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About the presence of paired helical filaments in dystrophic neurites participating in the plaque formation*

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Summary. Immunocytochemistry with monoclonal antibodies to the β-protein and to antigens associated with paired helical filaments (PHF) allows us to selectively stain two major components of neuritic (senile) plaques (NP): PHF and amyloid deposits. Using this method, the structure of NP in the brains of Alzheimer disease victims was compared to their structure in the brains of non-demented aged individuals selected for high numbers of NP. It is demonstrated that the dystrophic neurites participating in the plaque formation contain PHF only when cortical nerve cells in the same brain area form neurofibrillary tangles (NFT). People with many NP and many NFT were always demented, whereas people with many NP but few, if any NFT were not. It is speculated that there is individual susceptibility to the formation of PHF and that their appearance may represent a nonspecific response of the neuronal network to different kinds of injuries, like the deposition of amyloid in Alzheimer disease, or other pathogenic factors associated with various dementive neurodegenerative diseases. It is hypothesized that the deposition of brain amyloid in people resistant to neurofibrillary pathology may induce too little dysfunction for the development of dementia.

Key words: Alzheimer's disease – Amyloid – Immunocytochemistry – Neuritic plaques – Paired helical filaments

A neuritic (senile) plaque (NP) is a complex structure made of both neuronal and nonneuronal elements. The neuronal component of the NP consists of dystrophic degenerating and regenerating neurites, some of which may contain paired helical filaments (PHF). These profiles are particularly numerous in primitive plaques and in the corona of classical plaques. The other components of the NP are amyloid deposits, microglia, macrophages and reactive astrocytes. NP are found in normal aged human and animal brains [34, 36] and are particularly numerous in Alzheimer's disease [AD]. Similar lesions are also found in some cases of spongiform encephalopathies (Creutzfeldt-Jacob disease, Gerstmann-Sträussler syndrome, scrapie) caused by unconventional infectious agents [41]; however, the protein composition of the amyloid fibers in plaques found in these diseases is different from the β-protein making up brain amyloid in AD and normal aging.

PHF are uniquely human lesions. They are only found in nerve cells and, by and large, in unmyelinated nerve cell processes. Neurons bearing neurofibrillary tangles (NFT) made of PHF can be found in many and unrelated diseases. In some of them, few neurons with NFT are found while in others, e.g., AD, post-encephalitic parkinsonism, dementia pugilistica [6, 37] and Guam-parkinsonism-dementia complex [14, 15] there are numerous nerve cells with neurofibrillary changes.

As indicated above, animal neuronal perikarya never form PHF of the type described in AD and neurites of the plaques found in animals do not contain PHF [34, 36]. It was also reported that in cases of Pick's disease [35] where there were no neurons with NFT, the neurites of the NP did not show the presence of PHF.

At present it is not clear whether PHF can form in plaque neurites and neuronal cell bodies independently, or whether the presence of the pathological

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fibers at one site is always accompanied by their formation at the other. In other words, if there is no perikaryonal formation of PHF, will the neurites also be free of PHF, and vice versa? To address this question, we used immunocytochemical methods which allow us to selectively stain two major components of NP; amyloid deposits and PHF. Using this technique, we compared the structure of NP in the presence of NFT in the same brain area to their structure in brains free of neurofibrillary pathology.

Materials and methods

Five cases with clinically and neuropathologically confirmed AD (mean age: 70.6 ± 11.0 years) which had numerous NP and NFT in neocortex and hippocampus and five brains of aged individuals without a history of dementia (mean age: 81.8 ± 6 years) were chosen for this study. To underscore the difference between NP in areas rich and poor in neurofibrillary changes, we selected the non-demented cases for high numbers of NP in the neocortex. However, in contrast to the AD brains, they had no or only few NFT.

The brains had been fixed in 10% neutral formalin for 1–5 years. For Bielschowsky and immunohistochemical staining the following samples of the brain were taken: (1) frontal gyrus rectus (Brodmann's areas 11–12), 2 cm posterior to the frontal pole; and (2) hippocampal and parahippocampal gyri, at the level of the lateral geniculate body. The paraffin-embedded blocks were cut into 7 µm-thick sections.

For immunohistochemical staining, the following antibodies were used: (1) monoclonal antibody (mAb) 4G8 to amino acids 17–24 of the amyloid β-peptide [20] as a marker for amyloid. To enhance the immunoreactivity of amyloid deposits on formalin-fixed tissue, sections were pretreated with concentrated formic acid for 1 h prior to immunostaining [21]; (2) mAb Tau-1 to bovine tau [3, 13] as a marker for PHF. To obtain reactivity of PHF with this mAb, sections were pretreated with alkaline phosphatase (Sigma, Type VII, 400 µg/ml in 0.1 M Tris-HCl, pH 8) for 4 h at 37° C; (3) mAb 3–39 to PHF, recognizing an epitope residing on amino acids 50–65 of ubiquitin [25, 32]. The mAb to tau was used for the detection of NFT and plaque neurites containing PHF since among all PHF markers available to us, this antibody was the most sensitive for the detection of intracellular PHF [2].

Endogenous peroxidase was inactivated by exposure to 0.6% H_2O_2 in methanol for 30 min. Nonspecific protein bindings sites were blocked for 30 min by incubation with 5% horse serum in phosphate-buffered saline (PBS). The primary antibodies were diluted in 5% horse serum in PBS and incubated with the tissue sections for 16 h at 4° C. Biotinylated goat antibodies to mouse immunoglobulins in 5% horse serum in PBS were incubated with the tissue for 30 min at room temperature to bind to the primary mAb. The sections were then exposed to the avidin-biotin-horseradish peroxidase (HRP)–complex (Vectastain kit) for 30 min. Peroxidase was developed with diaminobenzidine (500 µg/ml in 0.05 M Tris-HCl, pH 7.4) containing 0.01% H_2O_2. The reaction was stopped by immersion of the slides in tap water and the sections were counterstained with hematoxylin.

To monitor nonspecific staining due to possible reactivity of the secondary antibodies, additional sections were processed without the primary antibodies.

NP and neurons with NFT were counted in the neocortex and the hippocampus. Five nonoverlapping fields were assessed at a magnification of × 100 and the mean number of lesions per mm^2 was defined. For each individual block, the same matched fields were chosen on serially cut sections for each immunohistochemical staining.

Results

In demented people where Bielschowsky's silver impregnation and the mAb to tau and PHF/ubiquitin showed the presence of NFT in neuronal perikarya, the neuritic component of the NP also contained anti-tau- and anti-PHF/ubiquitin-positive neurites (Fig. 1a, b; 2b, c; 3b; 4b). In addition, a network of neurofibrillary changes in the neuropil corresponding to neuropil threads of Braak et al. [4, 5] was observed outside of the plaque area with the tau antibody and also, but to a lesser extent with the mAb to PHF/ubiquitin. Even in cases with numerous NFT, the mAb to the β-peptide revealed more plaques than anti-tau, and many more than anti-PHF/ubiquitin (Table 1).

The nondemented group had similar numbers of NP as the group of demented cases by Bielschowsky and immunostaining for the β-peptide. However, few or no neurons with NFT were present (Table 1). In these cases the PHF/ubiquitin and tau antibodies did not reveal the presence of NP or neuropil neurofibrillary changes even though the mAb to the β-peptide showed numerous plaques in the same area in serially cut sections (Fig. 1a, b; 5a, b).

In the majority of the cases, immunostaining with the mAb to the β-protein on formic acid-treated tissue revealed more NP than the Bielschowsky method. The tau antibody and the Bielschowsky method stained similar numbers of neurons with NFT. More neurons with NFT and NP were seen using the tau antibody than the PHF/ubiquitin antibody (Fig. 2a–c) (Table 1). In both demented and nondemented cases, in addition to NFT, some apparently normal neurons stained with the mAb to tau. This stain was diffuse or showed fine granular patterns resembling conditions of pretangle formation or "Stage 0 tangles" recently described by Bancher et al. [2]. Stage 0 tangles were never observed in brain regions devoid of NFT.

Discussion

In cases with many neurons with NFT in the neocortex (Fig. 3a, b) and hippocampus (Fig. 4a, b) the mAb to tau showed the presence of positively stained neurites in 19% to 100% of the plaques detected by the mAb to the β-peptide. In cases with few or without neurons with NFT, the mAb to tau neither revealed the presence of the NP nor of the neuropil neurofibrillary

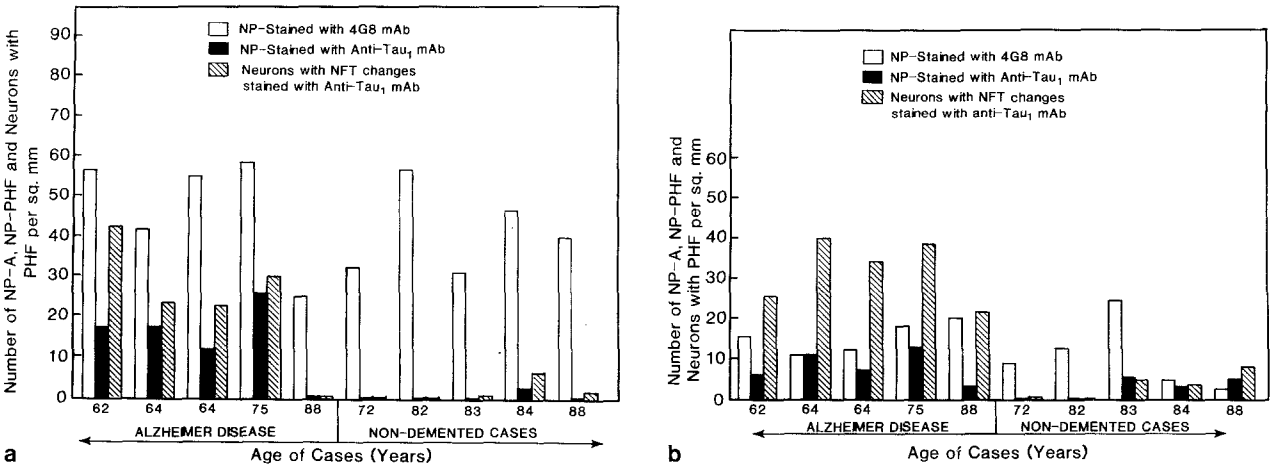

Fig. 1a, b. In cases with neurofibrillary tangles (NFT), the plaques (NP) are stained with both, the β-protein and tau antibodies. Note however, that the β-protein antibody detects more NP than the tau antibody

Table 1. Number of neurofibrillary tangles (NFT) and neuritic (senile) plaques (NP) in Alzheimer's disease (AD) and nondemented (ND) as seen by immunocytochemistry

Case	Clinical diagnosis	Age	Anti-amyloid 4G8		Anti-tau Tau-1				Anti-PHF/ubiquitin 3-39			
			Neocortex NP	Hippocampus NP	Neocortex		Hippocampus		Neocortex		Hippocampus	
					NFT	NP	NFT	NP	NFT	NP	NFT	NP
1	AD	62	57.63	15.64	43.01	17.68	26.86	5.95	1.53	2.21	3.74	1.19
2	AD	64	42.67	11.05	22.59	17.51	40.29	11.05	2.21	0	3.06	2.21
3	AD	64	54.74	13.09	23.29	10.88	34.51	7.31	10.03	4.59	28.73	3.57
4	AD	75	58.82	18.02	29.07	16.15	38.93	12.75	9.01	10.20	10.71	7.99
5	AD	88	24.99	20.57	0	0	21.42	2.72	0	0	4.08	0.34
6	ND	72	32.64	9.69	0.17	0.17	0.51	0.34	0	0	0.17	0
7	ND	82	47.73	13.09	0	0	0.	0	0	0	0	0
8	ND	83	31.11	24.4	0.51	0.17	5.61	6.29	0	0	0.17	0
9	ND	84	57.8	4.93	7.31	2.55	4.08	3.06	0.17	0	0.34	0
10	ND	88	40.46	8.2	1.87	0	8.84	5.27	0.17	0	7.31	0

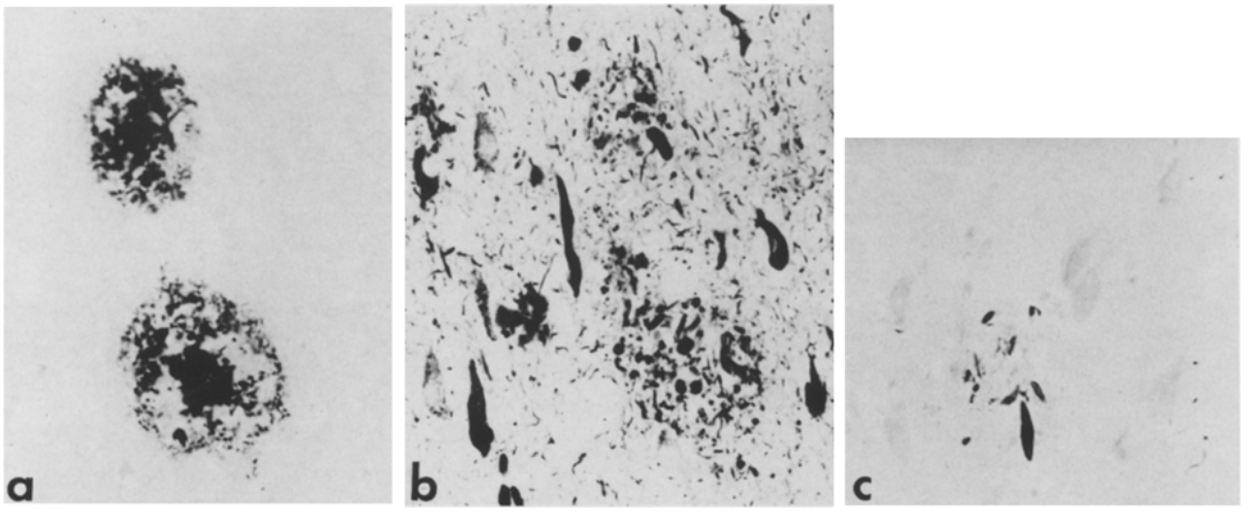

Fig. 2a–c. Alzheimer's disease (AD), hippocampus, **a–c** are serial section. **a** β-protein immunostaining. Two classical plaques in the pyramidal cell layer. **b** Tau antibody. Note the staining of neurons with NFT, neuropil threads and the neurites in the plaques. **c** PHF/ubiquitin antibody. Only one of two plaques stained. **a–c** $\times 87$

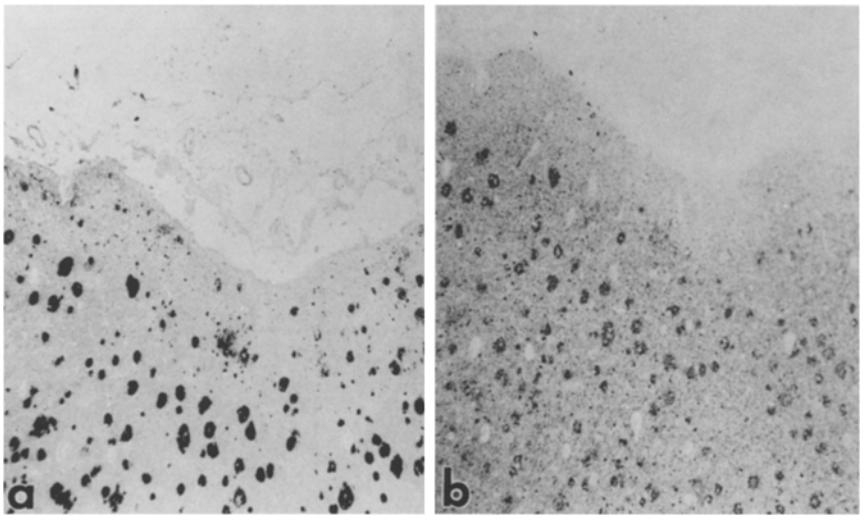

Fig. 3a, b. AD, neocortex. **a** β-protein immunostaining showing many NP mostly of the primitive tpye. **b** The same area, anti-tau stains almost the same number of NP. **a, b** $\times 11$

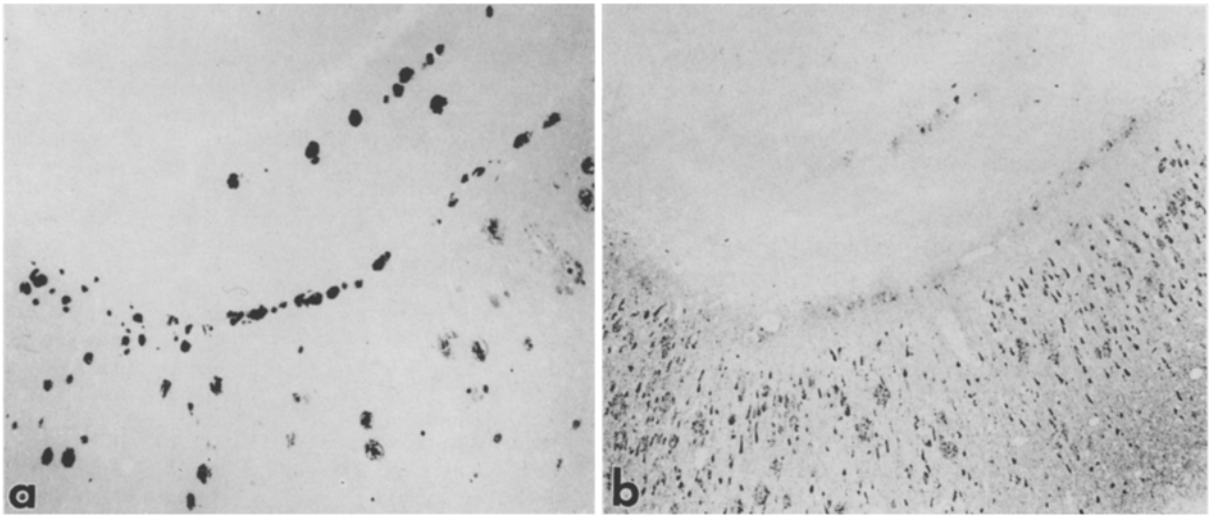

Fig. 4a, b. AD, hippocampus. **a** β-protein immunostaining showing many NP in characteristic location. **b** Adjacent section, anti-tau staining shows the presence of neurons with neurofibrillary changes; the neuritic component of the NP is also stained. **a, b** $\times 11$

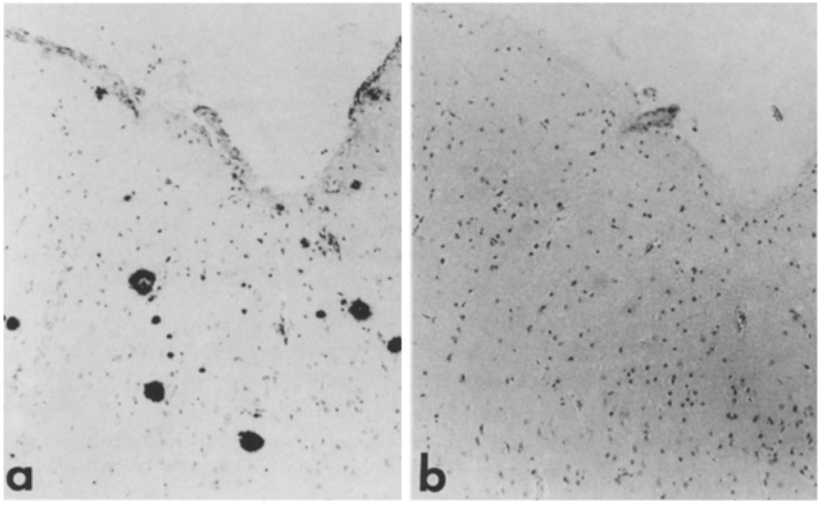

Fig. 5a, b. Nondemented case, neocortex. **a** β-protein immunostaining showing primitive and classical plaques. **b** In the same area, the tau antibody neither reveals the presence of the NP nor of neurofibrillary changes. **a, b** $\times 11$

changes (threads) (Fig. 5a, b). These data indicate that, if cortical nerve cell bodies do not make PHF, their neurites participating in the formation of NP are also free of PHF. In other words, if the nerve cell bodies do not produce PHF, their processes do not assemble PHF, or vice versa.

Previous studies indicate that the density of both NFT and NP correlates with the degree of dementia [28]. However, more recently several authors, [8, 9, 12, 19, 27] stressed that the existence of neurofibrillary changes is more closely associated with dementia than high numbers of plaques. Likewise, the studies of Crystal et al. [7] showed that NP counts did not distinguish between demented and nondemented subjects, but that every subject with numerous cortical NFT was demented. Wilcock et al. [33] emphasized that in patients with AD the reduction in choline acetyltransferase (ChAT) activity was significantly correlated to the severity of dementia and the numbers of NFT, but not to the numbers of NP present in the corresponding cortex. In contrast, Terry et al. [31] reported that in a series of 60 AD cases over age 74, a significant minority (30%) lacked neocortical NFT. According to these authors, senile dementia of Alzheimer type (SDAT) with neocortical NFT is the same disease as SDAT without them, although the presence of such tangles is associated with a tendency towards greater severity. However, it should be stressed that in all cases reported by Terry et al. [31] many neurons with neurofibrillary changes were present in the hippocampus. Since the hippocampus plays a key role in the AD type of dementia, [1, 16] the data cited above support the notion that the presence of neurons with NFT is of critical importance for the expression of cognitive deficiency in AD.

PHF accumulation appears to disturb nerve cell function. Evidence for abnormal functioning of neurons with neurofibrillary changes comes from studies of people with Guam-parkinsonism-dementia complex, dementia puiglistica [6], and reports of sporadic cases with extensive neurofibrillary changes and dementia [26]. Neurofibrillary changes are observed in many and pathologically unrelated conditions [43]. The topographic distribution of the neurons with NFT in many of these various diseases is similar, indicating regional (irrespective of etiology) susceptibility of specific nerve cell populations to the formation of PHF. For example, hippocampal pyramidal neurons are most susceptible, whereas neurons with NFT made of PHF are never found in the cerebellum or the spinal cord [39].

In addition to topographic susceptibility, there appears to be individual susceptibility to neurofibrillary pathology. Evidence for the individual susceptibility to tangle formation comes from our studies of hydrocephalic brains [42]. We examined 30 brains with various degrees and etiologies of hydrocephalus. In 47% of the cases, extensive formation of neurofibrillary changes was found. The other 53% of the brains were free of neurofibrillary pathology. Because there was no association between the age, degree, or etiology of the hydrocephalus and the occurrence or lack of neurofibrillary changes, we concluded that there must be individual differences in the susceptibility to PHF formation.

Accepting the hypothesis that there is both topographic and individual susceptibility, we postulate that the primary events in the pathogenesis of AD are the amyloid deposits. The primary amino acid sequence data [18], in vitro translation [11] and immunocytochemistry indicate that the amyloid precursor protein(s) may function as transmembrane receptors, adhesion molecules or secreted extracellular matrix proteins. Thus, in the susceptible brain, the aberrant catabolism of the amyloid precursor protein(s) might, possibly by disturbing the cytoskeletal phosphorylation/dephosphorylation system, lead to the formation of PHF. In this context, it is interesting to note that recent studies suggest that the accumulation of abnormally phosphorylated tau is one of the earliest changes in the process of PHF tangle formation [2].

Using the mAb Alz-50, Scicutella et al. [29] reported that one of the dramatic characteristics of this antibody is the extensive staining of neurites in the AD plaques as compared to plaques in normal old-age individuals. According to these authors, antibody Alz-50 recognizes a protein A68 with an apparent molecular weight of 68,000 daltons in the brains of patients who died with AD, but not in the brain tissue from individuals free of neurological disease. However, Alz-50 can apparently recognize tau [22, 23], a protein which is part of PHF. Recently Love et al. [24] showed that Alz-50 immunoreactivity is not limited to the brain tissue of patients with AD. They concluded that "it seems unlikely that the proteins recognized by this antibody are of primary etiologic significance in AD, although they may play a role in the formation of PHF". Both, Alz-50 and antibodies to tau stain neurons with PHF, some neurons without NFT and the neuritic part of NP [10, 17, 30, 44, 45]. Therefore, the extensive staining of AD plaques by the Alz-50 antibody and almost lack of staining of plaques in normal old-age people [29] may be due to the fact that in normal old people there were few, if any, neurons in the cortex with neurofibrillary changes and thus the NP were not demonstrated because they did not have a neuritic component containing PHF.

As indicated above, it appears that people who develop many plaques but are resistant to neuro-

fibrillary pathology are less likely to develop dementia than people with both plaques and tangles or tangles alone. There is no evidence that neurofibrillary pathology leads to amyloid deposits. However, there is evidence that amyloid deposits initiate neuritic plaque formation [38–41]. In our opinion, the amyloid formation, like trauma to the brain, is pathogenetically linked to PHF formation. Therefore, the assembly of PHF in susceptible neurons of susceptible individuals may represent a nonspecific response of the neuronal network to different kinds of injury to the brain tissue, like the deposition of amyloid in AD, an environmental factor in Guam-parkinsonism-dementia, mechanical trauma in dementia pugilistica, an infections agent in postencephalitic parkinsonism or the development hydrocephalus. However, since PHF are also associated with various other agents and conditions, i.e., viruses or trauma, it is also possible that the amyloid deposits and NFT made of PHF may be caused by etiologically different factors.

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