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Rapid Communication

Diffuse Plaques Do Not Accentuate Synapse Loss in Alzheimer's Disease

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Applying the relatively new technique of laser confocal imaging, vibratome sections which were double immunolabeled for amyloid beta protein and the presynaptic terminal marker synaptophysin were examined. It was found that while synaptic density was generally diminished in Alzheimer's disease (AD) cortical neuropil as compared to controls, the reduction was no greater within the diffuse plaques than outside them. Synapse loss was accentuated, however, within immature and mature plaques. These findings suggest that the pathogenetic process in AD might commence with synapse loss and neurodegeneration rather than with deposition of amyloid beta protein. (Am J Pathol 1990, 137:1293–1297)

The role of amyloid deposition in the pathogenesis of Alzheimer's disease (AD) is the subject of intense scrutiny. The diffuse senile plaque, which is characterized by deposition of amyloid beta protein in the neuropil unassociated with dystrophic neurites,[1] is particularly intriguing because its origin, significance, and destiny are all undetermined. Some authors claim that diffuse plaques (also called 'preamyloid deposits') are the earliest lesions of AD,[1–4] and that they progress to classical plaques associated with neocortical neurofibrillary tangles, thus forming the image of full-fledged AD. Other authors contend that diffuse plaques may be relatively harmless accompaniments of normal cerebral aging and are without ominous portent.[5] If diffuse plaques do initiate the brain damage in AD, then some measure of neuropil integrity would be expected to be altered within the plaques themselves while still unaffected in adjacent brain parenchyma. We recently showed, by quantitative immunochemistry and immunohistochemistry of the presynaptic terminal protein synaptophysin,[6] that the density of presynaptic terminals is markedly diminished in AD neocortex,[7,8] and in the present investigation we used novel imaging techniques to determine whether synapse loss was accentuated within the diffuse, immature and mature plaques.

Materials and Methods

A total of eight specimens from the Alzheimer Disease Research Center at the University of California, San Diego was used for the present study. Routine histopathologic examination was performed with paraffin sections from the formalin-fixed blocks of frontal, parietal and temporal cortex, hippocampus, and subcortical nuclei of the left hemibrain stained with cresyl violet, thioflavin S, and hematoxylin and eosin.[9] Four of these cases were diagnosed as AD, clinically, with histopathologic confirmation. The average age of the AD cases was 75 ± 10 years, with a postmortem delay of 6 ± 1.2 hours. The other four cases were clinically normal and free of any neuropathologic abnormalities. The average age of these control cases was 71 ± 11 years, with a postmortem delay of 5.2 ± 2.7 hours. From each of the eight specimens, blocks from the midfrontal (MF) cortex[9] of the right hemibrain were fixed in paraformaldehyde 2% (in PBS pH 7.4) for 72 hours at 4°C, followed by vibratome sectioning at 40 μ.

The sections were double immunolabeled for amyloid beta protein (ABP) and synaptophysin with Texas Red and fluorescein isothiocyanate (FITC), respectively. Briefly, optimal and consistent results were obtained in vibratome sections previously treated for 5 minutes with 99% formic

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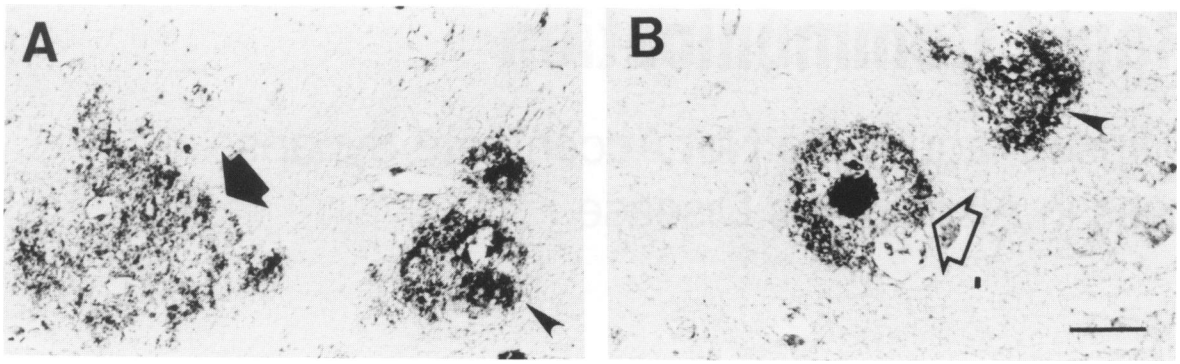


Figure 1. *Paraffin sections immunostained with anti-amyloid beta protein and developed with DAB, showing examples of the three different types of plaques.* **(A)** *Diffuse (arrow) and immature (arrowhead) plaques.* **(B)** *Immature (arrowhead) and mature (open arrow) plaques. Bar, 15 μ.*

acid that were incubated overnight at 4°C with a mixture of the monoclonal antibody against synaptophysin[6] (SY38, Boehringer-Mannheim Labs., Indianapolis, IN) at a concentration of 1 μg/ml and with the rabbit polyclonal antibody against ABP (region 11–22, courtesy of Dr. C. Masters) at 1:200 dilution, followed by incubation in a mixture of horse anti–mouse-FITC and goat anti–rabbit-Texas Red (Vector Labs, Burlingame, CA) at 1:100 dilution. All sections were treated simultaneously under the same conditions. Additional sections, double immunolabeled with anti-synaptophysin/anti-ABP, were incubated in a mixture of horse anti–mouse-Texas Red and goat anti–rabbit-FITC (Vector Labs.; 1:100). The immunolabeling protocol was repeated twice to assess the reproducibility of the results. These experimental conditions were established after testing a wide range of primary and secondary antibody concentations. The nonsaturating dilutions have been shown to yield reproducible, linear results in control microdensitometric studies,[8,10] as well as in studies using FITC-tagged secondary antibodies. The double immunolabeled sections were transferred to double-coated gelatin slides and mounted under glass cover slips with antifading media (n-propyl gallate 4%, Sigma Chemical Co., St. Louis, MO).

The double-immunolabeled vibratome sections were studied with the Bio-Rad MRC-600 laser scanning confocal microscope[11] (Watford, UK) mounted on a Nikon Optihot microscope (Tokyo, Japan). This system permits the simultaneous analysis of double-labeled samples in the same optical plane. The advantage of using confocal microscopy of 40-μ vibratome sections is that it allows sectioning through the whole thickness of a plaque, thus making it easier to identify and classify plaques according to their components. Images of serial 1-μ–thick optical sections of the neuropil harboring different types of plaques (diffuse, immature, and mature) were recorded, as was the corresponding serial images of the synaptophysin-labeled presynaptic terminals. Using amyloid beta protein immunohistochemical staining, senile plaques were assigned to one of the three categories, according to the following criteria for defining plaque types[12]: 1) Diffuse plaques: amorphous or polymorphous zones, vaguely defined positivity without neuropil disruption, cloudy or cotton-wool staining patterns lacking distinct boundaries or sharp edges; usually no swollen neurites, no compact amyloid cores. 2) Immature plaques: generally spherical and well defined with distinct parameters, containing swollen neurites, lacking compact amyloid cores. 3) Mature plaques: spherical, well circumscribed, with dense, compact, round amyloid cores and peripheral coronas of swollen, spherical and fusiform neurites. Examples of the three plaque types, as seen with the conventional diaminobenzidine (DAB) immunohistochemical system,[13] are illustrated in Figure 1. The plaque morphology was similar to the one obtained by using Texas Red-tagged secondary antibodies (Figure 3).

Quenching of the fluorescent images was minimized using the optical disk stored digitized video images for subsequent display, analysis, and quantification. The aperture, black, and gain levels initially were manually adjusted to obtain images with a pixel intensity within a linear range. Subsequently all sections were viewed with a 63× Nikon oil immersion lens (N.A. 1.4) and in all cases the image acquisition was done while maintaining constancy of the above-described parameters. The pixel intensity of the synaptophysin immunofluorolabeled neuropil, inside and outside (15 to 20 μ out of the border of the amyloid) of the plaques, was registered and averaged using the standard data analysis software of the MRC-600 system.

Results

Sections of control samples immunoreacted with anti-synaptophysin displayed the characteristic granular pattern in the neuropil (Figure 2A), with a denser immunofluorescent labeling in layers II, III, and V than in I, IV, and

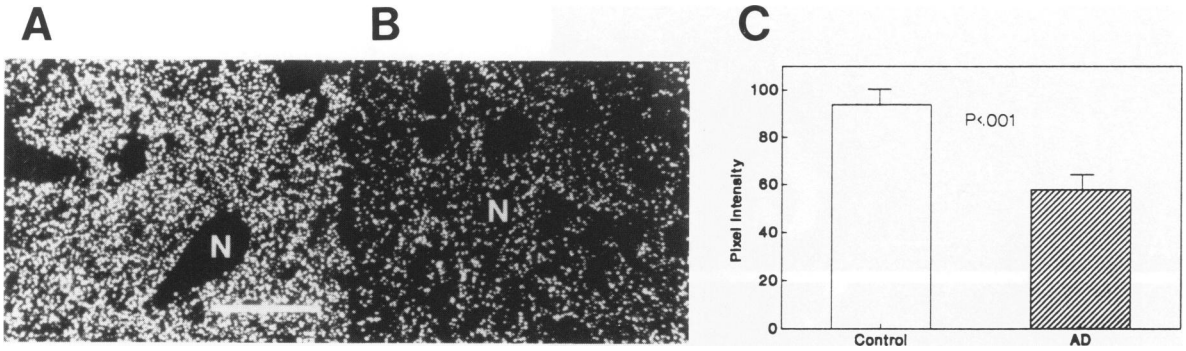


Figure 2. *Laser confocal imaging of the anti synaptophysin-fluorescein–labeled frontal cortex.* **(A)** *Control and* **(B)** *AD. The 1-μ optical sections of the amyloid-free AD neuropil displayed a significant reduction in the number of immunolabeled presynaptic terminals as compared to control (N, neuronal cell body; bar, 15 μ).* **(C)** *Quantification of the pixel intensity of the synaptophysin-immunolabeled neuropil showed an average 40% decrease in the AD samples (n = 4 controls and n = 4 AD cases, bars are SD).*

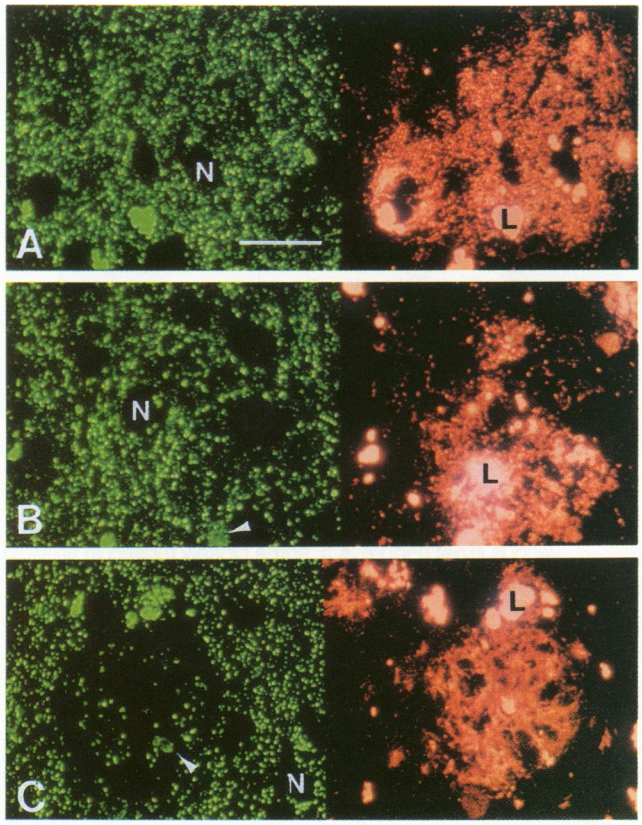
VI. The neuronal cell bodies and the white matter were not labeled (Figure 2A) and their pixel intensity was close to zero. The optical sections of the AD samples also presented a typical granular pattern of synaptophysinlike immunolabeling (Figure 2B). In the AD samples, the amyloid-free neuropil showed a 40% decrease in synaptophysinlike immunoreactivity (Figure 2C). This decrease in the pixel intensity of the AD neuropil was due to an overall reduction in the total number of labeled terminals (Figure 2B), while the average immunofluorescence of individual preserved terminals in AD was not diminished. That is, pixel intensity per terminal was the same in control and AD cases.

The AD neuropil within the diffuse plaques had synaptophysinlike immunofluorescence values similar to those outside the diffuse plaques (Figure 3A, D). Serial optical sections of the diffuse plaques revealed a finely granular or fibrillar appearance of the amyloid and often had a close association with the surface of neuronal perikarya (Figure 3A). In addition, serial optical sections showed that some diffuse plaques contained small foci of amyloid condensation, but cores or large dense amyloid deposits were not detected. Although no dystrophic neurites were identified in the diffuse plaques, some synaptophysin-immunolabeled presynaptic terminals were slightly dilated. Immature plaques showed some synaptophysin-positive dystrophic neurites accompanied by fibrillar amyloid deposits without core (Figure 3B). Inside the amyloid zone, the immature plaques presented a 47% presynaptic terminal loss, while the adjacent neuropil showed a 40% synaptic loss (Figure 3D). The mature plaques (Figure 3C) showed a 65% decrease in synaptophysin-labeled granules within the plaque amyloid zones (Figure 3D). In contrast, the peripheral coronal area surrounding the amyloid displayed a trend toward a mildly increased synaptic density as compared to the adjacent neuropil (Figure 3C). Serial optical sections of the mature plaques showed enlarged synaptophysin-positive immunolabeled terminals of various sizes (Figure 3C), some closely related to amyloid fibers.

Discussion

Synapse loss in AD has been documented by quantitative immunochemistry and immunohistochemistry[7,8,14,15] and electron microscopy.[16,17] The present investigation suggests that the decrease in presynaptic terminal density is unrelated to the concomitant presence of diffuse plaques but is accentuated within immature and mature plaques. Such findings are consistent with the ultrastructural compositions of the different plaque types. Diffuse plaques show only a few small scattered bundles of amyloid fibrils among focally blurred membranes of cell processes, and the neuropil within them appears almost normal morphologically.[1] These amyloid-related cell processes also have been demonstrated recently with immunoelectron microscopic techniques,[18] but their exact origin is unknown. The serial optical sections performed for the present study in the ABP-immunolabeled plaques suggests a possible involvement of neuronal cell processes in the different amyloid plaques. Immature and mature plaques, however, have greatly distended neurites containing PHF, granular degenerating mitochondria, and lysosomes, resulting in clearly evident neuropil distortion.[19]

The presence of synapse loss both within and outside the diffuse plaques may imply that amyloid deposition itself is not the inciting lesion in AD, but rather could be a response to pre-existing synaptic or neuronal pathology. The observation that synaptic density is diminished further within immature and mature plaques is to be expected, given the neuropil distortion seen by electron microscopy.[20] Because our data show that presynaptic terminal loss occurs equally in AD neocortex with and without amyloid deposition, the question arises as to the effect of



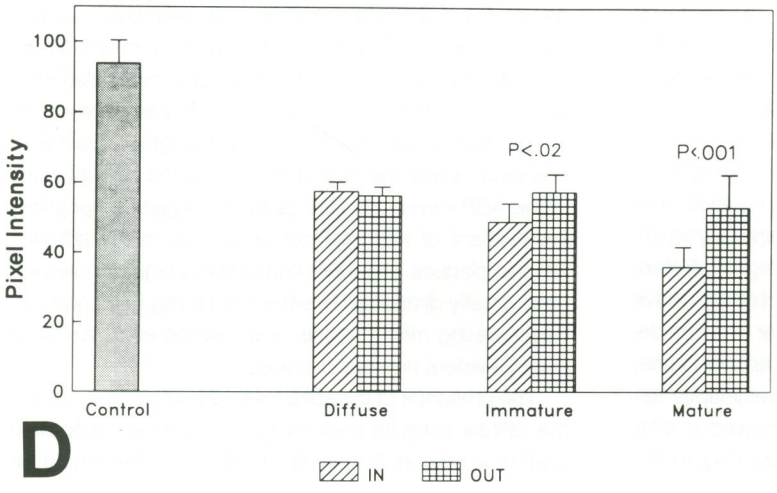


Figure 3. *Laser confocal imaging of the double-immunolabeled sections for synaptophysin (green) and amyloid beta protein (red).* **(A)** *The neuropil displayed a constant number of immunofluorescent terminals in the area corresponding to the diffuse plaques.* **(B)** *The immature plaques presented a moderate synaptic decrease and few distended neurites (arrowhead).* **(C)** *In the mature plaque the presynaptic terminals were significantly decreased in the amyloid zone and some of them were enlarged (arrowheads). Outside the amyloid zone the presynaptic terminals showed a mild trend toward increased density (N, neuronal perikarya; L, lipofuscin). Bar, 15 μ.* **(D)** *Quantification of the pixel intensity of the synaptophysin-immunolabeled neuropil in areas inside (in) and outside (out) the different types of plaques as compared to age-matched control cases (Bars are SD).*

the presence of amyloid in the neuropil. Recent investigations have shown that the amyloid beta precursor protein (APP) is a transmembrane neuronal protein[21] that undergoes fast anterograde axonal transport,[22] suggesting that APP is synthesized in neurons and delivered to nerve endings, and that subsequent alteration of local processing of APP results in deposits of brain amyloid. Further evidence suggests that a secreted form of APP is involved in growth regulation of fibroblasts.[23] Meanwhile other studies have shown that a specific portion of the C-terminal

region of APP has neurotoxic effects on hippocampal neurons *in vitro*.[24] The ABP enhances the survival of hippocampal neurons *in vitro*.[25] Together these data could imply that the APP, or even the ABP itself, serves as growth-promoting factors and are produced in AD in response to ongoing synaptic pathology. If so, rather than initiating brain damage in AD, amyloid deposition could be a response to synaptic and neuronal degeneration.

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