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Ultrastructure of the neuropil threads in the Alzheimer brain: their dendritic origin and accumulation in the senile plaques*

H. Yamaguchi[1,2], Y. Nakazato[3], M. Shoji[2], Y. Ihara[4], and S. Hirai[2]

[1] College of Medical Care and Technology, and Department of [2] Neurology and [3] Pathology, School of Medicine; Gunma University, Showa-machi, Maebashi, Gunma 371, Japan
[4] Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

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Summary. Thread-like structures immunoreactive with paired helical filaments and tau antisera were demonstrated as mesh-works in the neocortices of five brains with Alzheimer-type dementia, but not in those of five normal aged control brains. The ultrastructure of the threads was examined using paired routine electron microscopic ultrathin sections and adjacent 0.4-μm-thick semithin sections, immunostained for β protein. Outside the β protein-positive senile plaques, neuropil threads appeared sporadically as small slender neurites, containing either regularly constricted or straight filaments. These neurites often showed dendritic profiles. Similar threads were also seen within the senile plaques. The threads were accumulated in amyloid fibril-rich primitive plaques, but not in amyloid fibril-poor diffuse plaques. The presence of these threads was closely associated with neurofibrillary tangle formation. Our findings suggest that wide-spread change of the neuropil neurites, neuropil threads or curly fibers, both outside and inside of the senile plaques are dendritic in origin and play an important role in the clinical manifestation of dementia.

Key words: Neuropil threads – Alzheimer-type dementia – tau protein – Paired helical filaments – Senile plaques

Abundant formation of senile plaques (SP) and neurofibrillary tangles (NFT) are the hallmarks of Alzheimer-type dementia (ATD) including senile dementia of the Alzheimer type (SDAT) and presenile Alzheimer's disease. A sensitive method for detecting SP, β protein immunostaining, demonstrated a considerable number of SP, especially diffuse plaques [40, 41], in the brains of nondemented old people [5, 35, 40]. In brains with Down's syndrome, showing age-dependent pathology, deposits of β protein as diffuse plaques precedes NFT formation and further mental deterioration [22]. Although NFT formation has been shown in a wide range of diseases [38] and, therefore, is less disease-specific than SP formation, it is more closely associated with mental decline in aged people [6, 36].

Recently, some authors noted the significance of abnormalities of neuropil neurites in ATD by the Gallyas silver method [3, 4, 7, 8] and by immunostaining for paired helical filaments (PHF) [13], or microtubule-associated protein (MAP) tau [12, 21], or Alz-50 [11]. Massive formation of short, slender and distorted neurites, named "neuropil threads" [3, 4, 7, 8] or "curly fibers" [12, 21], has been demonstrated throughout the neocortex of ATD brains.

In this study we investigated the ultrastructure of neuropil threads, which have not yet been clearly examined in the literature. Threads which accumulated in the SP were also examined. The origin of these threads is discussed.

Materials and methods

We examined five autopsy brains from patients aged between 78 and 84 years with severe dementia. These brains fulfilled the clinical and pathological criteria of ATD [16]. As a control, five additional brains from normal aged between 75 and 85 years were also examined.

Tissue blocks taken from frontal, temporal, parietal and occipital cortices and hippocampal areas were embedded in paraffin. Serial 6-μm-thick sections were prepared and processed for modified Bielschowsky [43] and Bodian stains, and for immunohistochemistry for two kinds of MAP tau, PHF [13], and β protein [40]. One polyclonal antisera to MAP tau which was purified from normal human brain [12] mainly recognized epitopes on the N terminus of tau (tau-N) [14]. The other polyclonal tau antiserum was produced against synthetic peptide, tau-C4, which consisted of 16 amino acid residues compatible with the sequence of $tau_{254-269}$, and located at the carboxyl third of tau [20]. For tau-C4, PHF and β protein immunostainings, deparaffinized sections were treated with 99% formic acid for 5 min beforehand [19]. Moreover, sections for tau-C4

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Offprint requests to: H. Yamaguchi (address see [1] above)

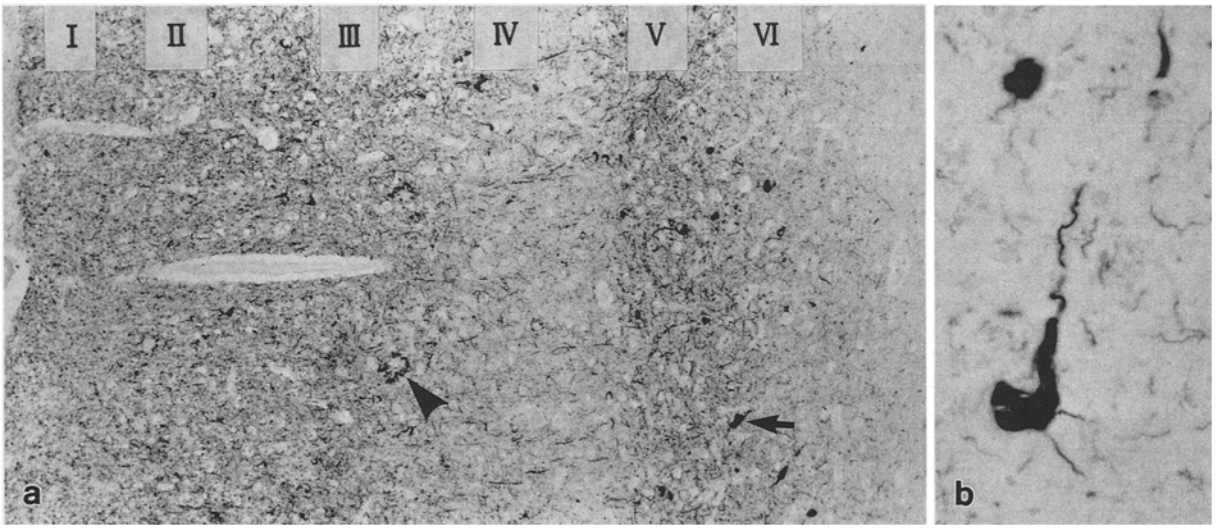

Fig. 1. **a** Distribution of the tau-N-positive threads in the occipital cortex of the Alzheimer's disease brain. Senile plaque (SP, *arrowhead*) and neurofibrillary tangles (NFT; *arrow*) are indicated. **b** Tau-C4-positive neuropil threads, showing the dendritic nature, and NFT forming pyramidal cell; temporal cortex. **a** ×80, **b** ×450

and PHF were treated with pronase (0.05% in phosphate-buffered saline, pH 7.4, protease type XIII, Sigma, USA) for 5 min at room temperature. These procedures highly enhanced the immunostaining of tau C4, PHF and β protein, whereas tau-N immunoreactivity was lost by the pronase treatment. Sections were reacted with primary rabbit antisera against tau-N (1:1,000), tau-C4 (1:500), PHF (1:500) and synthetic β peptide (1−28, 1:2,000), or normal rabbit serum (1:500), and then, stained using Vectastain ABC kit (Vector Lab, California, USA) [40].

For electron microscopic examination, small specimens of the frontal and temporal lobes were taken at autopsy from two SDAT patients (80 and 81 years old) and fixed in a 4% paraformaldehyde and 1% glutaraldehyde solution for 6 or 12 h, then postfixed with 2% osmic acid, and processed for the epon embedding. Alternative semithin (0.4 μm thick) and ultrathin (90 nm thick) sections were cut serially. After the removal of epon, semithin sections were immunostained for β protein as previously described [42]. The adjacent ultrathin sections were stained with uranyl acetate and lead citrate, and were examined under a JOEL 100 CX II electron microscope.

Results

Distribution of the threads

Aged control brains showed neither threads nor NFT in their neocortices. In contrast, ATD brains showed extensive threads formation together with NFT formation in the temporal (5/5), occipital (5/5), parietal (5/5), and frontal (4/5) cortices. One exceptional frontal cortex from among the ATD neocortices had no threads or NFT. The appearance of threads was closely associated with NFT formation. In the neocortex where threads appeared massively, they were encountered as mesh-works throughout the neocortical layers with a laminar arrangement (Fig. 1a). The density (incidence) was highest in the fifth neocortical layer, where NFT formation of pyramidal cell was also most common.

These threads appeared as short, slender, and distorted neurites in the sections immunostained for tau-C4 (Fig. 1b), tau-N (Fig. 2b), or PHF (Fig. 2c), and in those impregnated by modified Bielschowsky (Fig. 2a) silver method. The antiserum to tau-C4 showed the most distinctive staining of the threads, showing the dendritic nature of the threads (Fig. 1b). The density (incidence) of threads was somewhat high in the sections stained for tau-N than those for PHF.

When compared with the contiguous section immunostained for β protein, small and slender threads were demonstrated both inside and outside the β protein-positive SP areas (Fig. 2). The threads tended to accumulate in the plaque areas (Fig. 2b, c). This focal accumulation was prominent in the second and third neocortical layers of the temporal, parietal, and occipital lobes. However, in the frontal cortices, where diffuse plaques [40, 41] were predominant, this accumulation was not so frequent. There was no difference in the incidence of threads between the inside and outside of diffuse plaques.

Ultrastructure of the threads outside the SP

Semithin sections of the epon-embedded tissues of frontal and temporal cortices, treated with formic acid and stained for β protein, showed numerous SP. We found small slender neurites filled with pathological filaments both inside and outside the β protein-positive area in the adjacent ultrathin sections (Fig. 3). In this way, the ultrastructure of the threads outside the SP was examined (Fig. 4). The diameter of neuropil threads ranged between 0.2 to 1.5 μm (mean 0.62 μm; $n = 50$). The pathological filaments usually showed regular constriction with 80-nm periodicity and had a maximal diameter of 25 nm, corresponding to so-called PHF [17] (Figs. 3e, 4). These filaments were arranged loosely rather than intimately in the neurites. A minority of the neuropil threads consisted of packed straight 20-nm filaments in the temporal cortices (Fig. 3c, d). The cytoplasm of neuropil threads usually

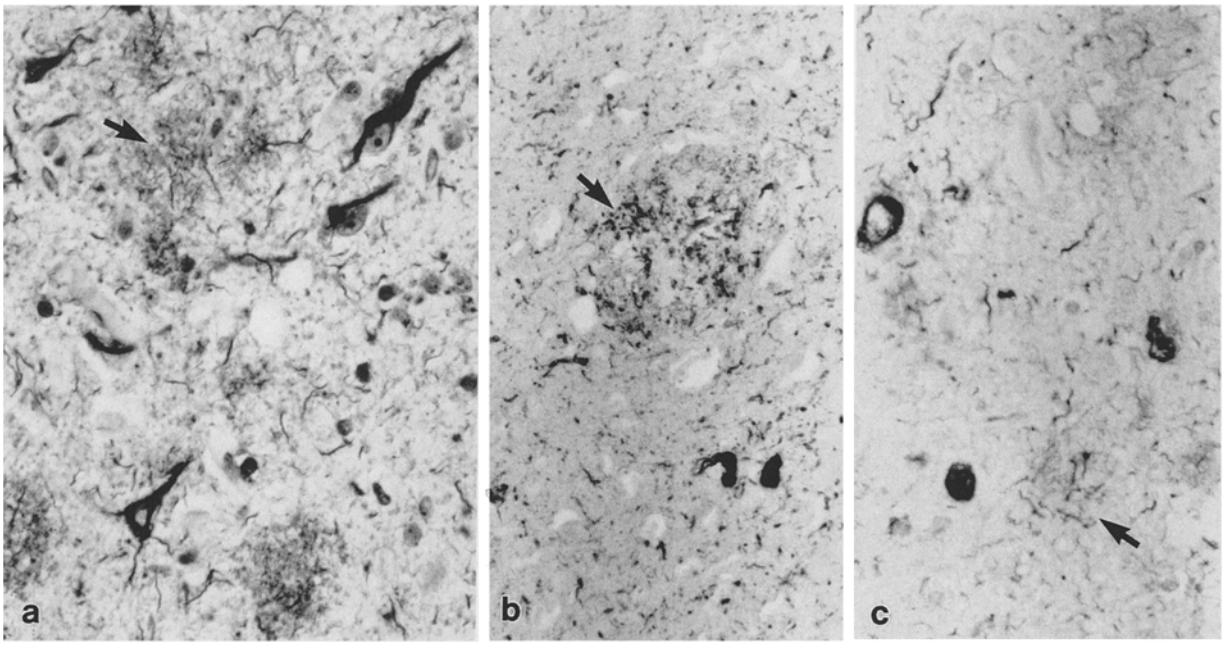

Fig. 2. Threads both inside (*arrow*) and outside the plaque areas, shown by the modified Bielschowsky stain (**a**), and tau-N (**b**) and paired helical filament (PHF, **c**) immunostains. Accumulation within the plaque was evident in **b** and **c**, but not in **a**. **a**–**c** ×400

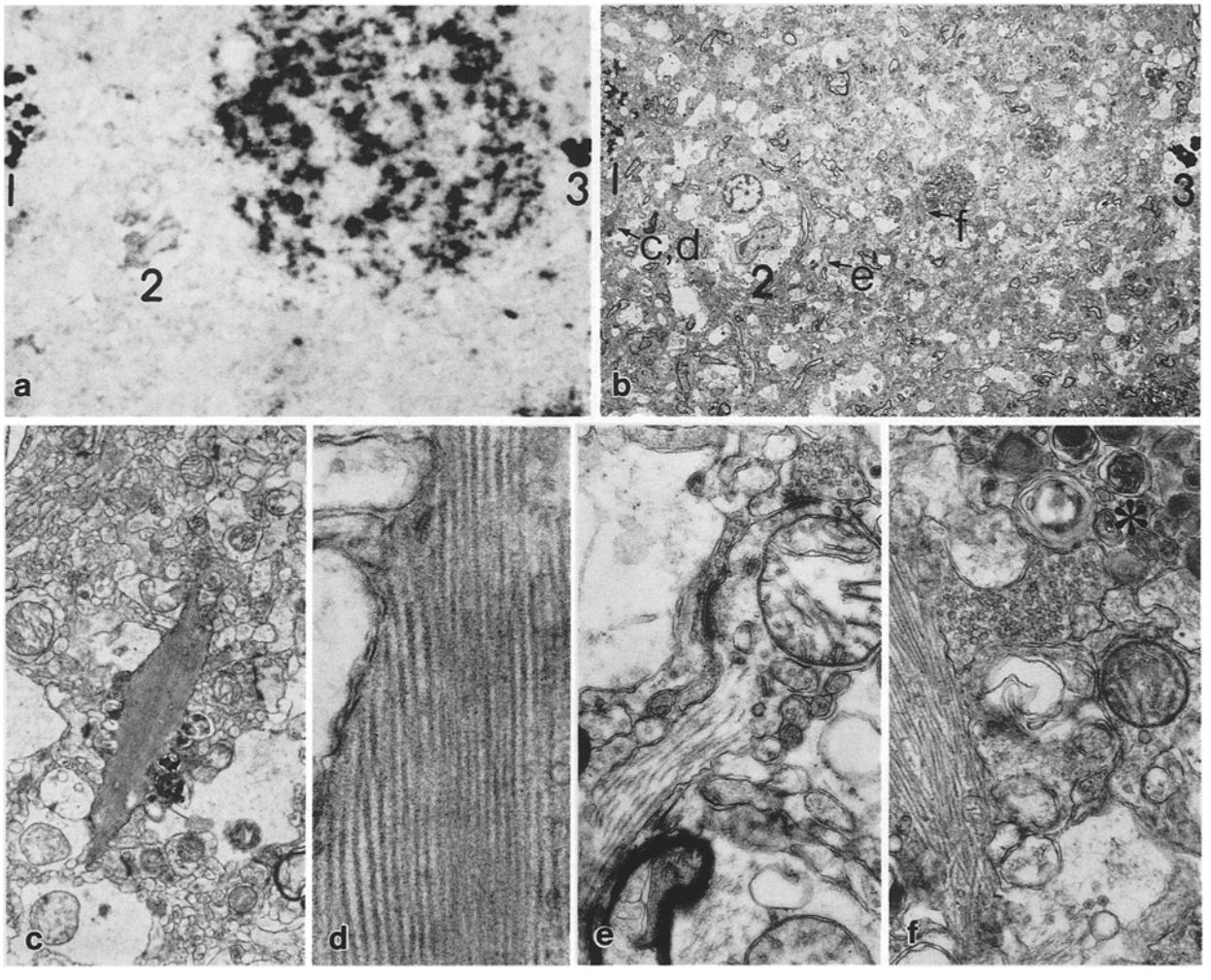

Fig. 3. Light (**a**) and electron (**b**–**d**) micrographs of a primitive plaque and surrounding neuropil. **a, b** *β* protein-positive plaque area of the semithin section (**a**) could be clearly identified in the adjacent ultrathin section (**b**). Numbers *1* to *3* indicate the same structure. Three threads, indicated with *arrows* and *letters*, located inside and outside the plaque areas; temporal cortex. **c**–**f** High-power view of the threads. Some threads consisted of straight filaments (**c, d**). Threads often showed profile of dendrites (**e**). Thread was associated with degenerated swollen neurite (**f**, *asterisk*). **a, b** ×1,100; **c** ×6,800; **d** 47,850; **e** ×18,400; **f** 14,940

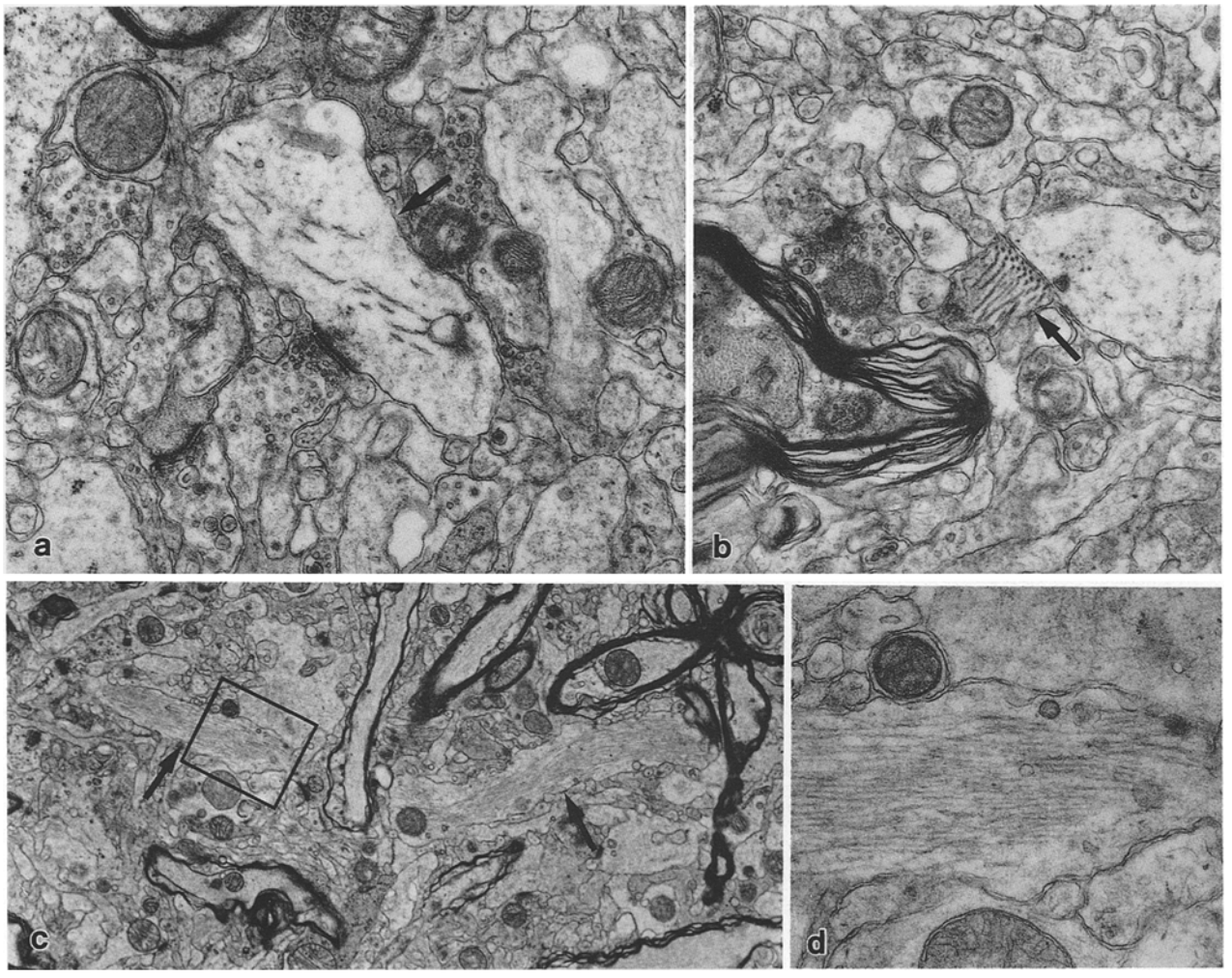

Fig. 4. Electron micrograph of the threads outside the SP (*arrows*) in the frontal cortex. **a** Somewhat swollen thread, showing postsynaptic density, corresponded to dendrite. **b** Thread contained straight and constricted filaments. (*c, d*) Tortuous thread composed of constricted filament. Rectangle in *c* indicates the location of high power view (*d*). **a** ×16,200; **b** ×15,000; **c** ×8,800; **d** ×19,000

had scanty subcellular organelles. The neuropil threads often showed postsynaptic density and, thus, were identified as dendrites (Figs. 3e, 4a). We could not find neuropil threads covered with myelin sheath.

Ultrastructure of the threads inside the SP

In primitive plaques, more abundant threads were seen together with scattering clusters of amyloid fibrils than in surrounding neuropils (Fig. 5). Many threads were filled with closely arranged constricted (Figs. 3f; 5a, b) and/or straight filaments. The threads also had a few cytoplasmic organelles. They often formed synaptic connections and identified as dendrites (Fig. 5b). Thus, the ultrastructure of the threads was not different between the inside and outside of the SP, but the diameter of threads tended to be wider in the primitive plaques than in the surrounding neuropil. The large swollen neurites, filled with degenerating mitochondria and dense bodies, were most typical form of SP neurites, but they rarely contained pathological filaments (Fig. 5a). In diffuse plaques, which had few or no apparent amyloid fibrils, the density and morphology of the threads were equal to those of threads seen in the surrounding neuropil.

Some presynaptic terminals (axon) had constricted filaments together with synaptic vesicles (Fig. 5). The pathological filaments were incidentally observed in myelinated axons. However, these axonal localization of pathological filaments were much less abundant than dendritic localizations.

Ultrastructure of the NFT

Although the NFT principally consisted of constricted filaments, some NFT contained straight filaments. The bundles of pathological filaments were associated with a small amount of vesicles.

Discussion

Some authors noted the presence of scattered, small, slender, tortuous, and thread-like structures in the neigh-

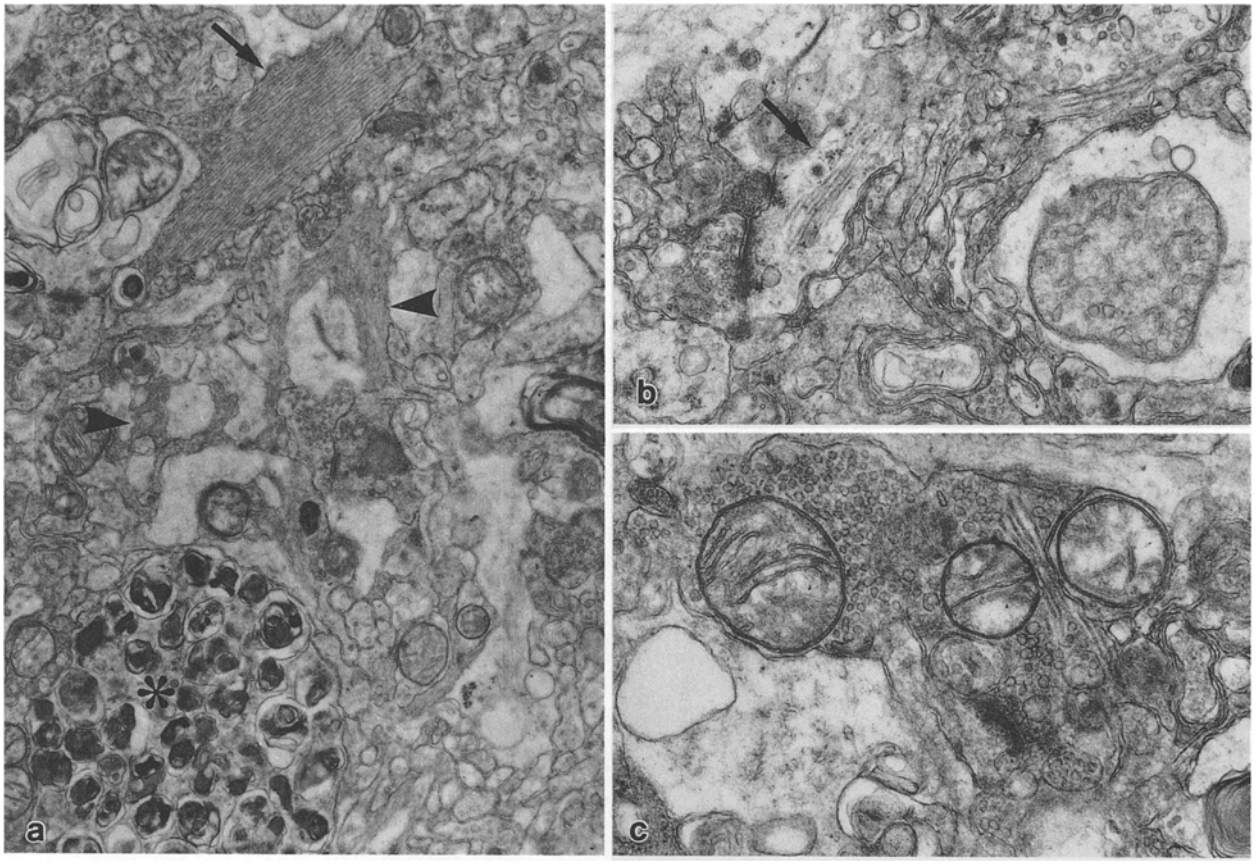

Fig. 5. Electron micrograph of the threads (*arrows*) in the primitive plaques which appeared in the temporal cortex. **a** Thread was associated with interwoven amyloid fibrils (*arrowheads*) and swollen neurite (*asterisk*). The swollen neurite, filled with dense bodies, contained no pathological filaments. **b** Thread (*arrow*) showed dendritic profile. **c** Some presynaptic terminals of the axon had constricted filaments. **a** ×14,000; **b** ×15,500; **c** ×20,000

borhood of plaques and NFT [3, 4, 7, 8, 11–13, 21]. The threads were observed only when NFT were present in the neocortex [2, 30]. The neuropil threads were not specific for ATD; they were observed in progressive supranuclear palsy (PSP) lesions in which abundant NFT were formed [29]. The appearance of threads was closely associated with NFT formation.

Kowall et al. [21] demonstrated aberrant localization of MAP tau in ATD brains by an immunohistochemical method. In the ATD brain tau accumulates in the dendrites and forms short and kinked "curly fibers", whereas it is located in axons in the normal human cortex. The occurrence of neuropil threads in dendrites of NFT-bearing pyramidal neurons was also demonstrated using double stain of Gallyas and Golgi [3] and using double-immunostaining for tau and MAP2 [12]. Our present study revealed that neuropil threads are often dendritic, confirming these observations.

Braak et al. [4] demonstrated PHF in neuropil threads by electron microscopy using vibratome sections which were impregnated by Gallyas silver method. However, routine electron micrographs of neuropil threads have not yet appeared in the literature. In 1964, Kidd [18] examined brain biopsy material by electron microscopy, and noted the presence of abnormal neurites having PHF in the neuropil which seemed normal when examined at low magnification. In this study, we revealed the ultrastructure of the neuropil threads. Additionally, we demonstrated that some SP were associated with similar threads. The border of SP was not clear and, moreover, diffuse plaques themselves could not be found by routine electron microscopic observation [42]. Therefore, we used neighboring semithin sections immunostained for β protein to clarify the exact plaque areas, and determined whether the threads were located inside the SP or outside. However, the ultrastructure of the threads, examined in this way, both inside and outside the SP, were similar, suggesting the same pathological processes.

The most common type of SP neurites were large swollen neurites filled with dense and lamellated bodies, and degenerating mitochondria. Recently, this type of degenerated neurite was experimentally produced in the rat brain, in which protease inhibitor, leupeptin, was infused continuously in the ventricular system [33]. However, threads were not observed in these experimental animals. In the present study, we demonstrated that the accumulation of threads in the plaque areas depends on the amount of amyloid fibrils detected within the plaques, suggesting that the accumulation is a reactive change induced by certain trophic factors associated with SP amyloid. Ihara et al. [12] revealed that the "curly fibers" resulted from the massive somatodendritic sprouting of

pyramidal cells, representing a regenerative reaction to neuronal degeneration. The neuropil threads, aberrant localization of MAP tau in the dendrites both inside and outside the SP, represent wide-spread changes of the neuropil throughout the neocortex, and may be closely related to the clinical appearance of dementia in ATD.

It is well known that tau antiserum labels pre-tangles or stage 0 tangles in the pyramidal cells, which synthesize tau protein in their cytoplasm but which have not yet formed pathological filaments [1, 15, 27]. In this study, tau-N antiserum labeled more abundant neuropil threads than PHF antiserum, suggesting that some tau-N-positive dendrites had not yet formed pathological filaments, and represented the early stage of neuropil thread formation which was nearly undetectable by electron microscopy. The tau-N immunoreactivity was completely abolished by the pronase pretreatment, whereas tau-C4 and PHF immunostaining were markedly enhanced by the pretreatment. Recently, it has been shown that pronase treatment removes tau-immunoreactive fuzzy coat from PHF [37], while the pronase-resistant core of PHF consists of the carboxyl third of tau [20, 37]. This explained the distinctive labeling of threads by the tau-C4 antiserum, directed toward the carboxyl third of tau.

In 1963, Kidd [17] demonstrated pathological filaments in NFT by electron microscopy, and referred to them as PHF, proposing a structure of double-helical filaments like the DNA model. However, many investigators have noted both PHF and straight filaments in the NFT of ATD [9, 26–28, 31, 32, 39], Guam parkinsonism-dementia complex [10], PSP [24, 34, 39], and lead encephalopathy [25]. We showed here that both neuropil and plaque threads consist of constricted and/or straight filaments. Recently, Miyakawa et al. [23] demonstrated eight protofilaments in NFT filaments, and found that PHF is not helical, but regularly constricted, proposing that the term PHF is not appropriate for expressing the pathological filaments of NFT. Moreover, recent immunoelectron microscopic studies [27, 28] have revealed that both PHF and tau antisera decorate straight filaments as well as PHF. The carboxyl third of tau may be the major constituent of both types of filament. Both filaments were components of the neuropil threads as well as NFT. Therefore, we now need a generic term such as "pathological tau filaments", which includes both constricted and straight filaments.

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