Neuropil Threads of Alzheimer's Disease Show a Marked Alteration of the Normal Cytoskeleton

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Abnormal neurites, neuropil threads, are a widespread and characteristic lesion of Alzheimer's disease likely to play a major role in the cognitive impairment of this disease. Contrary to normal neurites, neuropil threads contain straight and paired helical filaments that contain the microtubule-associated protein tau and ubiquitin. It is not known whether these abnormal filaments are added to or replace the normal cytoskeleton. In this study, we examined the fine structure of neuropil threads and carried out a morphometric analysis of the neurofilaments and abnormal filaments contained in the neuropil threads by using an antiserum to tau and colloidal gold immuno–electron microscopy. Almost 70% of the neuropil threads contained straight or paired helical filaments with no neurofilaments. The total number of filaments in each neuropil thread remained essentially unchanged either when straight or paired helical filaments were present alone or when they coexisted either together or with neurofilaments. When the three types of filaments were expressed as a proportion of the total, a linear inverse correlation was found between neurofilaments and straight filaments as well as between straight and paired helical filaments. Approximately 10% of the neuropil threads were found to be myelinated axons. It is concluded that straight filaments are likely to replace neurofilaments, that they in turn might be replaced by paired helical filaments, and that this process occurs in axons as well as dendrites.

Braak and co-workers (Braak et al., 1986) introduced the term "neuropil thread" to identify the abnormal neurites present in the cerebral cortex of subjects with Alzheimer's disease. Subsequent studies have shown that neuropil threads are a common and extensive alteration in this disease (Joachim et al., 1987; Kowall and Kosik, 1987; Ihara, 1988). Contrary to normal neurites present in autopsy tissue, neuropil threads are immunostained by antibodies to tau or ubiquitin, which immunodecorate either paired helical filaments (PHFs) or 14–16-nm

nonhelical filaments termed "straight filaments" (Onorato et al., 1989; Tabaton et al., 1989). Because no other cellular component is recognized by the tau and ubiquitin antibodies in neuropil threads, the presence of paired helical and straight filaments is the distinctive feature of these abnormal neurites and is the one that confers the abnormal antigenic features to them. Neuropil threads have been found to be closely correlated with the distribution, severity, and antigenic characteristics of perikaryal neurofibrillary tangles. Neurofibrillary tangles and neuropil threads are considered to be expressions of a widespread alteration of the neuronal cytoskeleton, referred to as neurofibrillary pathology, which has been shown to play a significant role in the expression of dementia in Alzheimer's disease (Tabaton et al., 1989; McKee et al., 1990).

An important question concerning the neurofibrillary pathology in Alzheimer's disease is whether the formation of paired helical and straight filaments is associated with concurrent changes of the normal cytoskeleton. We addressed this question with an immuno–electron-microscopic, morphometric study of neuropil threads. Analyses of these data show that straight filaments and PHFs replace the normal cytoskeleton in neuropil threads. These findings are consistent with the hypotheses that, in neuropil threads, straight filaments form either by the gradual transformation of preexisting cytoskeletal structures or by the utilization of important cytoskeletal components so that the normal cytoskeleton cannot be maintained.

Materials and Methods

Tissue source. Hippocampi and the adjacent entorhinal cortices from two cases aged 60 yr (case 1) and 71 yr (case 2), both with a definite history of dementia and fulfilling the National Institute of Aging (NIA) consensus criteria for Alzheimer's disease (Khachaturian, 1985), and corresponding tissue from the same region of two age-matched control cases were used. Tissue was fixed with Bouin's solution.

Antibody characterization. Rabbit antiserum to bovine brain tau (Connolly and Kalnins, 1980) was immunoaffinity purified by chromatography on Sepharose column containing tau coupled by cyanogen bromide activation of Sepharose 4B. Tau was prepared by glycerol precipitation of a perchloric acid extract of bovine brain (Lindwall and Cole, 1984). The specific antibodies were eluted using 0.1 M glycine (pH, 2.5) and standard procedures. Specificity of the affinity-purified antiserum to tau was proved by two means: (1) immunostaining of immunoblots prepared from 10% SDS-polyacrylamide gels (Laemmli, 1970) according to Towbin et al. (1979) containing either microtubule protein (Perry et al., 1982) or heat-stable microtubule-associated proteins (Herzog and Weber, 1978) and (2) immunoabsorption of the affinity-purified serum (0.25 µg/ml) performed with bovine brain tau (50 µg/ml; Lindwall and Cole, 1984) for 16 hr at 4°C. On immunoblots, the crude antiserum to tau recognized tau-protein bands and microtubule associated protein 2 (MAP2) and showed some background, but the
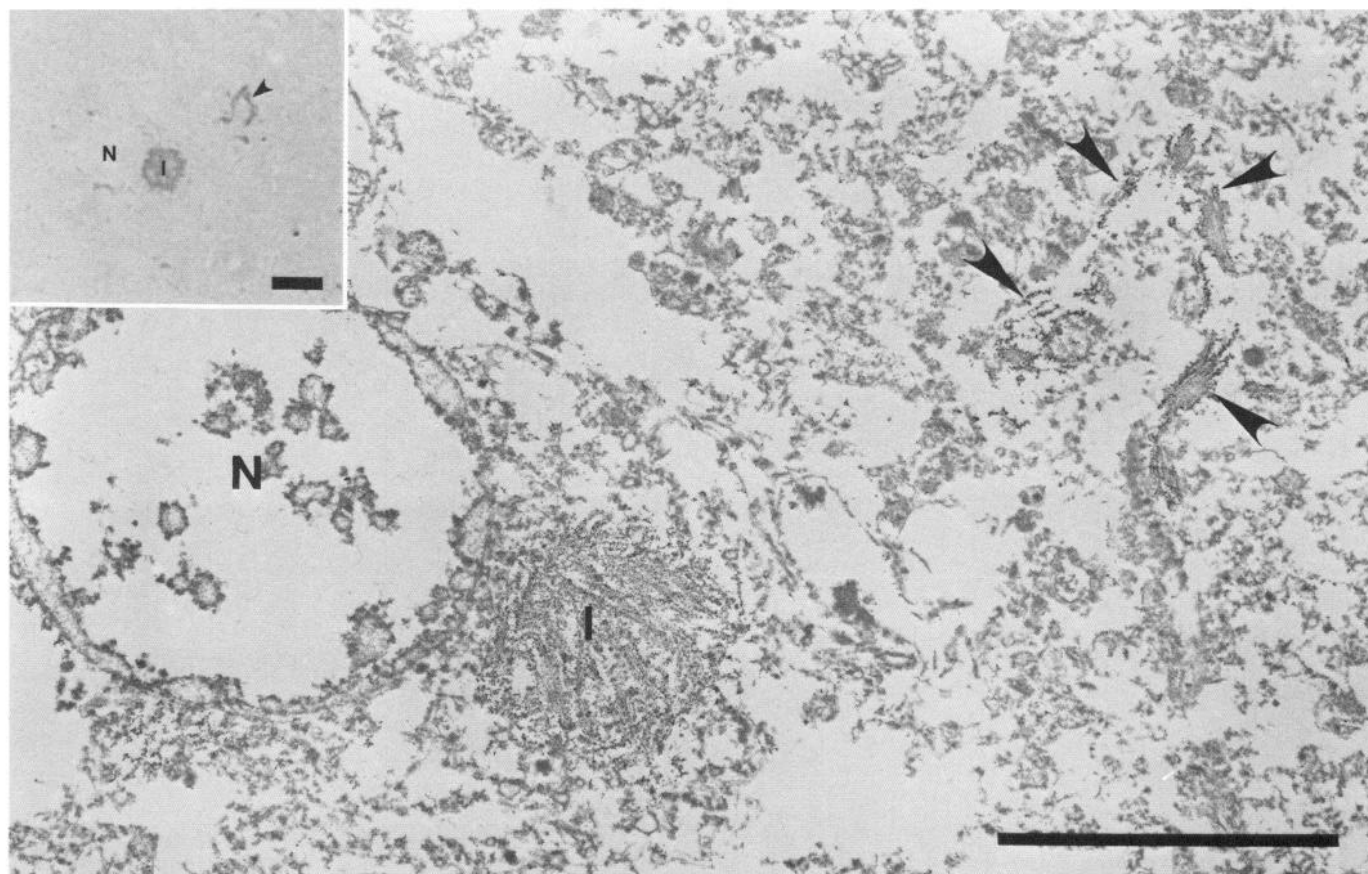
Figure 1. The ultrastructural correlate of the immunostained neuropil thread was demonstrated by correlative light and electron microscopy of the same specimen. The *inset* is a phase-contrast light micrograph of a 1-μm-thick plastic section with an immunostained neuropil thread (*arrowhead*) adjacent to a neuron containing an unusual juxtanuclear (*N*) immunostained inclusion (*I*). The next section (60 nm) was examined in the electron microscope. The same nucleus (*N*), immunodecorated inclusion (*I*), and immunodecorated neuropil thread (*arrowheads*) were again found. Antiserum to tau was used. Scale bars, 5 μm.

immunoaffinity-purified antibodies were selective for tau, showing only a weak reaction for MAP2 and no background (not shown). Absorption with purified tau abolished the reaction of the immunoaffinity-purified antiserum with tau bands in immunoblots and neurofibrillary pathology in tissue sections.

Light- and electron-microscopic immunocytochemistry. Immunocytochemistry for the light microscope was performed by the peroxidase–anti-peroxidase method (Sternberger, 1986) on tissue from all four cases after embedding in paraffin and sectioning at 6 μm. Vibratome (Oxford) sections (60 μm) for immuno–electron microscopy were prepared from the two cases of Alzheimer's disease without prior embedding of the tissue. Vibratome sections were treated with 0.5% Triton X-100 in 0.15 M NaCl and 50 mM Tris-HCl (pH, 7.6) at room temperature for 10 min, before incubation with the primary antibody for 2 d at 4°C. The sections were then incubated for 2–3 d with colloidal gold ($\simeq$17 nm) complexed to affinity-purified anti-rabbit IgG (Cappel Laboratories; DeMey, 1983). Sections were subsequently fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH, 7.2) for 2 hr followed by postfixation in 2% OsO_4 and flat embedding in Spurr's medium. Normal rabbit serum was substituted for primary antibody in order to evaluate background staining. Sections were viewed at 60 kV in a JEOL 100CX electron microscope.

Immuno–electron microscopy and quantitative analysis. Only immunodecorated neurites were considered neuropil threads and examined in both Alzheimer's cases. The neuropil threads were positively identified at the ultrastructural level with comparative light- and electron-microscopic examination of consecutive 1-μm-thick and 60-nm-thick sections, respectively, obtained from immunogold-decorated tissue. As shown in Figure 1, the immunodecorated neuropil thread observed in a 1-μm-thick section under the light microscope was then identified using tissue landmarks in the consecutive 60-nm-thick section viewed with the electron microscope. There was a close correlation between

neuropil threads detected at light and electron microscopy because even neuropil threads with only three immunodecorated filaments (not shown) were visible in the light microscope. For this reason, control cases that did not show any neuropil thread at light-microscopic examination following immunostaining with the antiserum to tau were not studied with immuno–electron microscopy.

Quantitative analyses were carried out only in Alzheimer case 2. Sections were scanned at 20,000 magnification so that, if even only one labeled filament was identified, the structure could be classified as a neuropil thread. Special attention was paid to exclude any filament bundles near amyloid fibrils and neuronal cell bodies. Micrographs at 20,000 and 40,000 magnification were made of each of the 205 neuropil threads found in the 0.44 μm² examined. The filament type could be determined unambiguously in 168 neuropil threads. The primary reason for excluding neurites was poor micrograph quality. Many neuropil threads were examined in tangential or cross sections, which allowed the evaluation of all the filaments. In neuropil threads cut longitudinally, only the filaments in the plane of sections could be evaluated. Moreover, in these threads the same filament might have been scored twice when coming in and out of the plane of section, yet even in the latter neurites, the bias should affect only the absolute number of filaments and not the type representative because all filament types should be equally affected. The distribution between tangentially and longitudinally cut neurites was entirely random and thus did not introduce any bias in the representation of each of the three types of filaments examined (see Results).

Results

The antiserum to tau-immunostained neuropil threads, neurofibrillary tangles, and dystrophic neurites in senile plaques
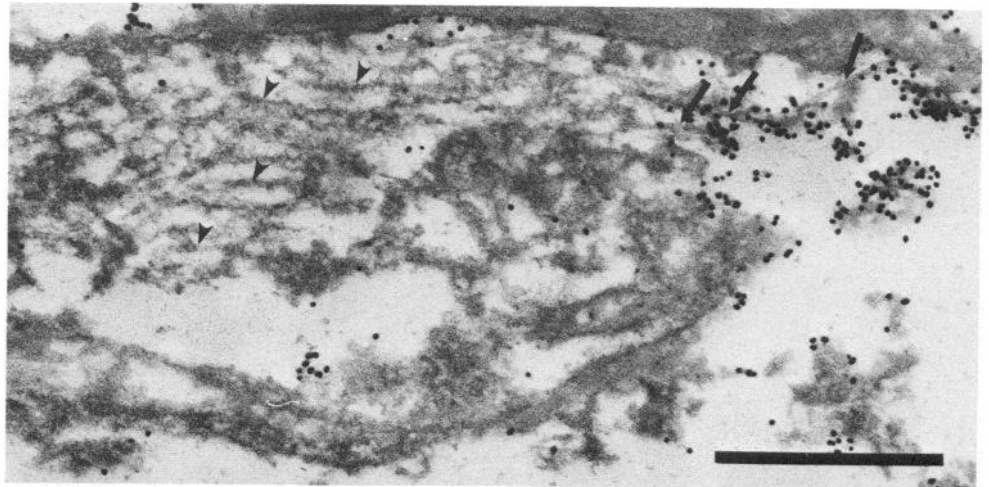
Figure 2. Immuno–electron micrograph of a myelinated neuropil thread in which neurofilaments predominate. Immunodecoration is associated with the straight filaments (*arrows*), while the abundant neurofilaments (*arrowheads*) contain few if any gold particles. Antiserum to tau was used. Scale bar, 500 nm.

within the gray matter of both Alzheimer's disease cases was examined. The antiserum did not react with any structures in sections prepared from the same brain regions of the two age-matched control cases (not shown), which were not investigated further (see below). Electron-microscopic examination of the immunodecorated tissue showed that the antibodies recognized both paired helical and straight filaments present in neurofibrillary tangles (not shown). In addition, immunodecoration of filaments organized in "bundles" was also prominent. These filamentous bundles were smaller and more irregular and elongated than neurofibrillary tangles. Moreover, they lacked lipofuscin and differed from dystrophic neurites of senile plaques, because they were not adjacent to amyloid fibers. Some of the immunostained filamentous bundles seen in the electron microscope were contained in processes that were clearly myelin-

ated, in which case the decoration on some filaments depended on their proximity to an opening in the myelin (see, e.g., Fig. 3). The filamentous bundles were positively identified as neuropil threads with combined light- and electron-microscopic examination of 1-μm-thick and 60-nm-thick consecutive sections, as demonstrated in Figure 1, where the immunodecorated neuropil thread observed in a 1-μm-thick section under the light microscope can be recognized using tissue landmarks in the consecutive thin section viewed with the electron microscope.

Electron-microscopic analysis showed that neuropil threads contained three major types of filaments: neurofilaments (Fig. 2), straight filaments (Fig. 3), and PHFs (Fig. 4). In addition, two intermediate types were occasionally observed, one displaying an equivocal helicity consisting of periodic constrictions in diameter (Fig. 5), different from that of PHFs, and another
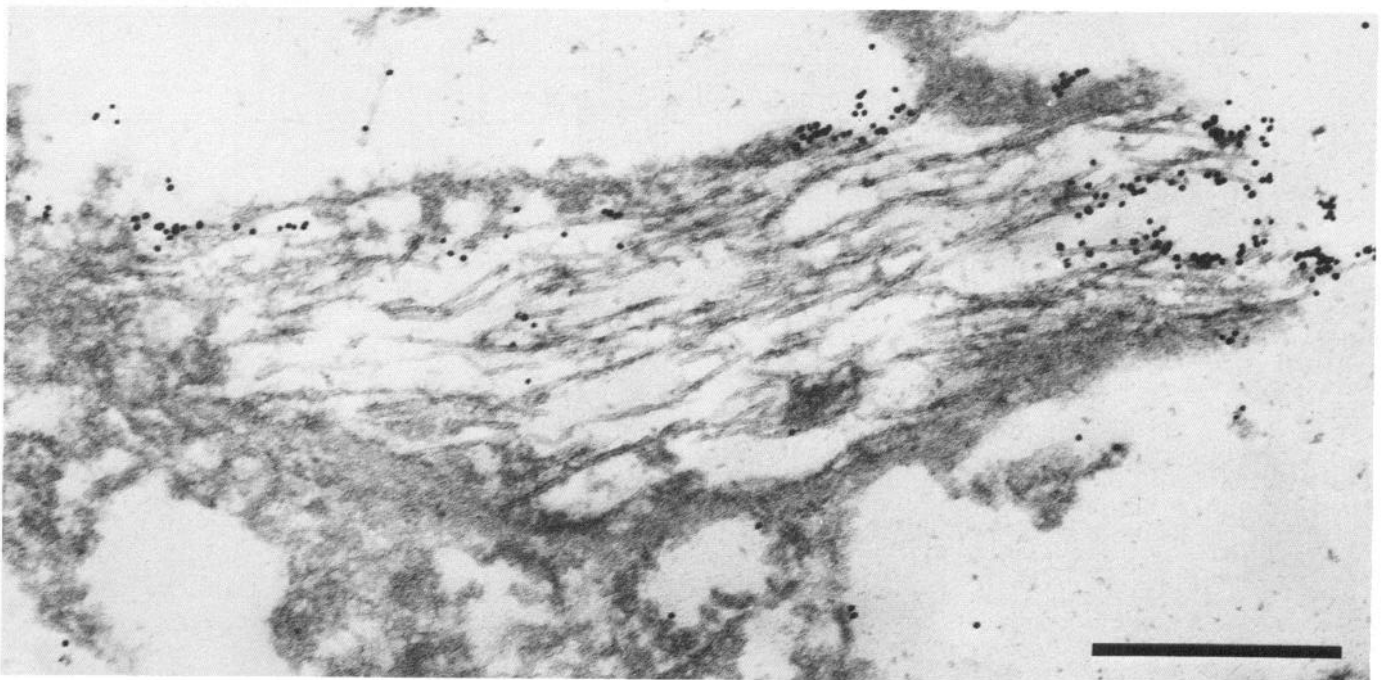

Figure 3. Over 10% of neuropil threads are myelinated. The heterogeneous decoration of straight filaments is probably due to poor penetration of gold particles through the sheath, presumably myelin, covering the filament bundles. Antiserum to tau was used. Scale bar, 500 nm.
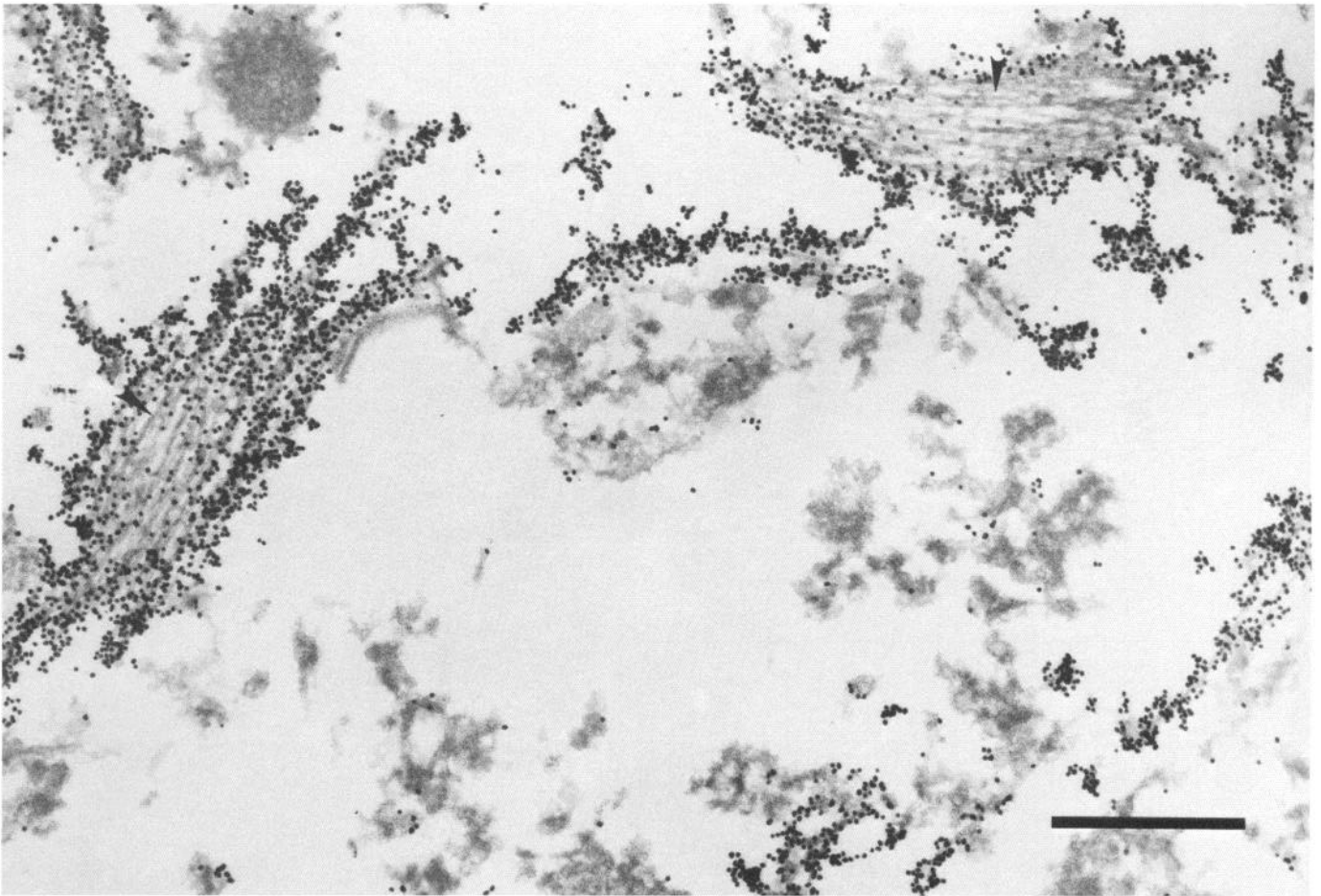
Figure 4. When viewed at high magnification, the neuropil thread shown in Figure 1 demonstrates abundant PHFs (*arrowheads*; see text). Antiserum to tau was used. Scale bar, 500 nm.

displaying a surface smoother than that of neurofilaments and yet having side arms and the size of neurofilaments (Fig. 6).

Morphometric analysis was carried out in neuropil threads to determine both in absolute as well as in relative number the prevalence and possible interrelationships of neurofilaments, straight filaments, and PHFs in neuropil threads. The few intermediate types of filaments encountered were counted according to their dominant feature.

In almost 70% of the neuropil threads, straight filaments, and/ or PHFs were present alone, without neurofilaments (Table 1). PHFs alone were seen only in the absence of neurofilaments (Table 1). The total numbers of filaments either when straight filaments and PHFs were present alone or when they coexisted with the neurofilaments were not significantly different ($p = 0.44$, Kruskal–Wallis; Table 1), suggesting that the addition of one filament type was associated with the loss of another; for example, neurofilaments were negatively correlated with straight filaments ($r = 0.33$; $p = 0.015$). When the three types of filaments were expressed as a proportion of the total, there was a linear inverse correlation between presence of neurofilaments and straight filaments on the one hand and between presence of straight filaments and paired PHFs on the other (Fig. 7). These findings indicate that straight filaments and PHFs replace, rather than are added to, the neurofilaments in neuropil threads.
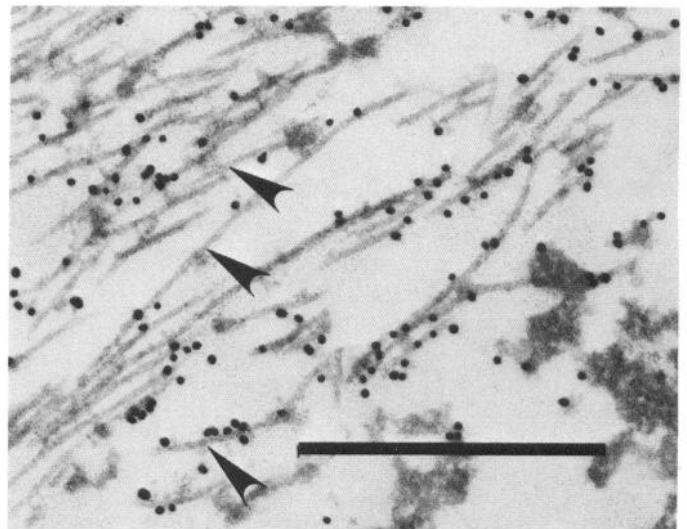

Figure 5. Helical filaments have a morphology intermediate between straight filaments and PHFs (see Results). *Arrowheads* indicate some of the filaments showing periodic constriction in diameter. Antiserum to tau was used. Scale bar, 500 nm.
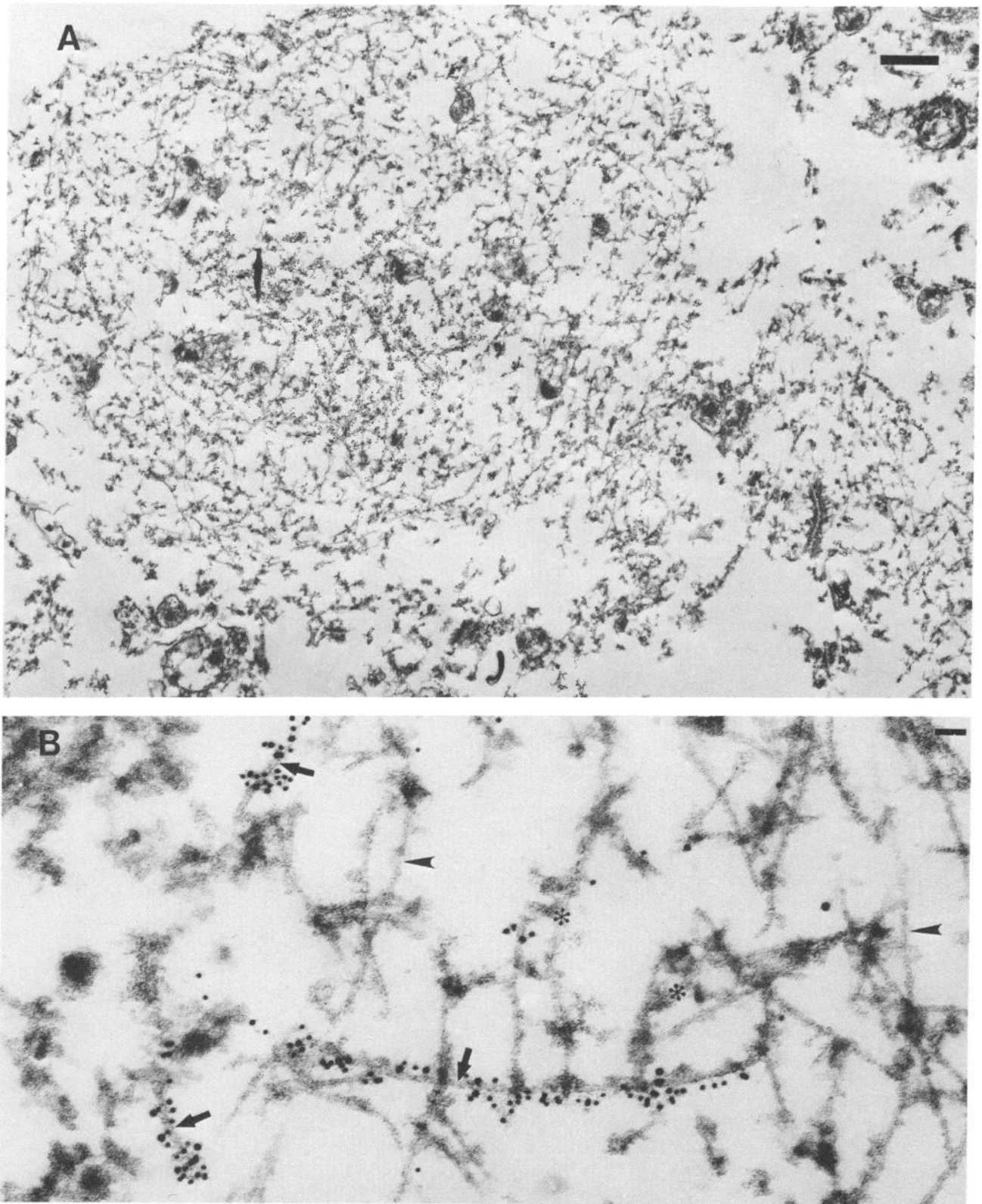
Figure 6. Straight filaments (14.6 ± 0.4-nm diameter) are appreciated in a neuropil thread in which they coexist with neurofilaments. *B,* Viewed at higher magnification, the intense decoration of the relatively more rigid and smoother-surfaced decorated straight filaments (*arrows*) is appreciated as distinct from the neurofilaments (11.3 ± 0.1-nm diameter; *arrowheads*). Some neurofilaments have less prominent side arms and look similar to straight filaments, though they were not decorated (*). Antiserum to tau was used. Scale bars: *A,* 500 nm; *B,* 60 nm.
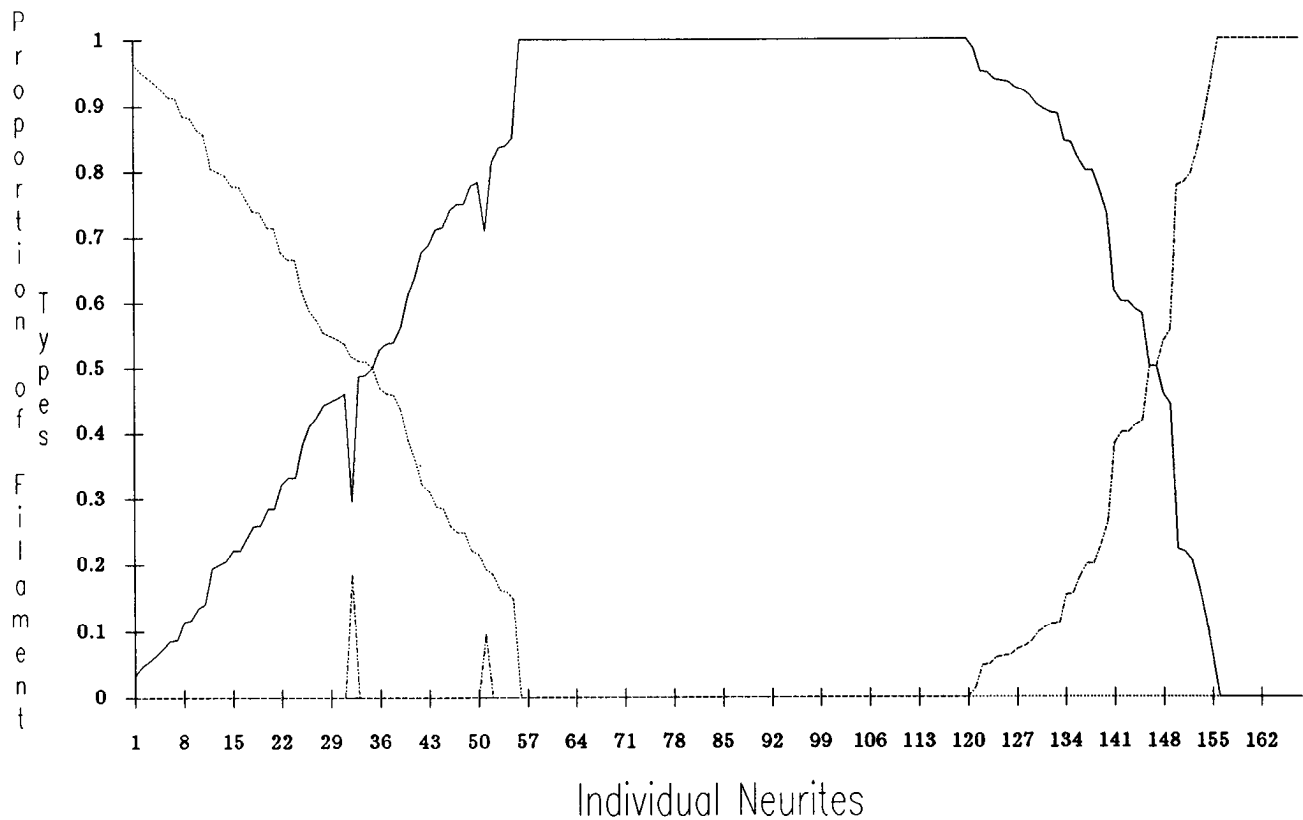
Figure 7. Proportion of filament types in individual neuropil threads for an area of 0.44 mm² in case 2. The neurites have been arranged according to decreasing neurofilaments, in their absence in increasing PHFs, and in the absence of both, in between. *Dotted line*, neurofilaments; *solid line*, straight filaments; *dot-dashed lines*, PHFs.

Although lacking PHFs, the 17 myelinated neuropil threads showed a similar inverse correlation between presence of neurofilaments and straight filaments ($r = 0.76$; $p < 0.01$).

Discussion

Despite the obvious limitations imposed on this study by the suboptimal preservation of the brain parenchyma due to postmortem autolysis, the present findings demonstrate that, in most neuropil threads of Alzheimer's disease, abnormal filaments, which include straight filaments and PHFs, replace the neurofilaments. Thus, in neuropil threads, the presence of straight filaments and PHFs is associated with a profound alteration of the preexisting cytoskeleton. A similar inverse correlation be-

tween PHFs and microtubules was observed (Ellisman et al., 1987; Metuzals et al., 1987, 1988) in a study of brain tissue obtained at biopsy, which, however, did not include morphometric analysis. Although microtubules could not be examined in our tissue samples, it is likely that straight filaments and PHFs replace not only neurofilaments but also microtubules in neuropil threads and that this profound alteration of the neuronal cytoskeleton is present also in the perikaryon of neurons containing neurofibrillary tangles. Thus, this study underlines the severe cytoskeletal alterations that neurons may undergo in Alzheimer's disease.

The modalities by which straight filaments and PHFs replace neurofilaments and possibly other cytoskeletal components such

Table 1. Neuropil threads divided by the constituent filaments and the number of various filament types in each

Constituent filaments	Number of neuropil threads	NF	SF	PHF	Total
NF and SF	53 (32%)	17.1 ± 1.6	15.2 ± 2.9	—	32.3 ± 3.7
NF and PHF	0	—	—	—	—
NF, SF, and PHF	2 (1%)	10.0 ± 5.7	15.0 ± 9.9	4.0 ± 1.4	29.0 ± 2.8
SF	65 (39%)	—	36.6 ± 3.9	—	36.6 ± 3.9
SF and PHF	35 (21%)	—	33.5 ± 6.7	14.7 ± 3.4	48.3 ± 7.8
PHF	13 (8%)	—	—	42.2 ± 16.4	42.2 ± 16.4

Values are mean ± standard error of the mean. NF, neurofilaments; SF, straight filaments.

as microtubules remain to be established. The simplest inter-
pretation of the present data is that straight filaments form either
directly by transformation of neurofilaments or indirectly by
the utilization of neurofilament or other cytoskeletal constitu-
ents so that the normal cytoskeleton cannot be maintained. The
formation of straight filaments does not necessarily take place
in the neurites because they could migrate to the neurites from
the cell body by axonal or dendritic transport. Three lines of
evidence support the origin of straight filaments from the normal
cytoskeleton. First, as our findings show, there is an inverse
relation between neurofilaments and straight filaments, and only
few neurites contain similar ratios of these two types of fila-
ments. Second, straight filaments share epitopes with the normal
cytoskeleton suggesting that these two structures have common
components. A third line of evidence comes from the findings
that straight filaments of Alzheimer's disease are insoluble in
ionic detergents (Perry et al., 1987) and that they contain epi-
topes of ubiquitin (Manetto et al., 1988). In an experimental
model, it was reported that abnormal ubiquitin is associated
with the filaments of the Mallory bodies and serves as a marker
for the intermediate filaments destined to be incorporated into
the inclusion (Ohta et al., 1988). Ubiquitination was associated
with an increase in diameter, a decrease in solubility, and a
change of antigenic characteristics of the cytokeratin interme-
diate filaments in Mallory bodies. These changes are similar to
the relationship we note here between neurofilaments and straight
filaments. Should this be the case, then straight filaments of
neuropil threads would derive from preexisting neurofilaments
through an extensive transformation in which the acquisition
of tau and ubiquitin and change in size, solubility, and antigenic
characteristics would be some of the steps.

PHFs alone were never seen concurrently with neurofilaments
in the same neuropil threads; they were seen either alone or in
the presence of straight filaments. This finding indicates that
formation of PHFs occurs when the preexisting cytoskeleton is
markedly altered, and therefore it is a late event in the patho-
genesis of the neuropil thread. Concerning the mode of for-
mation of PHFs, one may apply the same rationale used above
to postulate that PHFs derive directly or indirectly from the
straight filaments. Consistent with this hypothesis is the finding
that the presence of straight filaments is believed to precede
the appearance of PHFs in the formation of neurofibrillary tan-
gles (Tabaton et al., 1988). In the case of PHFs, however, these
abnormal filaments might well form independently from straight
filaments in a subpopulation of neurites.

Approximately 10% of the neuropil threads were found to be
myelinated (Figs. 2, 3). This may be an underestimation of the
actual number of axons that have become neuropil threads be-
cause not all gray matter axons are myelinated. The finding that
a significant number of neuropil threads are axons contrasts
with the suggestion that they form solely from dendritic sprout-
ing (Braak and Braak, 1988; Ihara, 1988; McKee et al., 1989)
but is consistent with the straight filaments shown in myelinated
axons of biopsy specimens (Metuzals et al., 1987).

In conclusion, though the origin of the straight filaments and
PHFs present in neuropil threads must remain speculative, the
present study definitely shows that the presence of these ab-
normal filaments in neuropil threads is associated with severe
decrease or disappearance of neurofilaments. On the basis of
the present findings, it can be postulated that straight filaments
and PHFs are the only structures related to the cytoskeleton left

in most neuropil threads of Alzheimer's disease. Such an alter-
ation of the cytoskeleton is likely to cause a marked reduction
of functions such as axonal and dendritic transport.

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