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Senile plaque neurites in Alzheimer disease accumulate amyloid precursor protein

(β-amyloid protein/τ/ubiquitin/neurofilaments/dystrophic neurites)

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ABSTRACT **Senile plaques are polymorphous β-amyloid protein deposits found in the brain in Alzheimer disease and normal aging. This β-amyloid protein is derived from a larger precursor molecule of which neurons are the principal producers in brain. We found that amyloid precursor protein (APP)-immunoreactive neurites were involved in senile plaques and that only a subset of these neurites showed markers for the abnormal filaments characteristic of neurofibrillary pathology. In the neocortex of nondemented individuals with senile plaques but spared of neurofibrillary pathology, dystrophic neurites in senile plaques showed only APP accumulation. In contrast, in the brains of Alzheimer patients, virtually all APP-immunoreactive neurites also showed immunoreactivity with ubiquitin, τ, and phosphorylated neurofilaments. The presence of τ and neurofilament epitopes in dystrophic neurites in senile plaques was correlated with the extent of neurofibrillary pathology in the surrounding brain tissue. Accumulation of APP and the formation of neurofibrillary pathology in senile plaque neurites are therefore distinct phenomena. Our findings suggest that APP accumulation in senile plaque neurites occurs prior to τ accumulation and is therefore more closely related to appearance of neuritic dystrophy.**

Senile plaques (SP) are extracellular amyloid deposits found in the brain most prominently in Alzheimer disease (AD) but also in normal aging (1). Their 6- to 10-nm-wide filaments consist of a 39- to 42-amino acid β-amyloid protein (β-AP) (2), which is derived from a much larger transmembrane amyloid precursor protein (APP) (3–5). The morphology and size of SP are highly variable and are regionally dependent (6). Recent studies have addressed the pathogenetic role of neurons, astrocytes, microglia, and capillaries in the development of SP (7–11). Another prominent lesion of AD is the accumulation of straight and paired helical filaments in neuronal cell bodies and in neurites (12). Studies of Down syndrome patients, who invariably develop pathology like that in AD, have shown that amyloid is probably deposited before any neurofibrillary pathology occurs (13). The most frequent form of dystrophic neurites in AD are neuropil threads that are not confined to the SP (14). Furthermore, neuropil threads are also found in progressive supranuclear palsy (15) and subacute sclerosing panencephalitis (16) in the absence of β-AP deposits. Neuritic SP contain dystrophic neurites that accumulate paired helical filaments and membranous dense bodies (17). It is not established whether these dystrophic neurites are a reactive phenomenon or if they are actually contributing to the amyloid deposit. The primary role of dystrophic neurites in producing the amyloid deposit has been questioned because τ-immunoreactive dystrophic neurites are rare in diffuse-type SP, which may be the earliest stage of SP (18). Recently, however, τ-negative, but synaptophysin- and ubiquitin-immunoreactive, neurites have been demonstrated in preamyloid deposits (19).

APP has been localized to cell processes that were tentatively identified as neurites in AD brains (20–23). We reported previously that APP-positive cell processes occur in the absence of β-AP deposits, in diffuse SP, as well as in fully developed β-AP core-containing plaques (24). These findings suggested that APP accumulation in cell processes could contribute to the deposition of β-AP in SP.

The present study was undertaken to define the cellular origin of APP-immunostained cell processes in SP and to define their relationship to neurofibrillary pathology in AD and normal aging.

MATERIAL AND METHODS

We studied the hippocampus and the temporal and frontal cortex of 24 AD patients (mean age 77, range 65–87, mean postmortem interval 2.8 hr, range 2–9 hr) and 18 aged and clinically normal controls (mean age 66, range 31–82, mean postmortem interval 10 hr, range 2.5–48 hr). Clinical and pathological diagnoses were made according to established criteria (25), which included a documented progressive cognitive decline in the AD patients. Brain tissue of mentally intact controls was obtained from the Cuyahoga County Coroner's Office. Most of these subjects died as a result of a motor vehicle accident or from cardiovascular disease. In no case was there any history of mental impairment.

Coronal slices of hippocampus and temporal cortex at the level of the corpora mamillaria and of the middle third of the superior frontal gyrus were fixed in methacarn for 16 hr. Six-micrometer paraffin sections were bleached in 3% hydrogen peroxide in methanol for 30 min, washed in 0.05 M Tris-buffered saline with 1% normal goat serum, and, after blocking with 10% normal goat serum, incubated with primary antibodies (Table 1) at 4°C for 16 hr. We used the unlabeled antibody bridge technique (26) to detect immunoreactivity.

We determined the density of neurofibrillary tangles (NFT) and SP in different regions (frontal cortex, entorhinal cortex, subiculum) in AD and controls. For this, sections were immunostained with Alz-50 and the number of immunostained NFT, SP, or both in three fields of 0.198 mm^2 was determined at ×250.

To study the relation of APP accumulation and cytoskeletal abnormalities in SP neurites, we selected three AD patients

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Abbreviations: AD, Alzheimer disease; β-AP, β-amyloid protein; APP, amyloid precursor protein; NFT, neurofibrillary tangles; phNF-H, heavy molecular weight subunit of phosphorylated neurofilaments; SP, senile plaques.

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Table 1. Antibodies

Antibody	Antigen*	Dilution	Ref.
Anti-Bac695	APP695	1:300	24
Anti-Bac770	APP770	1:300	24
RGP-3	APP-(45–62)	1:100	20
RGP-8	APP-(638–658)	1:100	20
RGP-9	APP-(597–638)	1:500	24
597	APP-(597–609)	1:20	27
2A1/10B10	APP-(597–606)	1:20	†
5E2	τ	1:5	28
Alz-50	τ	1:50	29
R26	NF	1:500	30
1.1.1	NF-H	1:200	31
SMI-34	phNF-H	1:1000	‡
4.2D8	Ubiquitin	1:100	32

*APP695 and APP770 are full-length recombinant APP; APP-(45–62) is residues 45–62 of APP, etc. NF, neurofilament; NF-H, heavy molecular weight subunit of NF; phNF-H, phosphorylated NFH.
†Gift of G. Glenner (University of California, San Diego) and J. Zuckermann (DuPont, Newark, DE).
‡From Sternberger Monoclonals (Baltimore).

and three controls that had at least some SP in hippocampus and neocortex. We first immunolabeled with a rabbit antibody to APP by using the avidin-biotinylated alkaline phosphatase technique with naphthol AS-MX phosphate (Sigma) and fast red TR salt (Sigma). We photographed the sections and removed the phosphatase reaction product with ethanol and xylene. The sections were then incubated with a second antibody (to ubiquitin, τ, or phNF-H) and subjected to the immunoperoxidase technique with diaminobenzidine (Sigma) as cosubstrate. This result was also photographed, so that pairs of photographs could be compared. In this way, we could study the colocalization of several antigens in the same neurites. We counted the SP neurites that were immunoreactive for APP alone and for τ, ubiquitin, or phNF-H alone or together with APP. In total, we studied 106 pairs of photographs and approximately 2500 neurites in three controls and three AD patients. As a control, we reversed the order of the immunoreactions to exclude blocking of epitopes and interference with subsequent antibody binding.

Characterization and absorption controls for the APP antibodies have been described (24). The references in Table 1 contain the data on characterization of the other antibodies used in this study.

RESULTS

APP and β-AP Immunostaining. All the APP antibodies directed to regions outside the β-AP domain (anti-Bac695, anti-Bac770, RGP-3, RGP-8) recognized fine granular structures in neurons and neurites (Fig. 1*A*). In the subiculum of the AD patients, there were also rare pyramidal neurons (Fig. 1*C*) that showed intense and homogeneous cytoplasmic immunoreactivity for APP. Some of these neurons carried NFT and many were shrunken, with their nucleus no longer detectable. In several control brains and in all the AD brains, we found small neuritic clusters and single APP-immunoreactive neurites (Fig. 1*B*). By double immunostaining with APP and β-AP antibodies, we demonstrated that some of these clusters and single neurites were surrounded by small amyloid deposits, while others were unrelated to amyloid (results not shown). In AD patients as well as in controls about one-half of the diffuse and virtually all neuritic and core-containing SP (Fig. 1*D*) contained APP-immunoreactive neurites. Microvessel walls, astrocytes, oligodendrocytes, and microglia remained unlabeled. We found no qualitative difference in immunostaining with antibodies to recombinant APP695, APP770, synthetic peptides APP-(45–62) (sequence as in ref. 3) and APP-(638–658) (Table 1). However, the latter two stained neurites less intensely.

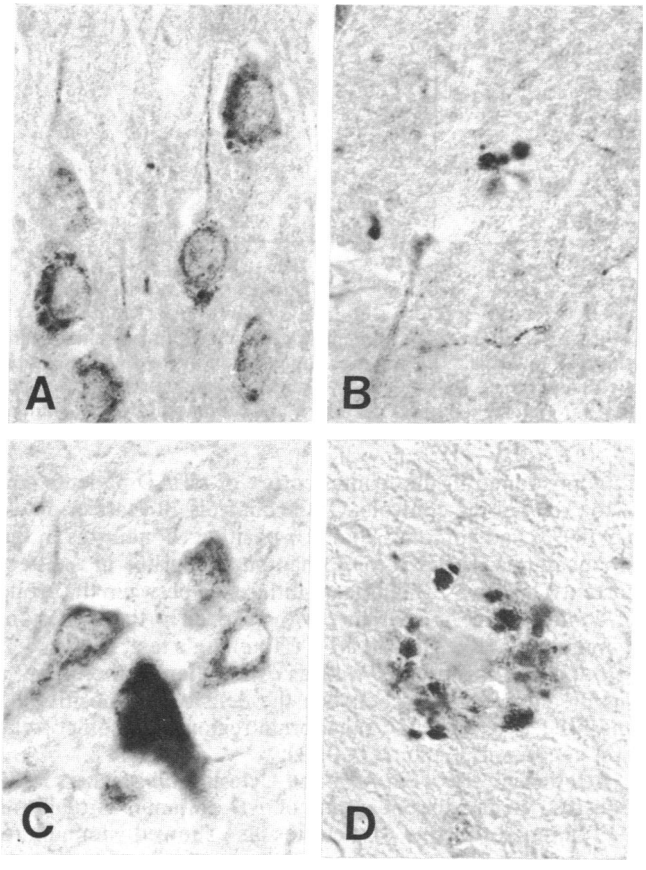

FIG. 1. (*A*) Normal neurons and neurites show a granular immunostain with anti-Bac695. (*B*) Neurites of normal caliber and neuritic clusters are immunostained with anti-Bac695. (*C*) In addition to normal neuronal staining, rare degenerating neurons are intensely immunoreactive for APP (anti-Bac695). APP-immunoreactive dystrophic neurites surround a core containing SP (anti-Bac695). (*D*) The SP core itself is not stained. (All ×580.)

Using antibodies directed to the β-AP region (RGP-9, 597, and 2A1/10B10), we found numerous SP in 24/24 (100%) of AD brains and in the hippocampus of 9/18 (50%) of the control patients. The youngest controls did not show any amyloid deposits and no abnormal neurites were found. Variable numbers of amyloid deposits were present in the neocortex of 5/16 (31%) of the controls. Of the controls over 70 years old, 8/9 (89%) showed β-AP deposits in the hippocampus and 5/8 (63%) in the temporal and frontal neocortex. In the controls, these SP were predominantly of the diffuse type, especially when present in neocortical areas.

τ, Ubiquitin, and Neurofilament Immunostaining. Normal immunostaining of τ (5E2) and neurofilaments (SMI-34, 1.1.1) was present in all brains: τ immunolabeling consisted of a fine network of immunoreactive neurites in layers I and less in II and in subcortical white matter. Normal immunostaining for neurofilaments was present as an extensive neuritic network and also as white matter axonal staining. Ubiquitin (4.2D8) immunostaining was present in all the brains as intense labeling of SP neurites and NFT when present, diffuse vessel staining, and focally as nuclear staining. Also, corpora amylacea and small rounded corpuscles in gray and less in white matter were intensely stained.

In all the AD patients numerous τ-immunoreactive neuropil threads were present in the hippocampus. They were much less numerous but were present in 9/18 (50%) of the control hippocampi. Also, variable numbers of NFT were

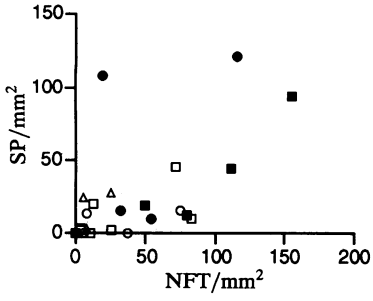

FIG. 2. Presence of τ immunoreactivity in SP is correlated with τ-immunoreactive NFT in the same region (Pearson correlation coefficient 0.680, $P < 0.001$). □, Control subiculum; ■, AD subiculum; ○, control temporal cortex; ●, AD temporal cortex; and △, control frontal cortex.

immunostained in the frontal cortex of all AD patients and 5/16 (31%) of the controls. Of the controls 70 years or older, 8/9 (89%) had some NFT- and τ-positive SP neurites in the hippocampus, and 5/8 (63%) had such neurites in the neocortex. There was a significant difference between the group of controls and AD patients with respect to the density of τ-immunoreactive SP and NFT (Hotelling's test, $F = 7.08$, $P = 0.02$). In controls as well as AD patients, there was a significant correlation between the density of τ-positive SP and NFT (Fig. 2) in the same brain region (Pearson correlation coefficient 0.680, $P < 0.01$).

Relationship Between APP and Cytoskeletal Markers in SP Neurites. In the hippocampus of AD patients, virtually all APP-immunoreactive SP neurites also showed τ immunoreactivity (Fig. 3). On the other hand, not all τ-immunoreactive neurites were also APP-immunoreactive. In particular, many τ-immunoreactive neuropil threads in the corona of SP remained unlabeled by APP antibodies. In contrast, in brains of mentally intact controls, the proportion of neurites that was APP positive and τ negative was much higher (Fig. 4). When no NFT were present in the surrounding cortex, the SP neurites showed only APP accumulation and no τ positivity (Fig. 5). The presence of τ in SP neurites varied with the degree of neurofibrillary pathology. Most of the τ-negative neurites in control brains also remained unlabeled with antibodies to ubiquitin or phosphorylated neurofilaments.

There were two remarkable types of abnormal neurites, both prominent in AD brains: first, swollen and solely APP-immunoreactive neurites that were not related to amyloid deposits (not shown); second, the numerous neuropil threads that were mostly τ immunoreactive but APP negative (Fig. 3).

DISCUSSION

Several important conclusions can be drawn from our study: First, by the presence of normal and altered neuronal cytoskeletal components, APP-immunoreactive cell processes could be unequivocally identified as neurites. Second, the presence of cytoskeletal alterations in APP-immunoreactive SP neurites is related to neurofibrillary pathology in the surrounding brain.

It is well established that in Down syndrome, amyloid deposits are found in a younger age group than NFT and τ-immunoreactive neurites (33, 34). A similar phenomenon occurs in a cross-sectional autopsy study that includes mentally intact elderly people and Alzheimer patients. As the incidence of AD in the control group is high, some of the control patients could have developed dementia had they lived longer. Also, mild dementia in some of the control patients could have gone unnoticed (35). Therefore, in this population, different degrees of pathology would be expected, some corresponding to normal aged brains, others to preclinical AD, still others to fully developed Alzheimer pathology. It is reasonable to assume that these different degrees of abnormality would reflect the development of lesions in a single individual over time. At one end of this spectrum, we found that τ-immunoreactive SP neurites and NFT were most prominent in AD patients and that APP-immunoreactive neurites in these cases consistently displayed τ immunoreactivity. In controls, SP neurites were always APP positive, but only a small proportion were also τ immunoreactive. In this group, there was a tendency for SP neurites to show τ immunoreactivity, but only when NFT were present in the neurons of the same brain region. These results confirm and expand on

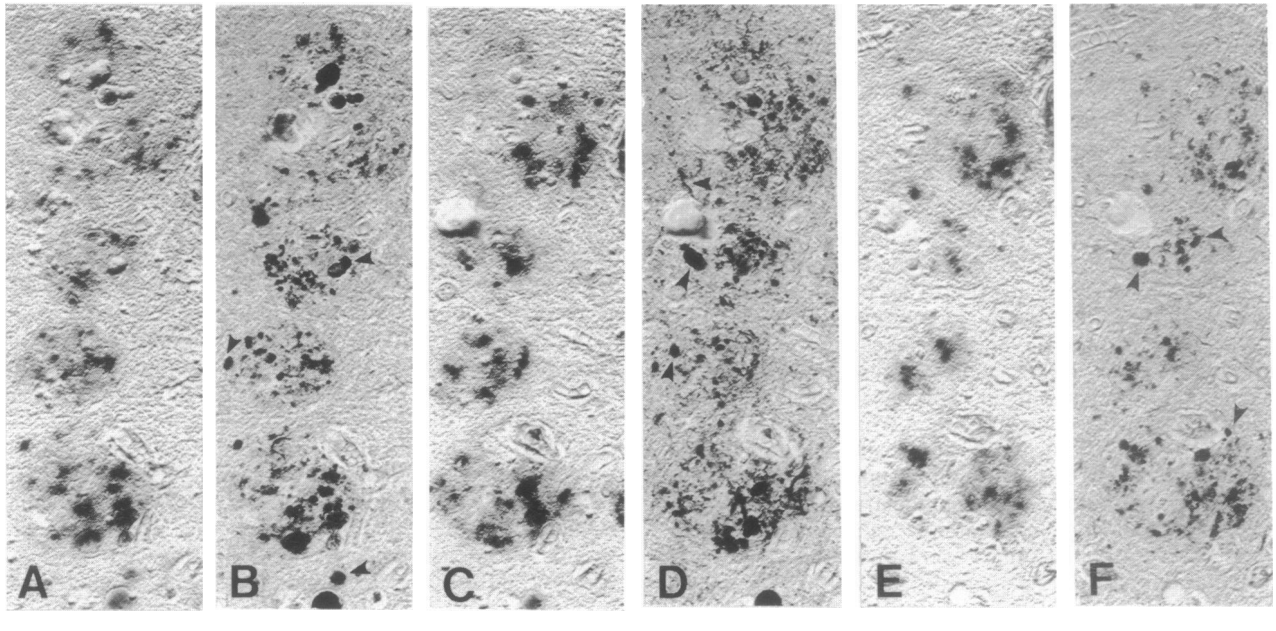

FIG. 3. Three serial sections of SP in the dentate gyrus in AD hippocampus. First, the sections were immunostained with APP antibody (anti-Bac695) (*A*, *C*, *E*), then we removed the chromogen and immunostained for ubiquitin (42D8) (*B*), τ (Alz-50) (*D*), or phosphorylated neurofilaments (SMI-34) (*F*). All the APP-immunoreactive neurites also show positivity for the second marker. In addition, some structures are immunoreactive only for ubiquitin, τ, or neurofilaments (arrowheads). *D* also shows many APP-negative, τ-immunoreactive neuropil threads. (All ×250.)

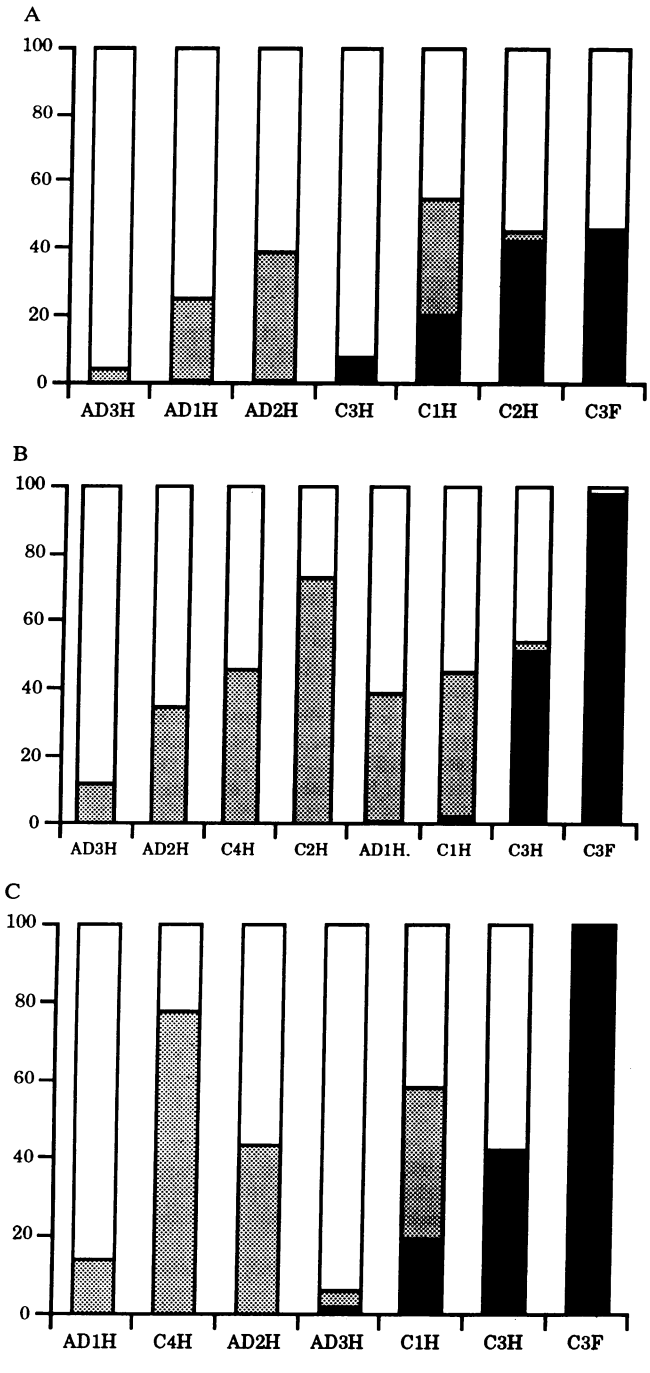

FIG. 4. Percentage of neurites that show immunoreactivity for APP and ubiquitin (*A*), τ (*B*), and phosphorylated neurofilaments (*C*) in AD and control hippocampus and control frontal cortex. The columns are ordered from left to right according to increasing proportion of APP-positive neurites, which is lowest in AD hippocampus and highest in control neocortex. For comparison, the numbers in graphs refer to identical cases. AD, Alzheimer disease; C, control; H, hippocampus; F, frontal cortex. Columns: black, APP immunoreactivity only; gray, colocalization of APP and, respectively, ubiquitin, τ, and neurofilaments; white, respectively, ubiquitin, τ, or neurofilament immunoreactivity only.

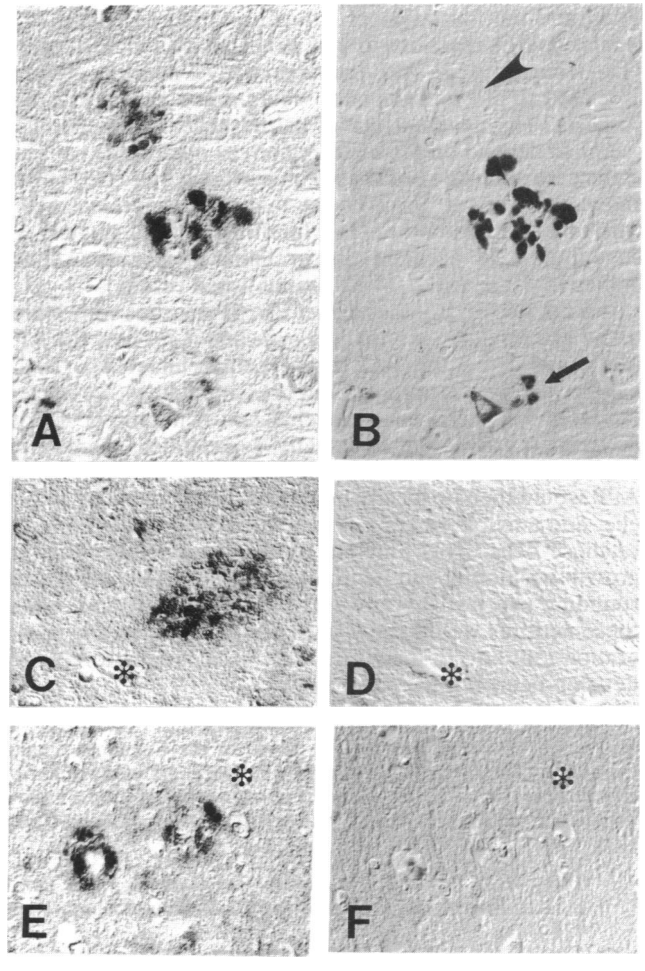

FIG. 5. Amyloid precursor protein immunostain (anti-Bac695) of control hippocampus (*A*) shows two SP, one of which is also τ (Alz-50) immunoreactive (*B*). The second SP is not recognized by the τ antibody, but the structure can be appreciated when Nomarski interference contrast is used (arrow head). Two APP-immunoreactive neurites (arrow) close to a tangle-bearing neuron are τ positive. (×340.) SP in control frontal cortex are immunostained with APP antibody (anti-Bac695) (*C, E*) but remain unlabeled with τ (Alz-50) (*D*) or neurofilament (SMI-34) antibodies (*F*). The asterisks are provided for topographical orientation on the photograph pairs *C–D* and *E–F*. (×170.)

previous studies that showed a correlation between τ-containing SP neurites and NFT (36, 37). An important proportion of our nondemented controls showed low numbers of τ-immunoreactive NFT and SP. These findings agree with the studies of Tomlinson *et al.* (38) and Mann *et al.* (39), who, respectively, found that 55% and 92% of their patients over age 70 had SP and NFT in hippocampus and neocortex. In a recent study, Crystal *et al.* (40) demonstrated that 6/9 prospectively followed mentally intact controls showed numerous NFT and SP. It is therefore clear that in a population over 70 years old, there may be a dissociation between the clinical findings and qualitative structural lesions and that quantitation is mandatory for pathological diagnosis of AD. Another reason why we found such frequent τ immunostaining in controls could have been the use of methacarn-fixed tissue. Kowall and Kosik (28) stressed the importance of optimal fixation to demonstrate normal τ immunoreactivity. It has also been suggested that different forms of τ could be differentially sensitive to fixation (41).

Our findings suggest that APP accumulates early in SP neurites. The whole APP molecule is probably accumulated in these neurites, as they were immunostained with antibodies to N- and C-terminal regions. Later on, paired helical filaments accumulate along with APP. This could be due to the fact that APP undergoes fast axonal transport (42), while τ and neurofilaments, important components of NFT, are transported more slowly. The APP accumulation by itself may be toxic to the neurite and cause further metabolic and cytoskeletal derangement. Alternatively, both APP accumu-

lation and the formation of straight and paired helical filaments could be reactions to the extracellular amyloid deposit. The occurrence of APP-immunoreactive neuritic clusters unrelated to amyloid deposits would argue against this hypothesis. The development of paired helical filaments also seems to depend on the brain region, as dystrophic neurites in the cerebellum do not show τ immunoreactivity (43) but do show membranous dense bodies that can be immunolabeled with ubiquitin antibodies (44). In ultrastructural studies, we have found APP accumulation in similar membranous dense bodies in SP neurites (M.K., unpublished results).

It is not known whether the APP in SP neurites contributes to the extracellular amyloid deposits and if so, how the APP is released so that the β-AP region remains intact (45). On the basis of previous reports and our present findings, we suggest the following pathogenetic mechanism: A neuron or neurite accumulates APP and eventually degenerates, thereby releasing APP together with numerous enzymes (46, 47). This APP would then be locally processed, presumably by microglia, and amyloid fibers would be formed in close proximity to these cells. This amyloid protein would then exert its growth-promoting and neurotoxic activities (48, 49) on surrounding neurites that are recruited into the lesion, where these neurites would show signs of degeneration and regeneration (50) and in their turn start to accumulate APP and develop neurofibrillary pathology.

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