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Deposition of β/A4 protein along neuronal plasma membranes in diffuse senile plaques

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Summary. The origin of the extracellular β-amyloid protein (β/A4) found in senile plaques and the cellular mechanisms responsible for its deposition in cerebral tissues are still an unresolved issue in Alzheimer's disease. In this study we analyzed in detail the distribution of various epitopes of β/A4 in relation to local cellular elements in diffuse plaques of the hippocampal region. We also correlated our findings with the presence and distribution of non-β/A4 epitopes of the amyloid precursor protein (APP) and with synaptophysin immunoreactivity in the cortical neuropil. Discontinuous β/A4-immunoreactive deposits were found along dendrites, and around the soma of neurons included in the plaques. Furthermore, increased synaptophysin reactivity with slightly dilated synaptophysin-immunolabeled presynaptic terminals were found in diffuse plaques. APP epitopes could not be found in diffuse plaques. However, some of the APP antibodies, mainly those to the C-terminal portion of APP, and antibodies to β/A4 recognized clusters of flat vesicular profiles (0.6–1.4 μm in width and 2–3 μm in length) in the neuropil of cortical areas where plaques had developed. Our findings are compatible with a neuronal origin of β/A4 in diffuse plaques and with a primary release of β/A4 at synaptic sites along the immunostained neurites. They also suggest that diffuse plaques might be preceded by minute lesions of the neuropil where β/A4 is not yet released from the precursor molecule.

Key words: Diffuse plaques – Dendrites – Synapses – β/A4 protein – Amyloid precursor protein

The deposition of amyloid occurs in pathological markers of Alzheimer's disease (AD) such as senile plaques (SP) and vascular amyloid. In recent years the problem of SP formation has been approached through the chemical characterization of amyloid, one of the main components of SP. Amyloid plaque cores from AD brain tissue have been found to consist of a 4- to 5-kDa protein (the "A4" protein) [20, 27], a peptide with virtually identical amino acid sequence to a 4-kDa protein previously isolated by Glenner and Wong [10] from the amyloid of meningovasculature (the "β-protein"). The pathogenesis of SP and especially the origin of the extracellular β/A4 protein found in SP remain unclear. β|A4 is derived from one or several members of the large amyloid precursor protein (APP) family and is supposed to be released as an abnormal cleavage product of APP [8]. Whether APP from which the β/A4 protein of SP derives is synthesized by neurons [3, 11, 12, 19], by other brain cells [28] or is derived from a systemic source [26] remains controversial.

Recent histochemical and immunohistochemical studies, have revealed important new information on the pathogenesis of SP by drawing attention to a special type of SP which are difficult or impossible to detect with Congo red or conventional silver strains. These lesions consist of weakly β/A4-immunoreactive deposits called "diffuse plaques" [36], amorphous plaques [25], "pre-amyloid deposits" [31] or "pre-plaques" [17] of which many morphological subtypes have been described [13, 23, 34]. This type of SP lacks any evidence of amyloid deposits, glial reaction or degenerating neurites and similar lesions are seen in the absence of typical SP in the brain of individuals with Down's syndrome between 30 and 40 years of age. This has led to the conclusion that "diffuse plaques" represent SP at a very early stage of development. Therefore, looking at the precise localization of β/A4-immunoreactive deposits in diffuse plaques could provide some information on the primary sites of β/A4 accumulation and deposition in the neuropil. The same applies to another type of putative "pre-amyloid" or early plaque which, however, differs from diffuse plaques by its spherical shape, sharp outer delimitation and by the presence in its center of a microglial cell ("plaque A") [24]. In this study we analyzed in detail the distribution of various epitopes of β/A4 in relation to

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local cellular elements in diffuse plaques and plaques A of the hippocampal region. We also correlated our findings with the presence and distribution of non-β/A4 epitopes of the APP in the cortical neuropil and with synaptophysin immunoreactivity in diffuse plaques.

Materials and methods

Tissue samples

To study the distribution of β/A4 in diffuse plaques, we examined the hippocampal area of 20 human subjects: 11 subjects (between 72 and 96 years of age; mean ± SD, 84.1 ± 6.6 years) had clinical histories of AD that were confirmed at autopsy [15]; 9 subjects were clinically free of neurological disease: 4 of them (84, 87, 95 and 100 years of age) had at least some cortical SP as shown by periodic acid-methenamine silver staining (PAMS) [14]; 5 other subjects (ages 6 month, 18, 25, 48 and 51 years) were without demonstrable SP. Brains with SP (demented and non-demented patients) were selected from a large number of cases because they had many diffuse plaques and plaques A. Brains were obtained within 2–25 h after death and immersed in 4% formaldehyde for 8–10 days. Small tissue blocks were cut from several cortical areas, the hippocampal region and various subcortical areas, embedded in paraffin and further processed for routine neuropathological examination. Special investigations on SP were carried out on serial 3- to 4-μm-thick sections of paraffin-embedded tissues from the hippocampal region (including the cornu ammonis, the entorhinal cortex and parts of the adjacent temporal cortex).

Antibodies and staining procedure

As primary antibodies we used anti-β/A4 sera raised against synthetic peptides corresponding to residue 1–10 [5] (dilution 1/100), 12–28 [5] (dilution 1/500) and 1–42 [20] (dilution 1/500) of the β/A4 peptide[1]. Anti-APP sera were raised in the rabbit, against keyhole limpet hemocyanin-conjugated synthetic peptides corresponding to different regions of the APP [2]. Antiserum R1 corresponds to cytoplasmic C-terminus residues 729–751 of APP_{751} and antisera R3 and R7 to extracellular APP_{751} amino acid sequences 628–652 and 296–315, respectively. The peptide R7 is a part of the 56 amino acid Kunitz protease inhibitor (KPI) insert specific to APP_{751} and APP_{770}. As presynaptic terminal marker we used a monoclonal antibody to synaptophysin (clone SY 38) [32] (purchased from Progen, dilution 1/200 in PBS).

One section of each series was stained with PAMS, which reliably stains diffuse plaques among other plaque types [35]. Two other sections were used for thioflavine S and Holmes silver impregnation for axons. The remaining sections of the series were processed for β/A4, APP and synaptophysin immunohistochemistry. After blocking intrinsic peroxidase with 0.3% hydrogen peroxide in methanol (15 min) sections were treated with normal horse (for monoclonal antibody) or normal goat serum (polyclonals) to depress unspecific background. To enhance β/A4 staining, the endogenous peroxidase blocking step was replaced by 15-min treatment of the deparaffinized sections with 98% formic acid. Sections were incubated overnight with the primary antibody, for 30 min with the secondary biotinylated antibody and for 45 min with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). The reaction was visualized by developing the sections in 1% diaminobenzidine containing 0.003% H_2O_2. The sections were then counterstained with hematoxylin or PAS, dehydrated and mounted. Negative controls included sections incubated with normal rabbit or mouse serum as primary antibody. Diffuse plaques in paraffin sections were photographied with a Zeiss-Axiophot microscope equipped with differential interference contrast optics. Selected immunolabelled structures were drawn using a drawing tube attached to a microscope (Wild) under an oil immersion × 100 objective.

[1] Antisera to residues 1–10 and 12–28 of β/A4 were kindly provided by Brian Anderton, London and antiserum to residues 1–42 by Collin Masters, Melbourne, Australia

Results

Diffuse plaques were defined as PAMS-positive and β/A4-immunoreactive neuropil lesions without argyrophilic neurites or brightly thioflavine S-fluorescent deposits. Diffuse plaques were further characterized by their ill-defined borders and their variability in shape and size, the smallest being only a few micrometers in diameter. In contrast, plaques A were large spherical lesions with sharp borders. Both types of plaques were immunostained by all three anti-β/A4 antibodies, although the staining was less intense with anti-$\beta/A4_{1-10}$ than with anti-$\beta/A4_{12-28}$ and $\beta/A4_{1-42}$. Diffuse plaques and plaques A, identified by PAS counterstain or anti-β/A4 immunostaining on an adjacent paraffin section, remained unstained using antibodies to different APP domains. In a high percentage of diffuse plaques and plaques A, the neuropil structure was obscured by large amounts of β/A4-positive material. For this reason we have chosen to investigate plaques in which immunostained material was more loosely distributed. Structural analysis of plaques was also extended to some very large "cloud-like" plaques [34] of the hippocampus (see Fig. 3A).

Diffuse plaques and plaques A essentially consisted of innumerable ring-shaped and tubular β/A4-immunoreactive deposits (Figs. 1A–C; 2A; 3C). Most of these β/A4-immunoreactive profiles were of very small size but some were of much larger diameter depending on the area of the hippocampus in which the plaque had developed. Large immunostained tubular profiles could often be followed up to the cell body of a nearby pyramidal cell and could, therefore, be identified as dendritic (Figs. 1A, B; 3C). In sections perpendicular to the axis of apical dendrites, dendritic profiles could be recognized as immunostained ring-shaped profiles of large diameter (Fig. 1E). β/A4-reactive material along longitudinally cut dendrites presented as parallel oriented rows of dots or horseshoe-like deposits disposed tangentially to the dendritic surface (Figs. 1D; 2A). Dendritic profiles which were immunostained in the plaque, could often be followed for some distance outside of the plaque as unstained processes (Fig. 1C). Among the β/A4-immunolabeled structures in diffuse plaques, there were also delicate nerve cell processes, probably axonal terminals, making contacts with dendrites of pyramidal neurons (Fig. 1A, B). Immunostained deposits also showed peculiar predilection for the proximal portions of basal and apical dendrites of pyramidal cells (Figs. 3C; 4A, B) but were also found around neuronal somata included in the plaque (Figs. 3B, D; 4A, B).

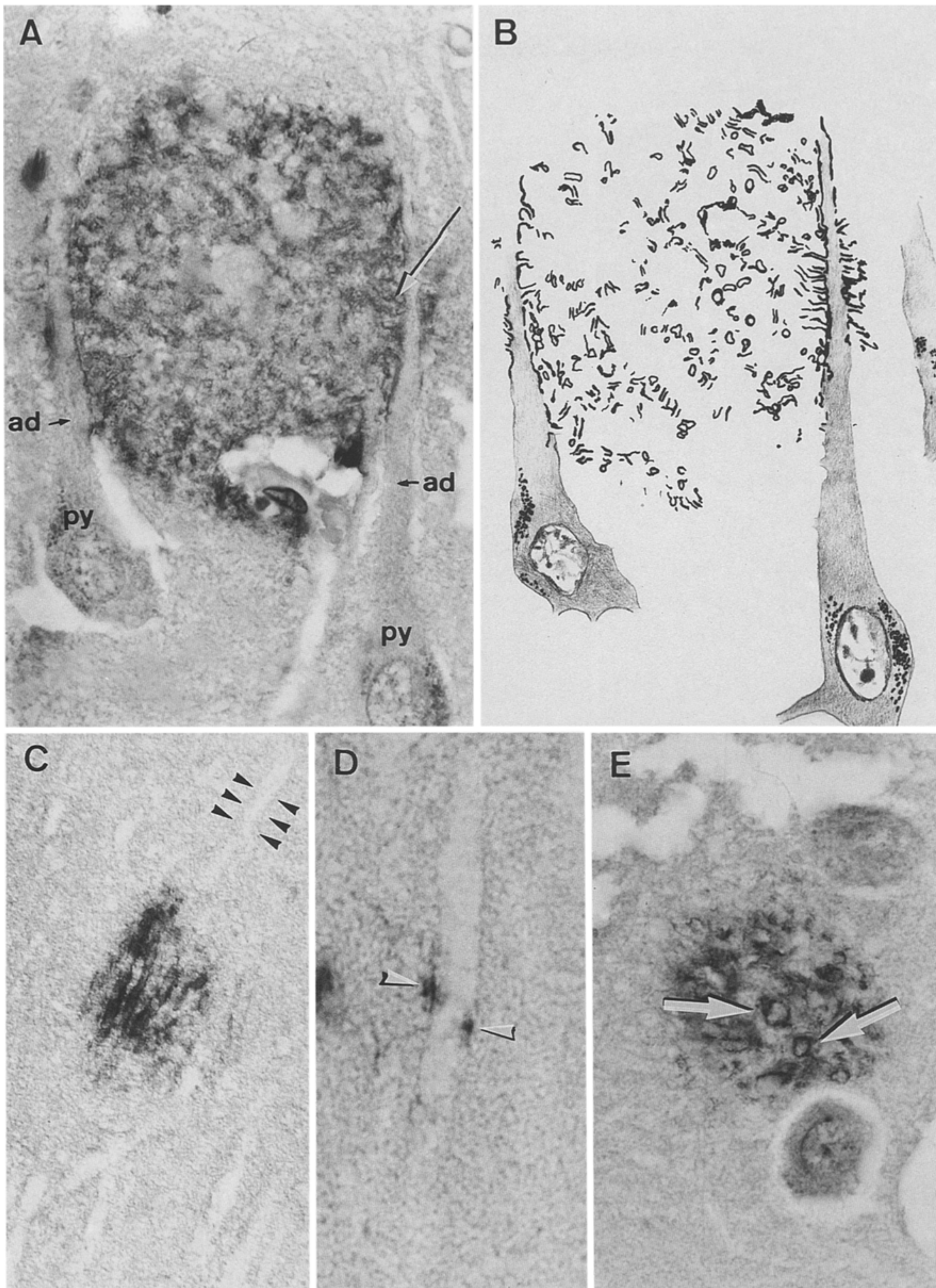

Fig. 1. **A** Paraffin section immunostained with an antiserum to β/A4. Diffuse plaque between the apical dendrites (*ad*) of pyramidal cells (*py*) in hippocampal CA1. Nomarski optics. Numerous immunoreactive ring-shaped and tubular profiles are seen at this magnification. Delicate nerve cell processes are making contact with the apical dendrites (*arrow*). Some immunoreactive products are also seen in neuronal perikarya. The same plaque is reproduced in a camera lucida drawing (**B**) where β/A4 deposits are indicated by black lines. **C** Small-sized diffuse plaque consisting of immunostained neurites, probably only dendrites. Dendrites can be followed outside of the plaque as unstained structures (*arrowheads*). **D** Small β/A4 immunostained deposits on an apical dendrite (*arrowheads*). **E** Small diffuse plaque in a section perpendicular to the main axis of pyramidal neurons in the subiculum. Large β/A4 immunostained ring-shaped structures (*arrows*) probably correspond to cross sectioned apical dendrites. **A, B** × 650; **C** × 640; **D** × 1370; **E** × 400

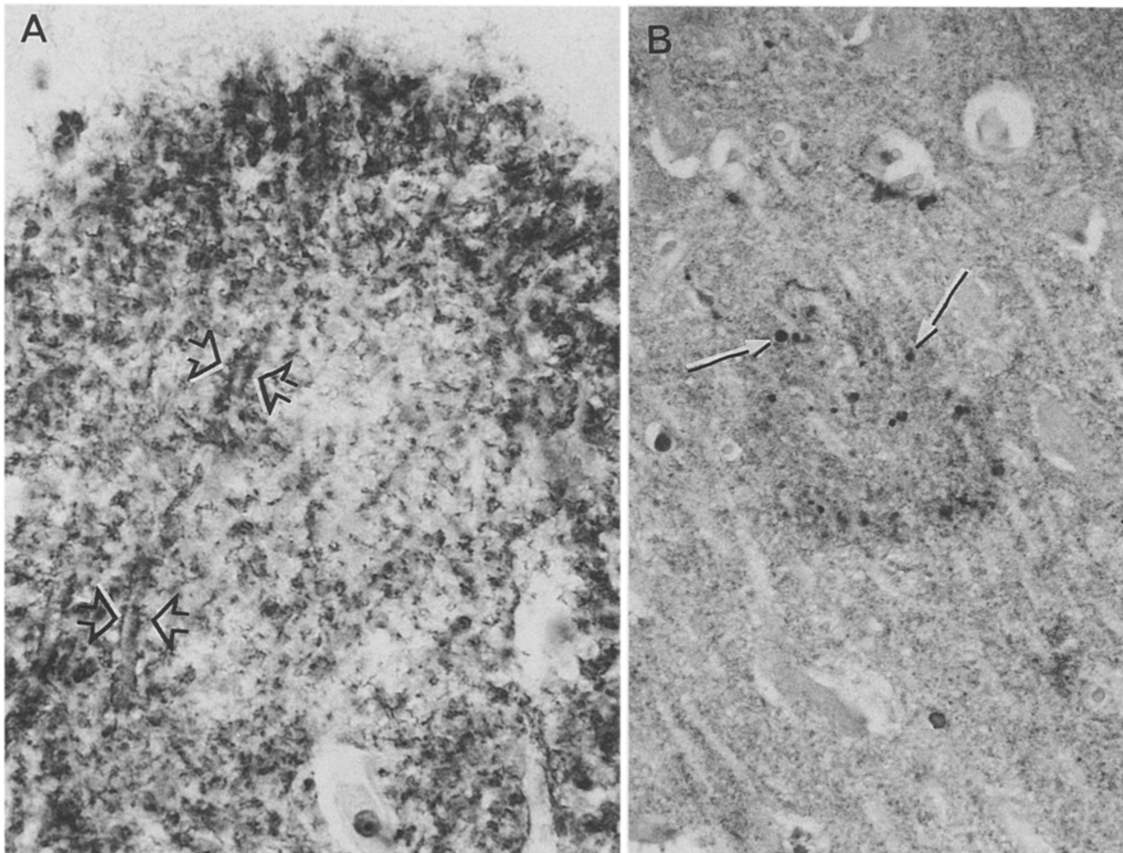

Fig. 2. **A** Immunostaining with the antiserum to β/A4 residues 12–28. Detail of a plaque A showing numerous small ring-shaped and tubular profiles. Some larger tubular profiles, probably segments of dendrites are seen to be made up of dot-like or horseshoe-like deposits (*between arrows*). **B** Immunolabeling with anti-synaptophysin antibody showing increased neuropil immunoreactivity and enlarged presynaptic terminals (*arrows*) in a small diffuse plaque. **A** × 1350; **B** × 400

Our findings in diffuse plaques and plaques A were quite different from the distribution pattern of β/A4-reactive material in "classical" plaques or in neuritic plaques of the hippocampus. In these latter plaques, enlarged dystrophic neurites did not demonstrate immunolabeling of their plasma membrane but were embedded in a meshwork of strongly β/A4-immunoreactive fibers apparently deposited in the extracellular space (not shown).

The neuropil of the hippocampal region, when immunostained with antibody to synaptophysin, displayed a characteristic granular pattern, with the densest reactivity in the molecular layer and the hilar region of the dentate gyrus, the pyramidal cell layer of the CA1 sector, the subiculum and the entorhinal cortex. Areas corresponding to diffuse plaques and plaques A, as seen on adjacent sections immunostained for β/A4, were characterized by increased synaptophysin reactivity of the neuropil and by slightly enlarged presynaptic terminals (Fig. 2B). Much larger and more intensely stained neurites were found by anti-synaptophysin labeling at the periphery of "classic" or "neuritic" plaques.

Looking at sections immunostained with the R1 antibody, we found various numbers of small vesicular clusters (approximately 20 to 50 μm wide) throughout the cortical neuropil (Fig. 5A–C). These clusters were composed of strongly immunostained flat vesicular profiles, 0.6–1.4 μm in width and 2–3 μm in length (Fig. 5C, inset). Some vesicular profiles were disposed along the main dendritic trunks of pyramidal cells (Fig. 5D, E) or in close proximity to a neuronal soma (Fig. 5A). Under oil immersion (obj. ×100) individual vesicular profiles were made up of approximately 0.3–0.5 μm immunostained dots (Fig. 5C, inset). Similar clusters, but in lesser number and less intensely stained were seen in sections stained with antibodies to β/A4 and to exo-domains of APP, including the Kunitz domain (antibody R7). The vesicular clusters were found to occur together with diffuse plaques, plaques A and typical plaques. However, some clusters were seen in the absence of β/A4-positive plaques in two patients (48 and 51 years old). Neither SP of any type nor vesicular clusters were found in the cortex of the three younger subects (6 months and, 18 and 25 years old).

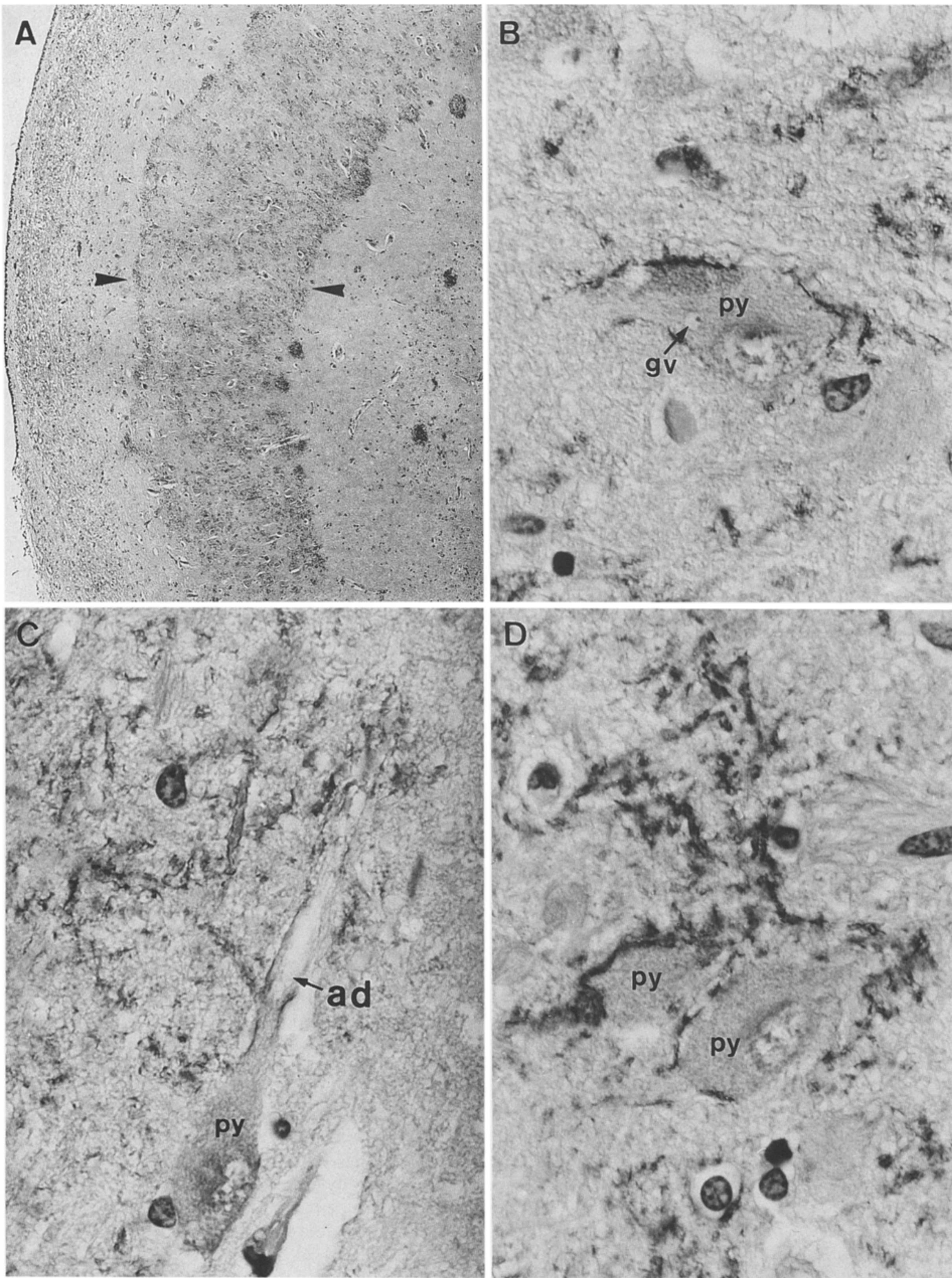

Fig. 3. **A** Large cloud-like diffuse plaque (*between arrowheads*) in the CA1 sector of the hippocampus in an Alzheimer patient. Paraffin section immunolabeled for β/A4. **B–D** Details of the plaque shown in **A.** Sheet-like β/A4 deposits are seen along the surface of the cell soma in **B** and **D,** along dendritic branches and small neurites in **C** and **D.** *py:* pyramidal neurons; *ad:* apical dendrite; *gv:* granulovacuole. **A** × 50; **B–D** × 1000

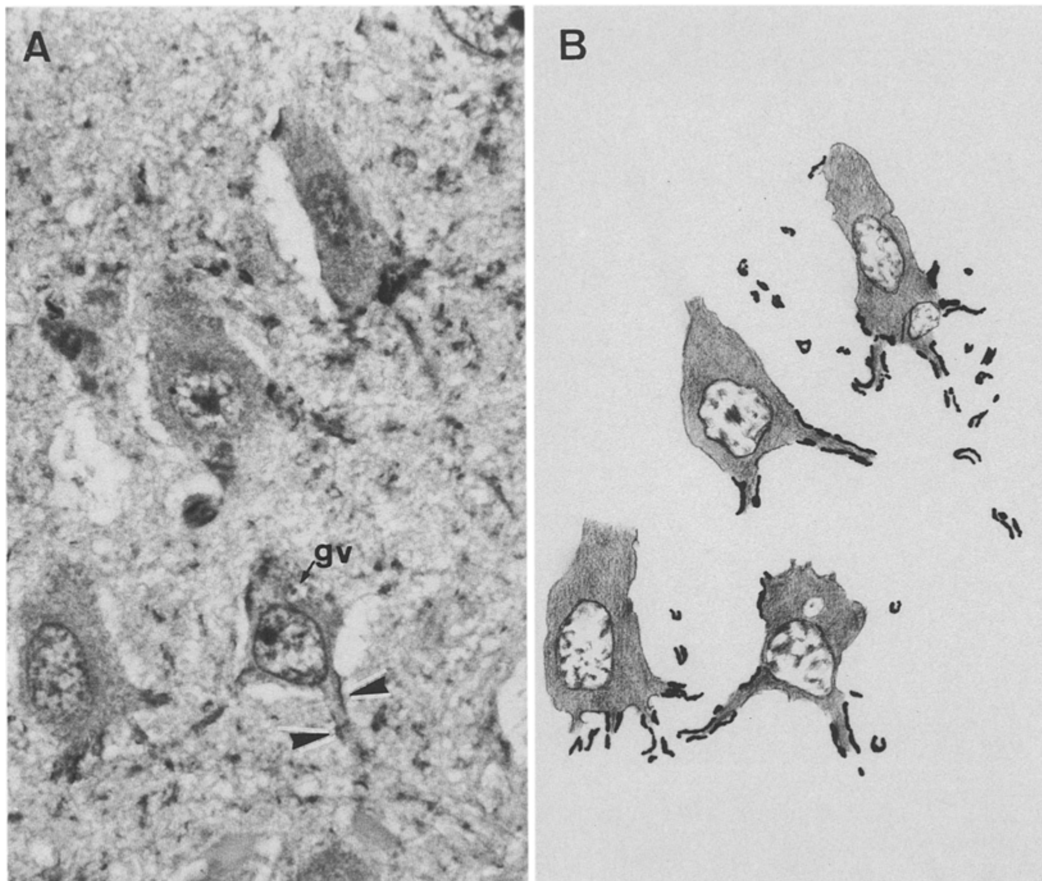

Fig. 4. **A** Detail of a large diffuse plaque in CA1 immunostained with the antiserum to β/A4 12–28. Note immunoreactive deposits along the basal dendrites of pyramidal neurons (some of them indicated by *arrowheads*). **B** β/A4 deposits are represented by *black lines* in the camera lucida drawing of the neurons shown in **A.** gv: Granulovacuole. **A, B** × 850

Discussion

Several attempts have been made to find the incipient lesion leading to plaque formation by investigating the spatial relationships between the plaques or their β/A4 deposits and other tissue components. Thus, emphasis has been put on tangle-bearing neurons [20, 29] or congophilic blood vessels [9, 22] as the most likely "nidus" of plaque formation. Observations by Allsop et al. [1] suggest that β/A4 deposits of "pre-plaques" precede the degeneration of neurites and that they may originate from the proximal dendrites and possibly the cell body of morphologically normal neurons. According to other investigators, abnormal neurites are the most likely focus for the accumulation of β/A4 protein in the neuropil. Earlier ultrastructural studies have shown occasional amyloid wisps among collections of degenerating neurites in the neuropil [33]. Cork et al. [7] recently demonstrated the presence of β/A4 deposits in relation to abnormal neurites in aged monkeys. Furthermore, β/A4 has also been found to decorate the edges of neurites adjacent to amyloid deposits in more mature plaques [16]. Our own findings in diffuse plaques and plaques A also indicate an intimate relationship between β/A4 deposits and neuronal structures. β/A4-immunoreactive material was mainly found around dendritic processes of neurons and around the soma of neurons included in the plaques. At a higher magnification, β/A4 was often distributed in dots or horseshoe-like curvilinear profiles at the dendritic surface. One likely explanation for such a distribution is that β/A4 is primarily released at synaptic sites along the neuronal soma and dendrites, thus giving the final impression of a more or less continuous β/A4-immunoreactive sheet at the neuronal surface.

We have found altered synaptophysin-immunoreactive presynaptic terminals in diffuse plaques, thus confirming recent observations in three other laboratories [5, 6, 18]. These observations, together with ultrastructural findings by Yamagushi and co-workers [37, 38] in diffuse plaques, suggest the view that the deposition of β/A4 is closely related to presynaptic terminals in early plaques. However, it remains unsettled whether a primary neuritic dysfunction is responsible for β/A4 deposition or is itself initiated by β/A4 deposition. Masliah et al. [18], using the presynaptic terminal marker synaptophysin, found that while synaptic density was diminished in AD cortical neuropil, the reduction was not greater within diffuse plaques than outside them. These findings suggest that the pathogenic process

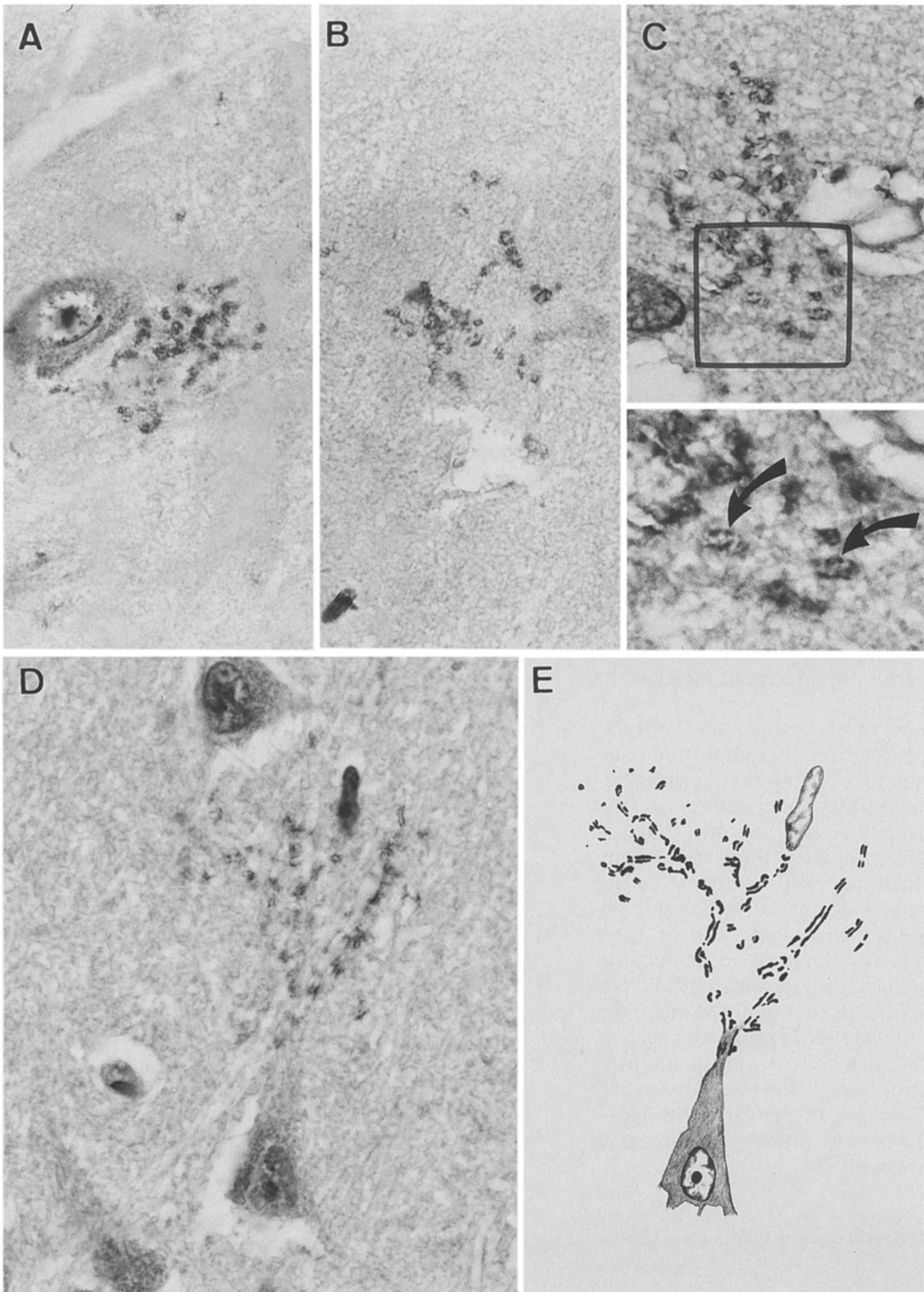

Fig. 5. A–E Anti-C-terminal antiserum R1 immunolabels small vesicular aggregates in the neuropil. **C** *inset:* Details included in the frame: vesicular profiles consist of small dots, approx. 0.3–0.5 μm in diameter (*arrows*). **D** vesicular profiles and linear deposits along the dendritic branches of a pyramidal neuron (subiculum). Immunostaining with antiserum R3 (amyloid precusor protein, APP 628–652). **E** Camera lucida drawing showing details of **D. A–E** × 800; **C** inset × 1750

in AD begins with neurodegeneration and synapse loss rather than with deposition of β/A4 protein. Evidence for an early neuritic pathology in diffuse plaques also supports our view that the β/A4 along neurites originates from local presynaptic elements rather than being derived from another, possibly more remote source.

A further finding of our study consisted in small clusters of vesicular profiles in the cortical neuropil. In contrast to diffuse plaques, vesicular clusters, in addition to being reactive with anti-β/A4 antibodies, were found to react with antisera to various portions of APP, the most consistent labeling being obtained with the anti-C-terminal antiserum R1. The substructure of vesicular profiles and their frequent association with dendrites suggest that they might correspond to abnormal synaptic sites. Although the exact nature of the vesicular clusters remains to be established, their association with SP and their absence in the cortex of young subjects suggest that they are linked in some way to the process of SP formation, possibly as an initial lesion. Furthermore, immunohistochemical analysis suggests that APP in vesicular aggregates may be in a stage of its processing where the β/A4 fragment is not yet cleaved from the rest of the precursor molecule, particularly from the C-terminal domain. In contrast, absence of APP determinants in diffuse plaques and plaques A suggests that β/A4 is already cleaved from the precursor molecule at this stage.

Besides the questions of the origin and cellular distribution of β/A4 in plaques, another important issue concerns the mechanisms responsible for the distribution of β/A4 in patchy or roughly spherical lesions (the plaques) involving many different neurons. Such a distribution can hardly be explained without considering other cellular events which may well take place in the processing of the APP molecule and be responsible for the shape of the plaques. Microglial cells or their processes have been recently detected in association with β/A4 deposits or plaques of all morphological types [21, 35]. There is some evidence that ramified microglia may be capable of limited phagocytic properties such as synaptic stripping [4, 30]. Such activity may be intensified at abnormal synaptic sites thus leading to an increased processing of synaptic proteins. In this hypothesis β/A4 deposits may be a by-product of microglial processing of synaptic proteins [21].

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