Diffuse, Lake-like Amyloid-β Deposits in the Parvopyramidal Layer of the Presubiculum in Alzheimer Disease

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Abstract. A characteristic feature of the parvopyramidal layer of the presubiculum of 6 individuals with Alzheimer disease (AD) was the presence of large, evenly distributed amyloid-β (Aβ) deposits, which in the end stage of the disease occupy 80.9 ± 12.2 % of the parvopyramidal layer. The strong reaction of Aβ deposits with antibodies 4G8 (17–24 amino acids, α-antichymotrypsin, and heparan sulfate proteoglycan, which are promoters of fibrillization or stabilizers of Aβ in neuritic plaques, are absent; activated astrocytes, which are the source of these proteins, are also absent. The unchanged number and distribution and the resting appearance of microglial cells revealed with RCA-I histochemistry suggest that they do not respond to diffuse Aβ deposits. The source of nonfibrillar presubicular Aβ is probably local neurons or neuronal projections to the parvocellular layer of the presubiculum. Neuronal, lake-like Aβ deposition appears to be characteristic of AD pathology. The presubiculum is most likely the model brain structure for the study of amyloid of exclusively neuronal origin. The parvopyramidal layer of the presubiculum reveals only a small population of the neurons (2.5 ± 2%) affected by neurofibrillary pathology.

Key Words: Alzheimer disease; Amyloid-associated proteins; Amyloid-β; Fibrillization; Microglia; Neuron; Parvopyramidal layer of the presubiculum.

INTRODUCTION

Amyloid-β protein (Aβ) deposits in the gray matter and leptomeningeal and parenchymal vessels, neurofibrillary degeneration of neurons, and neuronal loss are pathological hallmarks of Alzheimer disease (AD). Amyloid appears both in nonfibrillar form, mainly in diffuse Aβ deposits, and in fibrillar form, in neuritic plaques and vascular walls. Because of its beta-pleated sheet conformation, fibrillar Aβ shows fluorescence when stained with thioflavin S, and applegreen birefringence when stained with Congo red and examined under polarized light. Both neuropathological and cell culture studies indicate a causal role of fibrillar Aβ in the degeneration and death of neurons in AD (1–5). Some investigators believe that because both diffuse and neuritic plaques exist simultaneously in the brain parenchyma, nonfibrillar Aβ in diffuse plaques fibrillizes and diffuse plaques progress to become neuritic, fibrillary plaques (6–11). An alternative hypothesis is that nonfibrillar Aβ in diffuse plaques is of neuronal origin, whereas the harmful fibrillar Aβ in neuritic plaques is a product of microglial cells (12–15).

In vitro studies of Aβ properties have shown that Aβ1-42 is more fibrillogenic (16–19) than Aβ1-40 (17, 18, 20) and Aβ17-42 (P3 molecule) (20–22). Several amyloid-associated proteins have been shown to have an impact on Aβ fibrillation. Apolipoprotein E (apoE), which is present in significant amounts in plaques (23, 24), binds directly to Aβ (25–27) and promotes both Aβ1-42 and Aβ1-40 fibril formation (19, 28). The protease inhibitor α-antichymotrypsin (ACT) accelerates Aβ1-42 fibrillation (19), but inhibits Aβ1-40 fibrillation (29, 30). Heparan sulfate proteoglycan (HSPG) (31–35) and apolipoprotein AI (apoAI) (36, 37) promote Aβ fibrillation, whereas apolipoprotein J (apoJ) prevents fibril formation (36, 38–40).

In brains of individuals with AD, but not in age-matched control cases, the parvopyramidal layer of the presubiculum accumulates a marked amount of Aβ (41–43). Despite this accumulation, neurofibrillary degeneration in the parvopyramidal layer is minimal (41, 42, 44, 45), and neuronal loss is insignificant (44, 46). Both the entorhinal cortex and the subiculum neighboring the presubiculum suffer from severe neuronal loss caused by neurofibrillary degeneration and significant disorganization of the cytoarchitecture caused by neuritic plaques (44, 45). Heavy amyloid load with insignificant neuronal
loss and degradation of cytoarchitectonics in this part of the hippocampal formation, which is among the brain structures affected the earliest and most severely over the course of AD, may indicate the presence of Alzheimer disease.

The aim of this study is to characterize Aβ deposits in the parvopyramidal layer of the presubiculum. We hypothesize that the properties of these diffuse deposits are related to their unique pathogenesis and that the deposits are of neuronal origin. Because Aβ is released only by neurons, presubiculum deposits have a set of specific features—they are diffuse, nonfibrillar, and free of activated astrocytes, microglial cells, and amyloid-associated proteins, and they do not produce significant damage to the local cytoarchitectonics as neuritic plaques do.

MATERIALS AND METHODS

Our study was performed on tissue blocks from the brains of 6 subjects (80.6 ± 6.9 years old; mean age ± standard deviation, SD) who died in the end stage of AD (Table 1), and 4 nondemented control subjects (75.5 ± 5.8 years old). The dementia of AD patients was assessed by the Global Deterioration Scale (GDS) as GDS stage 7 and by the Functional Assessment Staging (FAST) procedure as FAST stages 7a to 7f (47, 48). The duration of disease from the onset of clinically manifest symptoms in GDS 3 stage until demise ranged from 11 to 19 years.

Archival paraffin tissue blocks of the hippocampal formation and adjacent perirhinal cortex, medial temporal gyrus, and Brodmann’s areas 17 to 19 were deparaffinized with xylene, treated with absolute and 95% alcohol and polyethylene glycol 400 and 1000, and embedded in polyethylene glycol 1000. Slabs were cut on the fronto plane into 90-μm-thick serial sections. The free-floating technique was used for both histological and immunohistochemical stainings. Sections stained with cresyl violet were used to assess the cytoarchitectonics. Neurofibrillar tangles, neuritic plaques, and neuropil threads were visualized with the Gallyas modern silver technique (49, 50). Thioflavin-S and Congo red were used to detect fibrillary Aβ. To characterize Aβ deposits, monoclonal antibodies (mAbs) 6E10, which recognizes the first 17 N-terminal amino acids of Aβ; and 4G8, which is raised against the 17–24 amino acid sequence; and polyclonal antibodies (pAbs) R165, which recognizes the last 9 amino acids of Aβ1-40; and R165, which is raised against the last 11 amino acid sequence of Aβ 1-42, were used (Table 2). The presence of the amyloid-associated proteins apoe, apoJ, apoAI, HSPG, and ACT were tested by using of mAbs and pAbs. Astrocytes were detected by means of mAb to glial fibrillary acidic protein (GFAP). Sections used for immunodetection of Aβ or amyloid-associated proteins were pretreated with 90% formic acid for 30 minutes, incubated overnight with primary antibodies at 4°C, and incubated for 2 hours at room temperature with biotinylated secondary antibodies anti-mouse immunoglobulin from sheep (1:200, Amersham) or anti-rabbit immunoglobulin from goat (1:1000, Sigma). After 1-hour incubation with extravidin–horseradish peroxidase complex (1:200, Sigma), the reaction was visualized by means of 3,3-diaminobenzidine (DAB). Microglial cells were detected by using Ricinus communis agglutinin-I (RCA-I). Sections were incubated overnight with biotinylated RCA-I (1:500, Sigma), and on the next day, with extravidin–horseradish peroxidase complex.

Delineation of the edges of the parvopyramidal layer was based on the size and distribution of neurons. Modified pyramidal cells of the parvopyramidal layer are smaller and more densely packed than those in neighboring subiculum and parasubiculum. Parvopyramidal cells form a continuous layer medially and islands laterally (51, 52).

The density of neurons without and with neurofibrillary degeneration was measured by means of the dissector unbiased method (53) in sections stained with the Gallyas method and counterstained with cresyl violet. The total number of neurons and tangles in the sample (20 × 20 μm and 50-μm depth) were counted, and the percentage of affected neurones was calculated. The coefficient of error, as a measure of sampling efficiency, was calculated for any case, and was found to be less than 0.06 (54).

The amyloid burden (i.e., the percentage of the area of the parvopyramidal layer of the presubiculum covered by Aβ deposits), detected with mAbs 6E10 and 4G8 and pAbs R165 and R162, was quantified by using the “C IMAGING” (COMPIX)
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<tr>
<td>Aβ; 17−24 aa (4G8)</td>
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<td>IBR (93)</td>
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* HSPG = heparan sulfate proteoglycan. ** ACT = α,-antichymotrypsin.

Inc., Cranberry Township, Pa) image analysis system. A gray-level threshold was set separately for analyzed sections to distinguish the Aβ deposits from the background. The coefficient of error was calculated for any staining; for any case, it was less than 0.06. Differences in the amyloid burden between particular stainings were evaluated with an ANOVA test followed by a post-hoc Bonferroni test.

RESULTS

In the subicular complex of all 6 AD subjects who died in the late stage of disease, we discovered a similar pattern of Aβ deposition, distribution of amyloid-associated proteins, and neurofibrillary degeneration. Diffuse Aβ-immunopositive deposits that covered the entire parvopyramidal layer of the presubiculum (Fig. 1) were prominent. In 3 cases, the deposits exceeded the borders of the parvopyramidal layer and partially covered both the underlying deep layers and the molecular layer, reaching the surface of the cortex. The amyloid burdens in the parvopyramidal layer of the presubiculum revealed by mAbs 6E10 and 4G8 and by pAb R165 (32−42 aa) were comparable: 88.9% (SD 6.5%), 80.9% (SD 12.2%), and 65.9% (SD 15.1%), respectively (Fig. 1a−c). Statistical evaluation performed by ANOVA followed by the post-hoc Bonferroni test (p < 0.05) showed that the 9.2% (SD 11.7%) amyloid burden revealed by pAb R162 (32−40 aa) is significantly less than that shown with other antibodies (Fig. 1d). In all studied cases, Aβ deposits in the presubicular parvopyramidal layer were negative when stained with both thioflavin-S and Congo red (Fig. 2). Vessels in the presubiculum also were thioflavin-S-negative. In contrast to the negative staining in the presubiculum, subicular plaques were both thioflavin-S- and Congo red-positive.

The presubicular deposits were negative when stained with antibodies against apoE and apoAI (Fig. 3). In contrast to negative staining in the presubiculum, the subiculum contained numerous apoE-positive plaques and a few pale apoAI-positive plaques. The presubicular deposits also were negative when stained with the antibody anti apo J. In the presubiculum, this antibody stained some neuritide threads and microglial cells, whereas in the subiculum, hippocampus, and temporal cortex, numerous apoJ-positive plaques and neurons with and without neurofibrillary changes and microglial cells were seen. The Aβ deposits in the parvopyramidal layer of the presubiculum were negative when stained with pAb

**Fig. 1.** Strong reaction in diffuse Aβ deposits in the parvopyramidal layer of the presubiculum (PP) of an 84-year-old AD subject immunolabeled with mAbs 6E10 (1−17 aa) (a) and 4G8 (17−24 aa) (b) and pAb R 165 (32−42 aa) (c). pAb R162 (32−40 aa) detects only slightly immunopositive deposits in the superficial part of the parvopyramidal layer (d). All 4 antibodies stain Aβ plaques in the subiculum (S). Scale bar = 800 μm.

**Fig. 2.** No fluorescence in presubiculum Aβ deposits (a), but strong fluorescence in plaques and NFTs in the subiculum (b) in sections stained with thioflavin S. Scale bar = 200 μm.

**Fig. 3.** When stained with mAb against apoE, amyloid deposits in the parvopyramidal layer of the presubiculum (PP) are negative, whereas subicular plaques (S) are strongly positive. Scale bar = 800 μm.

**Fig. 4.** Gallyas silver staining shows only a few neurofibrillary tangles and neuritic threads in the presubicular parvopyramidal layer (a), and numerous neurofibrillary tangles, neuritide threads, and neuritic plaques in the adjacent subiculum (b). Scale bar = 200 μm.
against HSPG. Proteoglycan-positive materials were found in the vascular walls. In the neocortex, vessels and a subset of plaques were positive. The Aβ deposits in the parvopyramidal layer also were negative when stained with antibodies against ACT. In the subiculum and the neocortex, some neurons, glial cells, and a subset of plaques were ACT-positive.

In the parvopyramidal layer of the presubiculum, Gallya silver staining revealed that only 2.5 ± 2% of neurons were affected by neurofibrillary pathology (Fig. 4). A few neurofibrillary threads, but no neuritic plaques, were found.

In the presubicular parvopyramidal layer, RCA-I histochemistry revealed numerous uniformly scattered microglial cells with small round perikarya and multiple dendritic processes spreading in various directions, which is characteristic of resting microglia (Fig. 5a, c). In contrast to resting microglia in the parvopyramidal layer, numerous microglial cells in the subiculum, perirhinal cortex, and other regions of the hippocampal formation showed morphological features of activation (Fig. 5b, d). They had large oval or round perikarya and short cytoplasmic processes, and were gathered in clusters of several cells that corresponded to Aβ plaques.

Only a few small, GFAP-positive astrocytes were found in the parvopyramidal layer (Fig. 6a). In contrast to the weak presubiculal reaction in the subiculum and the cornu Ammonis, numerous hypertrophied astrocytes were found grouped in clusters, corresponding to primitive and classical plaques (Fig. 6b).

In 3 control cases, the parvopyramidal layer of the presubiculum was free of Aβ deposits. In 1 case, small diffuse deposits were observed. They covered 0.2% of the parvopyramidal layer when stained with mAb 4G8. In all control cases, a few classical and primitive plaques were observed in other regions of the hippocampal formation.

DISCUSSION

Hippocampal formation subdivisions such as the cornu Ammonis and the subiculum, which develop pathological changes in the early stages of AD (55), are considered the most atrophic brain structures in the end stage of the disease (56). Like the subiculum proper and the parasubiculum, the presubiculum is affected by Alzheimer type pathology; however, the pattern of amyloidosis-β, neurofibrillary degeneration, and neuronal loss indicates that
AMYLOID-β OF NEURONAL ORIGIN DOES NOT FIBRILLIZE

Fig. 6. Only a few astrocytes are GFAP-positive in the molecular (mo) and the parvopyramidal (pp) layers of the presubiculum (a). Clusters of enlarged GFAP-positive astrocytes are evident in the subiculum (b). Scale bar = 200 μm.

the course of AD pathology in the presubiculum is different from that in other hippocampal subdivisions.

Several features distinguish amyloidosis-β in the presubiculum from that in the subiculum proper and the parasubiculum. In AD, the whole parvopyramidal layer of the presubiculum is filled with diffuse amyloid deposits (41–43), whereas in the other subicular subdivisions, amyloid accumulates in classical, primitive and diffuse plaques. The amyloid burdens of approximately 89%, 81%, and 66%, visualized in the parvopyramidal layer by using antibodies detecting amino acids 1–17 (6E10), 17–24 (4G8), and 32–42 (R165) of Aβ protein, respectively, are several times greater than in other subdivisions of the hippocampal formation (57) or in the neocortex (58). The 9.2 ± 11.7% amyloid burden detected with pAb R162 shows that the area occupied by Aβ1-40 is about 8 times less than that occupied by Aβ1-42. Very strong reaction with mAbs 4G8 and 6E10 and pAb R165 and weak reaction with pAb R162 indicate that neuropil contains a marked amount of highly fibrillogenic form Aβ1-42, but a relatively small amount of less-amylodigenic Aβ1-40. However, the methodology used cannot exclude the existence of a small amount of Aβ17-42 (20). Sections treated with thioflavin-S and examined in fluorescence, and stained with Congo red and examined in polarized light prove the absence of fibrillar amyloid. The parvopyramidal layer of the presubiculum, despite the local prevalence of fibrillogenic Aβ1-42 after 11 to 21 years of symptomatic AD, still contains only amorphous, nonfibrillar Aβ. The lack of fibrillar amyloid in the presubiculum might be the result of the concentration of Aβ in the extracellular space being too low. There are no data characterizing the local concentration of Aβ in the presubiculum that is free of fibrillar amyloid and in the subiculum proper or the parasubiculum that has numerous fibrillar plaques; however, the similar intensity of immunocytochemical reaction detecting amyloid in these structures suggests that concentrations might be comparable. Amyloid burden in the range of 66% to 89% revealed with antibodies to Aβ1-42 suggests that the total amount of Aβ in the parvocellular layer might be even higher than in the hippocampal subdivisions. The lack of fibrillation of potentially fibrillogenic Aβ1-42 might be related to the origin of Aβ peptide and to the local conditions associated with the cell-specific mechanism of generation of Aβ and amyloid-associated proteins.

Several amyloid-associated proteins that may promote or prevent fibril formation were characterized immunocytochemically. ApoE and apoAI may interact directly with Aβ and promote fibrillation or may fibrillize spontaneously themselves and serve as a nidus for deposition of fibrillar Aβ (36, 59). Antichymotrypsin promotes aggregation of Aβ1-42, but inhibits aggregation of Aβ1-40 (29, 30). The absence in presubicular Aβ deposits of amyloid-associated proteins promoting fibrillation, such as apoE, apoAI, HSPG, and ACT, as well as of those inhibiting fibrillation such as apoJ, indicates that in a local environment free of amyloid-associated proteins, Aβ1-40 and Aβ1-42 present in the proportion of 1:8 do not fibrillize, even after many years of amyloid accumulation. The absence of amyloid-associated proteins in presubicular Aβ deposits appears to be related to lack of activation of astrocytes, which are regarded to be a source of apoE (60, 61), ACT (62, 63), and HSPG (64, 65).

Fibrillation of Aβ in the adjacent subiculum proper and parasubiculum and the lack of fibrillation in the presubiculum suggest that the key factor in Aβ fibrillation is the different origins of amyloid in subicular subdivisions. Our studies and others show that fibrillar Aβ deposition is associated with smooth muscle cells in the walls of arteries and veins in leptomeninges and parenchymal vessels (66, 67), with cells of monocyte-microglial cell lineage in the walls of gray matter capillaries (68), and with parenchymal microglial cells in classical and primitive plaques (12, 69–71). Diffuse amyloid deposits in human, dog, and transgenic mice brains are associated with neurons (7, 8, 14), which release Aβ at synaptic terminals (72). Staining with RCA-I in the
presubicular parvopyramidal layer revealed evenly dispersed microglial cells with a resting appearance, recalling intact structure, whereas in other hippocampal formation subdivisions, numerous clusters of activated microglial cells were colocalized with aggregates of fibrillar Aβ. Characteristics of Aβ and amyloid-associated proteins, astrocytes, and microglial cells, as well as the low numbers of neurofibrillary tangles and neuropil threads in the presubiculum, are in accord with the properties both of diffuse amyloid deposits and of properties of amyloid characterized as being of neuronal origin.

The lack of activated microglial cells in the parvopyramidal layer of the presubiculum suggests that these cells are not engaged in amyloid production or amyloid removal. The topography and distinct borders of Aβ deposits may indicate that the source of Aβ is neuronal projections to the parvopyramidal layer of the presubiculum or the parvopyramidal cells themselves. The presubiculum receives input from the anterior thalamic nuclei and the cingulate cortex (73–75). Projections from these structures also innervate deep layers of the entorhinal cortex (76–78), in which diffuse plaques prevail (52, 79). Aβ deposition in the presubiculum without activation of microglial cells and astrocytes and in the absence of the majority of glia-related factors would appear to be the model of the neuronal origin of amyloid.

The abundance of Aβ1-42 in comparison with other forms of Aβ and the lack of fibrillization in the presubiculum recall the properties of diffuse plaques in the brains of aged dogs (80). Trisomy of chromosome 21 is the cause of both the elevated expression of the amyloid precursor protein and the appearance of Aβ1-42 in brains as early as 21 weeks gestation (81). Subjects with Down syndrome develop nonfibrillar diffuse Aβ1-42-positive plaques in the second decade of the life, whereas fibrillar plaques appear more than 20 years later when the first microglia containing plaques appear (13). Experimental models show that elevated expression of the amyloid precursor protein in the neurons of transgenic mice results in accumulation of Aβ1-42 in the brain parenchyma; however, this amyloid has no tendency to assemble into fibrils (82–84). However, if the transgene is expressed not only in neurons, but also in non-neuronal cells, both diffuse nonfibrillar and neuritic fibrillar plaques are found (85).

The Aβ originating from microglial cells appears to fibrillize readily and to form deposits that cause degeneration in the surrounding neuropil (12). Fibrillar amyloid deposition associated with smooth muscle cells of brain vessels is the cause of degeneration and death of myocytes, which are the source of Aβ peptide (66). In contrast to fibrillar amyloid, nonfibrillar Aβ appears to cause no harm to the brain parenchyma (14, 86–88). The presubicural parvopyramidal layer contains no neuritic plaques and fewer NFTs or neurofibrillar threads than other regions of the hippocampal formation (41, 44, 45). According to Davies et al (46) and Fukutani et al (44), there is no neuronal loss in the presubiculum in AD.

Astrocytes react to neuronal degeneration and death by proliferation, hypertrophy, and secretion of trophic factors and mediators (89, 90). Their activation in areas of fibrillar amyloid deposition indicates that they respond to amyloid and probably participate in amyloid removal (91). The absence of astrocytic reaction in the parvocellular layer may indicate that presubicular Aβ deposits do not elicit astrocyte response. In AD, astrocytes also respond to neurofibrillary tangles, which in the late stages of cell degeneration are penetrated by astrocytic processes (92). However, neurofibrillary changes and ghost tangles are so sparse in the presubiculum that they do not activate astrocytes here the way they do in other brain subdivisions.

This study suggests that the uniqueness of Alzheimer-type pathology in the presubiculum is related mainly to the neuronal origin of Aβ. The parvocellular layer of the presubiculum appears to be the ideal region of the brain for study of Aβ deposition by neurons only. Preservation of this structure despite chronic accumulation of potentially amyloidogenic Aβ1-42 indicates that amyloid of neuronal origin without the cofactors typical for neuritic plaques is relatively neutral and harmless to the brain parenchyma. This study also shows that amyloid deposition by neurons is a separate pathological process that exists in the presubiculum in pure form without microglial cell and astrocyte activation or all of the pathological events related to Alzheimer-type glial activation.

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