Limited Effect of Neuritic Plaques on Neuronal Density in the Hippocampal CA1 Area of Alzheimer Patients

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**Summary:** Neurofibrillar tangles (NFTs) and neuritic plaques (NPs) are the classic neuropathological hallmarks of Alzheimer disease (AD). It is generally assumed that the pathogenic process of AD could start by local neurotoxicity induced by the β-amyloid core of plaques, followed by the appearance of NFTs and eventually cell death. To determine whether or not local neurotoxicity around NPs is indeed a major pathogenetic mechanism, we used an image analysis system to measure the neuronal density around Bodian-stained NPs in the hippocampal CA1 area of eight AD patients. Neuronal density, as measured within two arbitrary concentric circles around NPs with a radius of 74 and 123.5 μm, respectively, was on average 19% and 16% lower than the density in similar control circles without NPs in the same section. Furthermore, neuronal density around NPs was inversely related to their size. To investigate the impact of such a local reduction in cell density around NPs on the entire CA1 area, we also determined the proportion of the CA1 covered by the NPs and the arbitrary concentric circles around them. This appeared to be 16.3% of the total CA1 area, which means that the negative effect of NPs on the cell density can only explain 2.6% of cell death in the entire CA1 area. In conclusion, this study suggests that although NPs have a local negative effect on neighboring neurons, their contribution to the strong decrease in CA1 cell numbers is limited. **Key Words:** Alzheimer disease—CA1 area—Neuritic plaques—Hippocampus—apoE.

Alzheimer disease (AD) is a devastating neurodegenerative disorder and the major cause of dementia in elderly people. Clinical features of AD are loss of memory and impairment of other higher cortical functions, e.g., aphasia, apraxia, and personality changes, without clouding of consciousness. The final diagnosis of AD can be established only by neuropathological confirmation (McKhann et al., 1984).

Apart from neurofibrillary tangles (NFTs), one of the most characteristic features of AD is the presence of neuritic plaques (NPs) in a variety of cortical and subcortical brain areas. NPs are usually composed of an extracellular core of aggregated 6–10-nm proteinaceous fibrils primarily consisting of β-amyloid protein, complement, apoE and α1-antichymotrypsin, dystrophic neurites, and reactive glia (cf. Terry et al., 1994). It has been hypothesized that β-amyloid accumulation plays a key role in the pathogenesis of AD by starting a cascade leading to the appearance of NFTs and finally to cell death (Hardy and Allsop, 1991; Selkoe, 1994a, 1994b). The deposition of β-amyloid has been proposed to be the result of a metabolic imbalance between non-amyloidogenic and amyloidogenic processing of amyloid precursor protein (Caporaso et al., 1992).

The present study was conducted to determine in situ the possible local effect of NPs on cell loss in their immediate environments and the possible impact of such an effect on total neuronal loss in the CA1 area. For this purpose we measured neuronal density in two arbitrary concentric circles, either around NPs or around control points in the hippocampal CA1 area, a region known to show considerable neuronal loss in AD (West et al., 1994).
MATERIALS AND METHODS

The brains of eight AD patients (aged 73.6 ± 4 years) were obtained at autopsy (Table 1). The clinical diagnosis “probable AD” was established according to criteria of the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer Disease and Related Disorders Association (McKhann et al., 1984) and was confirmed neuropathologically by assessing the presence of a large number of NPs, NFTs, and dystrophic neurites in the hippocampus and a number of cortical areas. The following brain areas were studied: gyrus orbitalis, gyrus temporalis superior, hippocampus, corpus striatum, substantia nigra, thalamus, locus coeruleus, gyrus cinguli, and cerebellum. The hippocampi were dissected out and fixed in 4% formaldehyde for about 1 month, after which they were embedded in paraffin. Sections 6 μm thick were cut. Every 50th section was stained with 0.5% thionine for anatomical orientation. Silver staining was performed according to Bodian (1936), which showed NPs, NFTs, and dystrophic neurites. The Bodian staining is routinely performed in our center and gives highly reproducible results. To visualize nuclei and nucleoli, silver impregnation was followed by counterstaining with neutral red (0.05%) (Mann et al., 1981). The densities of NPs before and after the application of neutral red were measured in the hippocampi of six patients (patients 1, 2, 4, 5, 6, 8; Table 1) to determine whether counterstaining had any effect on the visualization of NPs. However, no statistically significant effect was found (p = 0.76). Subsequently, an image analysis system (Kontron) was used to perform the measurements, which consisted of two different procedures: (1) determination of the cell density within two concentric arbitrary circles around NPs or around control points that did not contain a NP, and (2) determination of the measured area within the CA1 area covered by NPs and their arbitrary concentric environments.

Determination of Neuronal Density

The size of the NPs was measured and the number of neurons containing a nucleolus was determined within two concentric circles around the NPs: circle 1 with a radius of 74 μm and circle 2 with a radius of 123.5 μm, i.e., the largest circular area fitting on the screen (Fig. 1). The seven stages of this procedure were as follows: (1) outlining the CA1 area of the hippocampus at low magnification (2.5 × objective); to prevent the circles from exceeding the CA1 area, the outlined area was made smaller than the CA1 area (Fig. 2); (2) superimposing a grid of rectangular areas “seen” through a 40 × objective by the TV camera over this area; (3) selection at high magnification (40 × objective) of all fields containing one or more NPs; (4) random selection of the same number of NP-free fields, the centers of which were assigned as control points; (5) manual outlining of NPs; (6) placing the plaques or the centers of the NP-free fields in the center of an area, demarcated by two circles (Fig. 1); and (7) counting the number of neuronal profiles containing a nucleolus inside this area by using a 25 × objective.

Moreover, to address the question of how far a local effect of the presence of NPs on neuronal density around them would continue, the area of circle 1 was subtracted from the area of circle 2. The neuronal density was measured within this ring-shaped area (ring 1) around every NP and control point (Fig. 1).

Measurement of the CA1 Area Covered by NPs and Their Environments

This procedure was designed to measure the total area of the CA1 covered by the arbitrary environments (radius 123.5 μm), taking their overlap into account. This procedure consisted of four stages: (1) outlining the CA1 area, (2) placing a grid over this area, (3) selecting 189 ± 14 fields per patient regardless of the presence or

<table>
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<th>Age (years)</th>
<th>Age of onset</th>
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Mean ± SEM 73.60 ± 4 64 ± 5 3.90 ± 0.3 1,138 ± 39

NBB, Netherlands Brain Bank; m, male; f, female; PMD, postmortem delay; GDS, Global Deterioration Scale (Reisberg et al., 1982); NA, not available.

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**FIG. 1.** Schematic representation of circles 1 and 2 and ring 1 around each plaque. Similar ones were used for control points. To correct the calculation of the neuronal density in circles 1 and 2, the area of each neuritic plaque (NP) was subtracted from the area of the circles.

**FIG. 2.** Representation of the outlined CA1 area. The grid shows the size of the fields studied at 40× and the size of the large circle (radius: 123.5 μm) around each neuritic plaque (NP). Note that the overlapping areas between the NP environments are subtracted from the total area. The total outlined area of CA1 was calculated on the basis of the number of selected fields. Areas of the circles beyond the outer border of the selected fields were not taken into account. G, granular layer. Scale bar = 500 mm.
absence of NPs on a total of 1,328 fields, and (4) selecting all NPs present within the outlined area seen in the 40× objective. Subsequently, the image analyzer calculated the size of the outlined area in the 2.5× objective, placed the centers of circles on control or NP points, and corrected for overlap and for those parts situated beyond the outer borders of the selected fields in the CA1 area (Fig. 2). APOE genotyping was performed on frozen tissue from the cerebellum of the AD patients. The genotype of each extracted DNA sample was determined by polymerase chain reaction (PCR) amplification using the primers 5'ATAAATAATATAAAATATAAATAACAGAAGATT CGCCCCGGCCTGGTACAC 3' and 5'TAAAGCTTGACCGTCTGCAAGGA 3'. The PCR product was then digested by CfoI, and fragments were separated by electrophoresis on a 5% agarose gel (Crook et al., 1994).

Statistical Analysis

An SPSS program (SPSS Inc., Chicago, IL, U.S.A.) was used for statistical analysis. One-way analysis of variance and multiple regression were employed to determine the relationship between the presence or absence of plaques and the size of the plaques and neuronal density.

RESULTS

A large number of NPs and NFTs were seen throughout the CA1 area of the hippocampus in sections stained with the Bodian silver-impregnation method. The number of plaques per area varied considerably per patient, but in no case did the number of NPs per field of measurement exceed three (40× objective). About 35 NPs and 35 control points were selected per patient, and in total 280 plaques and 292 control points were studied. The areas of the NPs varied considerably from 138 to 5,562 μm² (mean ± SEM: 1,583 ± 48 μm²). On average the NP area was 9.2% in circle 1 and 3.3% in circle 2. To correct the cellular density in the two circles, the area of each NP was subtracted from the area of the circle (Fig. 1).

Determination of Neuronal Density

There was a 19% (p = 0.01) reduction in the neuronal density around NPs within circle 1 and a reduction of 16% within circle 2 (p = 0.01). Furthermore, there was a 12.5% reduction in neuronal density in ring 1, which was not significantly different from that around the control points (p = 0.087; Table 2). The multiple regression analysis showed a negative relationship between NP size and neuronal density in circle 2 (p = 0.0001).

Measurement of the CA1 Area Covered by NPs and Their Environments

The measured CA1 area was 1.53 ± 0.1 mm² per section. The CA1 area covered by the circles around NPs, corrected for overlap, was 0.25 ± 0.04 mm² per section, which is on average 16.3% of the total outlined area. The amount of overlap between the environments of NPs was calculated by using the following formula: overlap factor = (CT – OV)/(n • C2), where CT is the total CA1 area covered by plaque environment, OV is the amount of overlap between plaque environments, n is the total number of NPs counted, and C2 is the area of circle 2, which was 46,333 μm². In our study the overlap factor was 0.77 ± 0.3, which indicates that on average around 23% of the total NP area was subtracted because of the overlap and suggests that NPs are not located as a clump in the CA1 area but are spread out over this area.

To test theoretically whether an accumulation of NPs in the area of ring 1 would result in a reduction in the neuronal density around NPs, the cellular density was calculated in the area of ring 1 subtracted by the area of three NPs (the maximum number of NPs present in one field

| TABLE 2. Neuronal density in two concentric circles and one ring around neuritic plaque (NP) points versus control points* |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                  | Radius (μm)     | Area (μm²)      | Control         | Plaque          | % Reduction     | F               | p*              |
| Circle 1         | 74              | 15,620          | 0.86 ± 0.05     | 0.69 ± 0.05     | 19             | 6.186           | 0.013           |
| Circle 2         | 123.5           | 46,333          | 0.70 ± 0.03     | 0.59 ± 0.03     | 16             | 6.606           | 0.010           |
| Ring 1           | —               | 30,713          | 0.62 ± 0.03     | 0.54 ± 0.03     | 12.5           | 2.942           | 0.087           |

*Control point: Center of an NP-free field in the CA1 area.

*Data are shown as mean ± SEM.

*The areas of circles 1 and 2 are subtracted by the mean area of NPs.

*An arbitrary circle with a radius of 74 μm centered in NPs or in control points. The area of the plaque in the center is subtracted as a correction.

*An arbitrary circle with a radius of 123.5 μm centered in NPs or control points. The area of the plaque in the center is subtracted as a correction.

*Area of circle 2 subtracted by the area of circle 1 (Fig. 1).

*One-way analysis of variance.
in our study). However, there was only a slight decrease in the significance of reduction ($p = 0.018$) in ring 1.

As shown in Table 1, two of the AD patients (91094 and 90118) showed APOE e4/e4 genotype. To test whether the presence of these two patients could influence our data, we excluded them from the statistical tests. However, there was still a significant difference in neuronal density between NP and control points in circles 1 and 2 ($p = 0.045$ in circle 1, $p = 0.046$ in circle 2, and $p = 0.193$ in ring 1).

**DISCUSSION**

The present study obtained evidence for a local neurotoxicity of NPs in AD. The cell density in the immediate environment of NPs in the hippocampal CA1 area was significantly lower than that of the control points. Furthermore, there was a negative relationship between the size of NPs and the neuronal density around them. However, the contribution of the local neurotoxic effect of NPs to the strong total cell loss in CA1 seems to be only marginal.

To our knowledge this is the first study that deals with the local effects of a neuropathological hallmark of AD, i.e., NPs, on cell density in situ brain sections. The same procedure can be used to study local effects, e.g., of NFTs, Lewy bodies, granulovacuolar degeneration, or amyloid depositions in neurodegenerative diseases. The present procedure not only allowed the study of local effects on the neighboring neurons but also enabled us to evaluate the contribution of local effects to alterations in the entire brain area. This makes it possible to identify a severe local effect that has a negligible effect on the entire area, e.g., because there are relatively few structures with a severe effect on adjacent neurons. Important in this method is the fact that the control areas are selected from the same patients and even from the same sections.

The histological hallmarks of AD are NFTs and NPs. There is still no agreement about the exact pathophysiological role of NPs in AD. Some investigators have hypothesized that the β-amyloid content of the core of NPs is the local initiator of a cascade leading to AD pathology (cf. Selkoe, 1994a, 1994b). It has also been suggested that NPs represent an endstage that does not participate in the pathogenesis of this disease (Roses, 1994). Our study was conducted to determine possible local neurotoxic effects of NPs on the density of neighboring neurons in situ, and we observed only a small effect.

The observations that there is a 19% reduction in cell density around NPs compared with control points and that a negative relationship exists between the size of the NPs and the neuronal density around them suggest that NPs exert a local neurotoxic effect on the neurons. Such a local neurotoxic effect, however, seems to contribute only a little to the strong total cell loss of CA1 area. Only 16.3% of the measured area of the CA1 was covered by circle 2 (radius 123.5 μm). Consequently, only 2.6% of the reduction in neuronal density can be accounted for by the effect of NPs. A local neurotoxic effect of NPs thus seems to be responsible for only a few percentages of the 70% cell death observed in the CA1 area of AD brains (West et al., 1994). An alternative possibility is that NPs exert an effect on neurons in the CA1 area over a distance by axonal or dendritic transport. The observation that the cellular density in ring 1 with an area of 30,713 μm² was not significantly different from that of the control is against this possibility. Of course, a 19% local reduction in neuronal density around NPs can theoretically be caused by many factors other than the β-amyloid content of the core of the NPs, i.e., by other compounds present in the core or by degenerating neurites. However, it can be concluded from our data that none of these putative plaque factors seems to induce a major neuronal loss in the CA1 area in AD.

Although it might be possible that the neurotoxic effect of NPs are transferred via neuronal processes, the present study deals only with the hypothesis that plaques might have local neurotoxic effects. Furthermore, reduction in cell number around plaques can theoretically be compensated for by dislocation of neurons from surrounding areas. However, our data do not support this possibility because we found a 19% reduction in the cellular density in the immediate environments around NPs. An alternative explanation that cannot be excluded is that NPs can be preferentially located in areas with lower cell density. In this sense our estimation that only 2.6% of the reduction in neuronal density can be accounted for by the effect of NPs (see above) should even be considered an estimation of the maximally possible effect of NPs.

Remarkably, as it appears in Table 2, cellular density in circles 1 and 2 and in ring 1 in both control and plaque areas are different from each other. A factor that contributes to this difference is the border effect. In our counting, each cell that was touched by the border of one circle was included in the smaller circle. For this reason the density of neurons in smaller circles is higher than the density in larger ones.

As shown in Table 1, two of the AD patients showed the APOE e4/e4 genotype, whereas the other six had the APOE e3/e3 genotype. It has been suggested that the binding of the β-amyloid peptide to APOE is isoform specific (Strittmatter et al., 1993). For example, the binding of β-amyloid peptide by oxidized APOE e4 is more sensitive to pH changes than to binding by oxidized
APOE ε3, a difference that has been suggested to play a role in the pathogenesis of AD. This idea is not supported by our findings, which showed that there is not a more severe effect of NPs on neuronal density in tissues from APOE ε4/4 patients than from APOE ε3/3 patients. However, more APOE ε4/4 AD patients have to be studied to support this point.

In the present study NPs were stained according to Bodian. Because there are large differences between the type of staining obtained by various NP stainings (Duyckaerts et al., 1990), it might be of interest to perform similar studies using other methods of NP staining and in different brain areas.

Questions of whether the observed decreased neuronal numbers in AD around NPs are due to a direct neurotoxic effect of NPs, whether NPs cause only an increased vulnerability of neurons to a more general AD insult such as decreased metabolic activity (Salehi et al., 1995), and whether β-amyloid is produced in response to ongoing neuronal pathology (Masliah et al., 1990) also remain to be answered. Furthermore, the possible role of NFTs in cell death in the AD brain will be subject of a subsequent study.

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REFERENCES


