tau-positive neurons than those in the controls. Thus, neuronal tau accumulation in the subcortical nuclei of AD is increased far more than that in the normal aging process. In patients with PSP, on the other hand, the distribution patterns of tau-positive neurons differed from those in the controls and AD. The globus pallidus, putamen, subthalamic nucleus and pontine nucleus, non-vulnerable sites in both controls and AD, also showed a

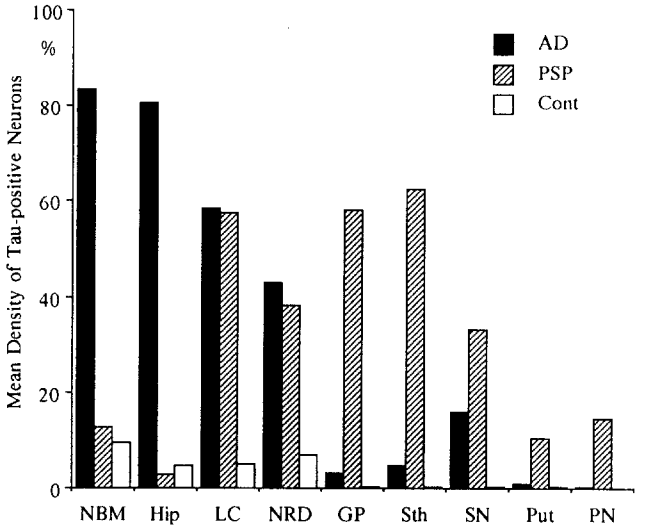

Fig. 4. Mean density (%) of tau-positive neurons in each site of Alzheimer's disease and PSP subjects and the age-matched controls (see Table 2). Sites are arranged from left to right roughly in decreasing order of the mean density for the controls. Abbreviations are the same as for Tables 1 and 2

conspicuous accumulation of tau. Therefore, PSP is not simply an increased state of tau accumulation in the normal aging process. These findings suggest that in PSP different mechanisms from those of controls and AD may be involved in tangle formation, which is in support of the proposal of Schmidt et al. [36].

Even in nondemented persons, tau-positive neurons were often present in subcortical neurons and the hippocampus. Thus, the presence of NFTs does not always represent a pathological process. When tau-positive neurons are found in the subcortical nuclei, a quantitative evaluation should be made for a pathological diagnosis of tangle-bearing diseases including AD and PSP. In addition, evaluation of the distribution pattern and density of tau-positive neurons will provide more insight into the pathogenesis of tangle-bearing diseases.

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