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Loss of the Presynaptic Vesicle Protein Synaptophysin in Hippocampus Correlates with Cognitive Decline in Alzheimer Disease

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Abstract. We tested the hypothesis that synaptic defects in the hippocampus of individuals with Alzheimer disease (AD) correlate with the severity of cognitive impairment. Three postmortem groups were studied: controls with normal and stable cognition; cognitively intact subjects with senile plaque densities diagnostic for possible AD (p-AD) and neurofibrillary changes characteristic of early AD (Braak stage III); and individuals with definite AD and neurofibrillary changes typical of incipient to severe AD (Braak stage III, V, or VI). Synaptophysin (a presynaptic vesicle protein) levels were quantified by immunoblotting of synaptic membrane fractions isolated from hippocampus, entorhinal cortex, caudate nucleus, and occipital cortex. Average synaptophysin levels were reduced in hippocampus when comparing definite AD to controls (55%, $p < 0.0001$), p-AD to control (25%, $p < 0.005$), and definite AD to p-AD (30%, $p < 0.05$), but levels in entorhinal cortex, occipital cortex, and caudate nucleus were either unchanged or less significantly altered than in hippocampus. By univariate analysis, hippocampal synaptophysin levels correlated with neuropsychological measurements, including Mini-mental state examination scores ($r = 0.83$, $p < 0.0001$) and Blessed scores ($r = 0.74$, $P < 0.001$), and with senile plaque densities ($r = 0.89$, $p < 0.0001$). We conclude that synaptic abnormalities in the hippocampus correlate with the severity of neuropathology and memory deficit in individuals with AD, and that this defect may predate neuropsychological evidence for cognitive impairment early in AD.

Key Words: Braak stage; CA1; Memory loss; Senile plaque; Synapse.

INTRODUCTION

Progressive impairments in memory and cognition occur with aging and, more profoundly, with Alzheimer disease (AD), but the mechanisms are not understood. A large proportion of the elderly population has impairments in memory and cognition (1). Many (15 to 20%) individuals >65 years of age will experience mild memory impairment (2). A significant proportion (5 to 7%) of these elderly individuals with mild memory problems will become demented, the majority having AD (1). The prevalence of AD in elderly individuals doubles every 5 years between 65 and 85 years of age (3, 4). Initially, it can be difficult to distinguish between benign, age-associated memory impairments and the early manifestations of AD. In this context, it is important to identify markers for early AD.

Synapses are the principal unit of intercellular communication in neural circuits, and long-term potentiation of hippocampal synaptic function is important in animal models of memory and learning (5, 6). Assessment of the regional integrity of synapses in the brains of elderly humans relies on prospective neuropsychological and neurological evaluations or, in postmortem brain, quantitative ultrastructural evaluation of synaptic density and immunological detection of synaptic proteins. Based on these approaches, synaptic loss in neocortex has been postulated to be associated with the cognitive and memory impairments in normal elderly individuals (7, 8) and in subjects with AD (9–15). However, only a few studies have identified correlations between mental status test scores and counts of presynaptic terminals in AD neocortex as determined by electron microscopy (12) or levels of presynaptic enyzmes in AD neocortex as determined by neurochemical assay of choline acetyltransferase (16), and only 1 study (14) has shown correlations between cognitive deficits and decrements in immunologically detectable synaptophysin in the neocortex of individuals with AD. These findings raise several questions. Does synaptic loss occur in aged individuals without clinical evidence of memory deficits, or is synaptic loss primarily an end-stage event associated with the profound neuropathology of AD? Is there a brain regional selectivity for the loss of synapses in normal aging and AD? Surprisingly, despite the well-established role of hippocampus in memory and learning (17, 18) and its vulnerability in AD (19), few studies have attempted to identify relationships between indices of synaptic integrity in hippocampus and neuropsychological function in elderly populations (20).

To address these questions, we used quantitative immunoblotting (21, 22) to test the hypothesis that abnormalities in the levels of the presynaptic vesicle protein

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TABLE 1
Autopsy Cases Used for Immunoblotting Assay

	Case no.	Age/Sex	Post-mortem delay (hours)	MMSE score[1]	Blessed score[3]	Free recall score	Braak stage	Cause of death
Control	732	93/M	5	29[2]	1	24	—	Pneumonia
	827	79/M	10	29	0	30	—	Cardiac arrhythmia
	1013	91/M	10	29	2	31	—	Prostate carcinoma
	1036	81/M	20	30	1	37	—	Pneumonia
	1172	77/M	13	26	8	29	—	Cardiac arrhythmia
	1189	89/M	5	27	2	26	—	Metastatic prostate carcinoma
P-AD	1101	79/F	11	26	4	13	III	Renal and cardiac failure
(early AD)	1104	82/F	14	27	1	n/a	III	Pancreatic carcinoma
	1139	87/M	11	28	1	26	III	Pneumonia
	1141	91M	16	30	2	33	III	Metastatic prostate carcinoma
AD	916	86/F	4	24	7	23	III	Pneumonia
	1143	83/M	n/a	24	7	24	III	AD
	1177	92/F	20	16	16	7	V	Abscess, sepsis
	400	77/M	5	22	8[4]	n/a	V	AD
	941	83/F	15	15	18[4]	n/a	VI	AD
	1164	74/M	19	13	21[4]	n/a	VI	Pneumonia

[1] The mean interval between last MMSE score and time of death was 25.8 months with a range of 1 month to 49 months.
[2] Derived from Blessed score (28).
[3] The mean interval between last Blessed test and time of death was 14.5 months, with a range of 1 month to 38 months.
[4] Converted from MMSE scores (28).

synaptophysin occur in the hippocampus of elderly humans with intact cognition and the neuropathology of early or possible AD (p-AD) and in demented individuals with AD. We used postmortem brain samples from elderly subjects who had undergone prospective longitudinal, neurological, and cognitive evaluations shortly before death and up to 5 years prior to death and who had received a detailed neuropathological evaluation (23). In individuals with definite AD, loss of synapse marker in hippocampus correlated strongly with the severity of cognitive deficit and with neuropathology. In individuals with p-AD and a Braak stage diagnostic for early AD, loss of hippocampal synaptophysin was detected, despite normal cognitive scores. We conclude that presynaptic defects in hippocampus could precede the clinical occurrence of memory and cognitive impairment in early AD.

MATERIALS AND METHODS

Subjects and Clinical Evaluation

Most subjects (13 of 16) used in this study were examined according to the Baltimore Longitudinal Study of Aging (BLSA) protocols (24), including a comprehensive medical history, complete neurological examination, and a battery of neuropsychological tests. The non-BLSA subjects (3 of 16) were enrolled in the Alzheimer Disease Research Center at Johns Hopkins. The mental status test scores that were used to establish correlation with synaptophysin levels in this study were the Mini-mental State Examination (MMSE), Blessed, Free Recall, Spatial Location, and Delayed Recall (23).

Neuropathology

Postmortem brain samples from 3 age-matched groups of individuals were analyzed (Table 1). Paraffin-embedded blocks of hippocampus (anterior and mid-hippocampus), occipital pole, and caudate from formalin-fixed brains (the left hemisphere) were sectioned (10 μm) and stained using the Hirano silver method, which in our experience is the most sensitive method for the detection of neuritic and diffuse SP (23). Relative densities of SP in CA1, entorhinal cortex, occipital cortex, and caudate were determined using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) protocol, which advocates the use of SP counts rather than neurofibrillary tangle counts for the neuropathological diagnosis of AD (25). Diffuse SP were identified as amorphous or fibrillary extracellular argyrophilic structures, and neuritic SP had readily identifiable swollen axonal/dendritic processes (23). In 3 different 1 mm^2 fields in each region, the number of diffuse and neuritic SP were counted. Average counts (representing combined types of SP) were expressed as SP per mm^2. In addition, the subjects were evaluated according to the Braak neuropathological staging scheme, which relies on neurofibrillary changes for the classification of AD (26). Neurofibrillary changes in Hirano silver-stained sections were evaluated at 3 levels to ascertain the Braak stage (26): anterior hippocampus (through the uncus), mid-hippocampus (at the level of the lateral geniculate nucleus), and occipital pole (including area 17 and the para- and peristriate regions).

Immunoblotting

Samples from the entorhinal cortex (Brodmann area 28), hippocampal formation (primarily CA1), caudate nucleus (rostral

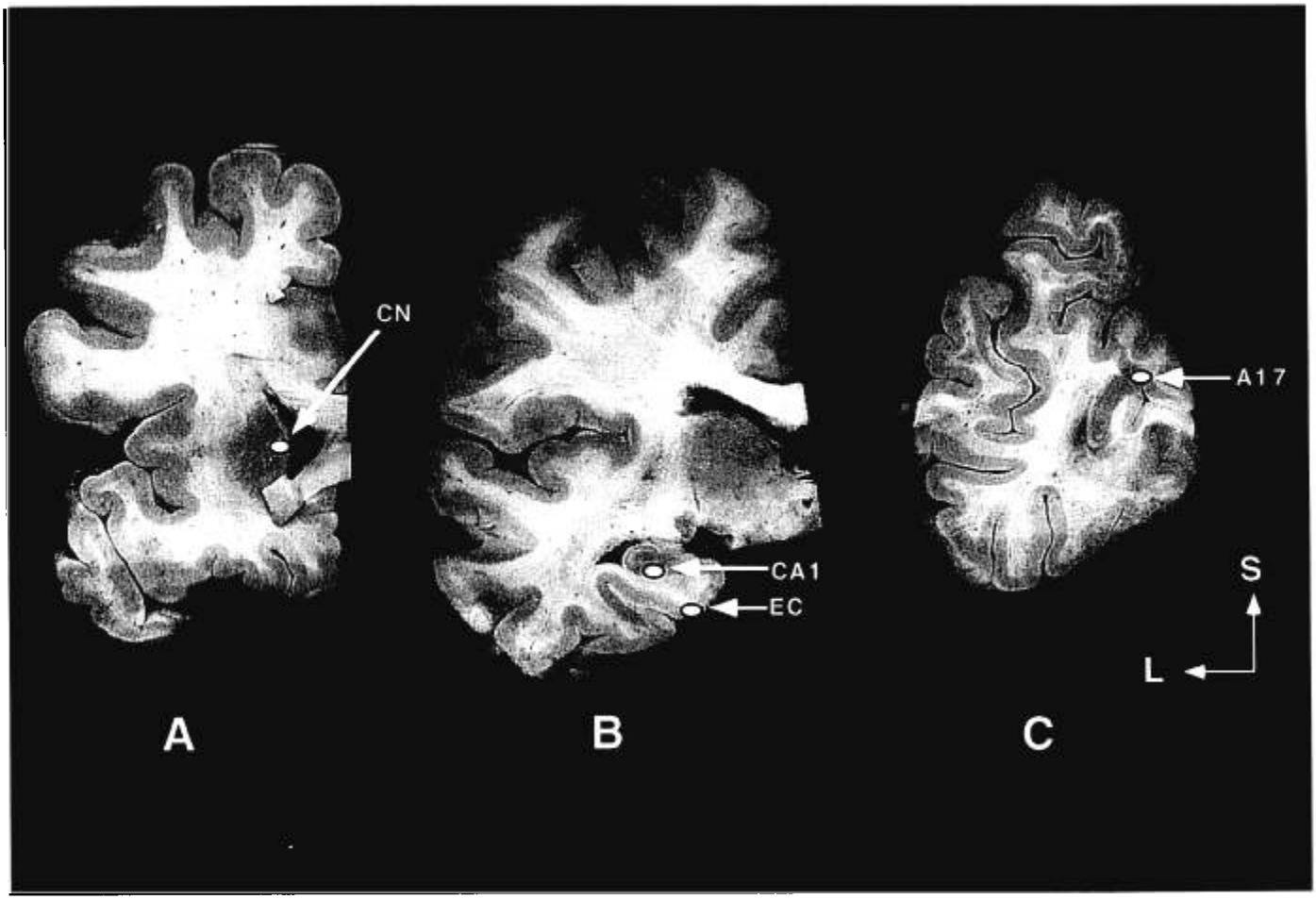


Fig. 1. Location of single punch samples that were taken for immunoblot analyses. Slabs A–C (from a formalin-fixed control brain) are shown to identify the precise location of the samples that were obtained from unfixed frozen hemispheres. Solid white ellipses indicate regions that were punched from the anterior caudate nucleus (CN, level A), hippocampus and entorhinal cortex (CA1 and EC, level B), and visual cortex (A17, level C). The orientation of the slabs is indicated by the arrows (S, superior; L, lateral).

head), and primary visual cortex (Brodmann area 17; identified by the line of Gennari) were used for quantitative immunoblotting (Fig. 1). Samples of caudate and striate cortex were intended for internal controls. Using a 2 or 4 mm Acu-Punch (Acuderm Inc., Fort Lauderdale, Florida), samples (0.3 to 0.5 g) were obtained from fresh-frozen postmortem brain slabs of the right hemisphere (Fig. 1) that were stored at −70°C and warmed to −20°C. As described previously (21, 22), samples were homogenized with a Brinkman Polytron in cold 20 mM Tris HCl (pH 7.4) containing 10% (wt/vol) sucrose, 20 U/ml aprotinin (Trasylol), 20 μg/ml leupeptin, 20 μg/ml antipain, 20 μg/ml pepstatin A, 20 μg/ml chymostatin, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, 1 mM EDTA, and 5 mM EGTA. Crude homogenates were centrifuged at 1,000 g_{av} for 10 minutes (min). The supernatant (S1 fraction) was then centrifuged at 114,000 g_{av} for 20 min. The resulting pellet (P2 fraction) was washed in homogenization buffer (without sucrose) 3 times by resuspension, followed by centrifugation at 114,000 g_{av} for 20 min. The final pellet (P2 fraction) was resuspended fully in this buffer supplemented with 20% (wt/vol) glycerol. Protein concentrations were measured by a Bio-Rad protein assay with bovine serum albumin as a standard.

The levels of synaptophysin (p38) and β-tubulin immunoreactivities were quantified by immunoblotting. To establish linearity of the immunoblot assay and, thus, demonstrate that synaptophysin and β-tubulin immunoreactivities were not saturated by loading excessive amounts of protein, a standard curve was created by loading 0.5, 1.0, 10, 15, 20, and 30 μg of P2 fraction protein to each lane. Based on the results of the standard curve (Fig. 2), 15 μg of P2 fraction protein was determined to be optimal for comparisons between samples. All samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane by electroblotting (21, 22). Blots were blocked with 2.5% nonfat dry milk with 0.1% Tween 20 in 50 mM Tris-buffered saline (pH 7.4), then incubated overnight at 4°C with mouse monoclonal antibody against synaptophysin (SY38, Boehringer Mannheim, stock concentration 1 mg/ml), diluted 1:10,000 (0.1 μg/ml). For β-tubulin levels, membranes were incubated with monoclonal anti-β-tubulin (Amersham,

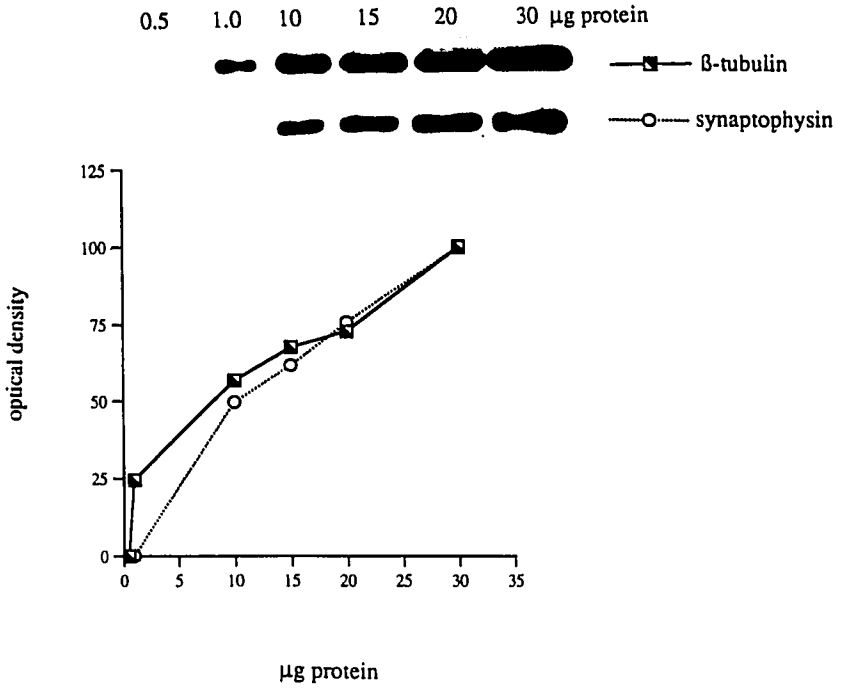


Fig. 2. Standard curve analysis of immunoreactivity for synaptophysin and β-tubulin demonstrates the linearity of the assay method. Immunoblots of synaptophysin and β-tubulin were generated by loading increasing amounts (0.5 to 30 µg) of total protein in extracts of crude synaptic membranes from human hippocampus (case 1036). The relative OD (y axis) of resulting immunoreactivity was plotted against protein amount (x axis). Corresponding immunoblots that were quantified are shown (top).

Arlington Heights, Illinois) at a 1:20,000 dilution (0.05 µg/ml). After the primary antibody incubation, membranes were washed and incubated with peroxidase-conjugated secondary antibody (0.2 µg/ml) and developed with enhanced chemiluminescence (Amersham).

The reliability of sample loading and electroblotting in each experiment was evaluated by staining membranes with Ponceau S before immunoblotting. Because β-tubulin levels in the brain have been shown to be unchanged in AD (27), each blot was probed for β-tubulin. Thus, as best as possible, we ensured that equivalent amounts of protein were loaded in each lane and that transfer was comparable.

To quantify synaptophysin and β-tubulin immunoreactivities, films were scanned using a Macintosh Adobe Photoshop program and an Agfa Arcus Plus scanner. Densitometric analysis was performed using signal Analytics IP Lab Gel software. Synaptophysin and β-tubulin protein levels were expressed as relative optical density (OD) measurements, determined by comparing the density and area of the immunoreactive band in each lane scanned to control lanes in the same blot. The relative values for each case were replicated in duplicate or triplicate experiments.

Statistical Analyses

The OD for synaptophysin and β-tubulin immunoreactivities, numbers of SP, and mental status measurements (i.e. MMSE, Blessed, Free Recall, Delayed Recall, and Spatial Location scores) were assessed by student *t*-test, ANOVA, simple and multiple regression, and Spearman correlation procedure to evaluate statistically significant changes among groups.

RESULTS

Characterization of Subjects

The groups of subjects evaluated in this study were characterized as definite AD, possible AD or early AD, and normal controls (Tables 1 and 2). Individuals with definite AD (n = 6; age range, 74 to 92 years; postmortem delay, 4 to 20 hours [h]) had a clinical history of dementia and high densities of SP in hippocampus, entorhinal cortex, neocortex, and caudate (Tables 1 and 2). The Braak stages of the definite AD cases were either stage III (cases 916 and 1143), stage V (cases 400 and 1177), or stage VI (cases 941 and 1164). Thus, the severity of neurofibrillary pathology was variable in the definite AD cases. In the entorhinal cortex of definite AD cases, neurofibrillary tangles were concentrated in layer 2; however, they could be found either uniformly throughout this layer (Fig. 3A) or in clusters (Fig. 3B), while deeper layers had varying densities of SP. The duration of cognitive dysfunction in cases of definite AD ranged from 2.1 to 10 years (Table 1). Subjects who had normal cognitive scores but sufficient numbers of SP in neocortex for the diagnosis of AD (Tables 1 and 2) were classified as p-AD (n = 4; age range, 79 to 91 years; postmortem delay; 11 to 16 h). The neurofibrillary changes in these 4 subjects were typical of Braak stage III and could thus be regarded as early AD (26). Throughout the entorhinal cortex of early AD cases, the

TABLE 2
Average Synaptophysin Levels and Senile Plaque Densities

	Hippocampus		Entorhinal cortex		Occipital cortex		Caudate	
	OD±SE[1]	SP±SE[2]	OD±SE	SP±SE	OD±SE	SP±SE	OD±SE	SP±SE
Control	95 ± 3.3	2.4±2.4	88 ± 5.9	3.3 ± 3.3	88 ± 5.6	1.6 ± 1.6	76 ± 10.5	0.3 ± 0.3
P-AD (early AD)	70 ± 6.0	14 ± 0.8	69 ± 10	32 ± 6.3	55 ± 10.9	16 ± 6.2	54 ± 15.8	26 ± 3.4
AD	40 ± 9.2	26 ± 3.2	62 ± 12	54 ± 12	68 ± 8.1	40 ± 4.7	68 ± 5.7	29 ± 4.9

[1] Synaptophysin values represent mean ± standard error (SE) of % optical density. Averages are based on 6 control cases, 6 definite AD cases, and 4 p-AD cases.
[2] Values represent average senile plaques (SP)/mm² ± standard error (SE).

neurofibrillary changes in layer 2 were not of uniform severity (even in the same case); some regions of entorhinal cortex had more frequent neurofibrillary tangles than other fields of entorhinal cortex (Fig. 3C, D). Normal controls (n = 6; age range, 77 to 93 years; postmortem delay, 5 to 20 h) had no evidence of neuropsychiatric disorders, had stable cognitive performance over the years, and had few SP in hippocampus, entorhinal cortex, neocortex, and caudate (Tables 1 and 2). Scattered neurofibrillary tangles were found in layer 2 of the entorhinal cortex in normal controls (Fig. 3E), consistent with our previous studies of the BLSA cohort (23). Based on autopsy reports, there was histopathological evidence of heart disease in 5 out of 6 control cases, 1 out of 4 p-AD cases, and 2 out of 6 definite AD cases.

Immunoblot Assay System

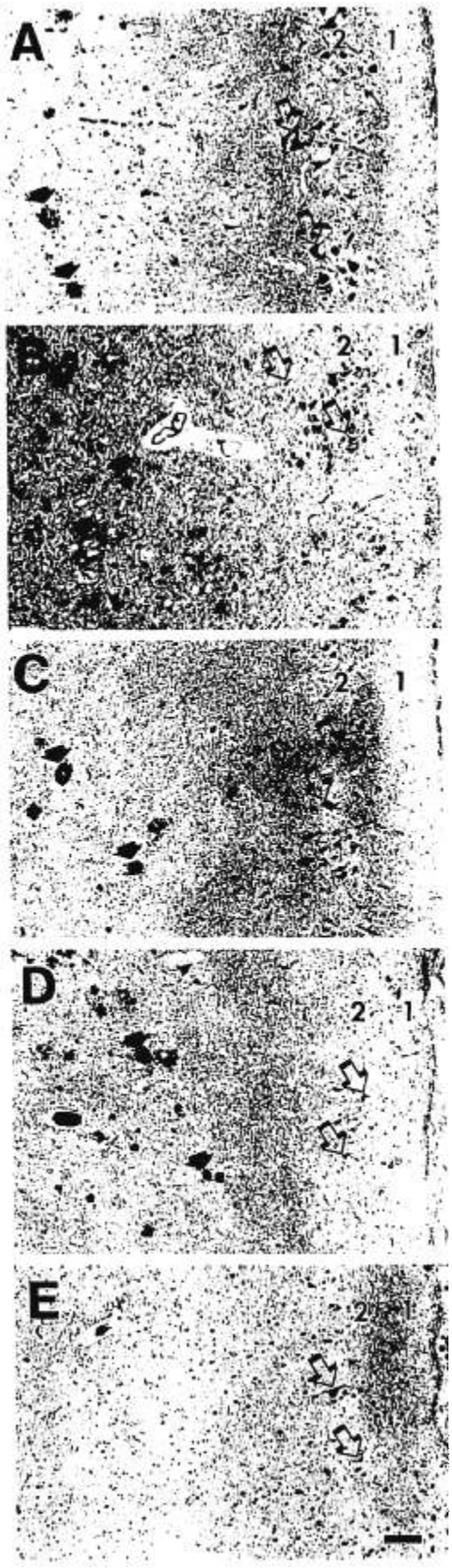
The detection of relative amounts of synaptophysin and β-tubulin immunoreactivities (determined by OD) in crude synaptic membrane homogenates with enhanced chemiluminescence was linear over a range of protein concentrations from 0.5 to 30 μg (Fig. 2). Therefore, the assay system was not saturated because of excessive amounts of sample and high concentrations of reagents. The monoclonal antibodies to synaptophysin and β-tubulin were highly specific, and this specificity in human brain was not altered by the presence of disease or postmortem autolysis ranging from 4 to 20 h. In hippocampus (Fig. 4), entorhinal cortex, occipital cortex, and caudate nucleus of controls and individuals with AD, the antisynaptophysin antibody detected a band of proteins at 38 kDa, and the anti-β-tubulin antibody detected a band of proteins at 55 kDa. The M_r of these proteins was unaltered in cases of AD compared with controls.

Abnormalities in Synaptophysin Levels Occur in the Hippocampus of Individuals with Definite AD and Early AD

A representative immunoblot of synaptophysin and β-tubulin levels in definite AD, p-AD, and control hippocampi is shown in Figure 4. In cases of definite AD and p-AD compared with controls, there were qualitative differences in the abundance of synaptophysin immunoreactivity, but the relative amounts of β-tubulin in hippocampus were similar (Fig. 4). The quantitative densitometric results of the regional levels of synaptophysin and β-tubulin immunoreactivities are summarized in Figure 5 and Table 2. When mean levels of synaptophysin immunoreactivity in hippocampus were compared between definite AD cases and controls, there was a large significant decrease of 55% ($p < 0.0001$). A 26% decrease in synaptophysin was found in entorhinal cortex of definite AD cases, but this change was not significant ($p < 0.1$) compared with control, most likely because of the greater variability in entorhinal cortical measurements as compared with the measurements for hippocampus. A 20% reduction in synaptophysin in the occipital cortex of definite AD cases was also not significant ($p < 0.1$) compared with controls. No significant changes were found in the caudate nucleus (Fig. 5). In p-AD cases, average synaptophysin values were significantly decreased 25% ($p < 0.005$) in hippocampus as compared with controls. Synaptophysin was also reduced significantly in the occipital cortex (33%, $p < 0.05$) of p-AD cases. However, there were no significant changes in entorhinal cortex or caudate. Comparisons of average levels of synaptophysin in definite AD and p-AD disclosed a significant difference in hippocampus, with a 30% ($p < 0.05$) reduction in definite AD vs p-AD, but no differences were observed in entorhinal cortex, occipital cortex, or caudate. Mean values for β-tubulin immunoreactivity were not significantly different among groups in hippocampus, entorhinal cortex, occipital cortex, or caudate (Fig. 5).

Synatophysin Levels Correlate with Cognitive Functions

We classified subjects with an MMSE score of 0 to 14 as severely demented and those with an MMSE score of 15 to 25 as moderately demented, while individuals with MMSE scores > 25 were classified as cognitively normal. The majority of definite AD cases evaluated in this study were moderately demented (Table 1). Only one subject (case 1164) was severely demented. By univariate analysis of all cases, MMSE, Blessed, and Free Recall scores



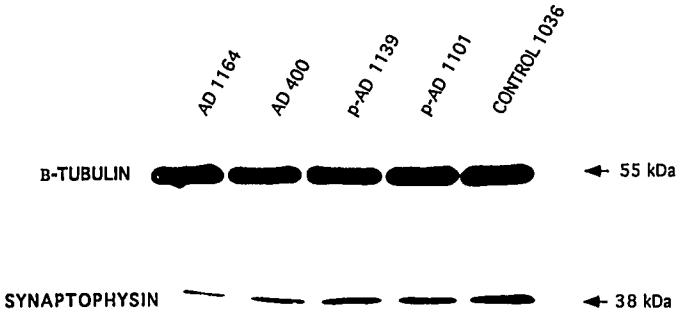


Fig. 4. Representative immunoblots of synaptophysin and β-tubulin immunoreactivities in hippocampal (primarily CA1) synaptic membranes (15 μg protein in each lane) from subjects clinically and neuropathologically diagnosed (case numbers are identified) as definite AD, p-AD (early AD), and controls. The monoclonal antibodies to β-tubulin and synaptophysin detected a single predominant band at 55 kDa and 38 kDa, respectively. Individuals with AD showed a visible reduction in the amount of synaptophysin immunoreactivity compared with controls. In contrast, the relative amounts of β-tubulin immunoreactivity in the same cases were similar.

(Table 1) were strongly correlated with levels of synaptophysin in hippocampus (Fig. 6). This correlation with MMSE scores also existed in definite AD vs p-AD cases ($r = 0.82$, $p < 0.01$, Spearman correlation = 0.82). A similar, but not statistically significant, trend was also present in the entorhinal cortex (not shown), but was not detected in the primary visual cortex or caudate nucleus (not shown).

A relationship between synaptophysin levels, MMSE scores and Braak stage was also apparent (Table 1; Fig. 6A). Definite AD cases at stage VI had MMSE scores of 13 (case 1164) or 15 (case 941), and those cases at stage V had MSSE scores of 16 (case 1177) or 22 (case 400). These cases had the lowest synaptophysin levels (10% to 40% of control levels). Definite and early AD cases at stage III had MMSE scores ranging from 24 to 30 and synaptophysin levels ranging from 55% of control to control levels.

Synaptophysin Levels Correlate Inversely with SP Densities

The results of the analysis of densities of SP in the 3 groups are summarized in Table 2. By univariate analysis

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Fig. 3. Neurofibrillary pathology in Hirano silver-stained sections of entorhinal cortex of definite AD (A, B), early AD (C, D), and control (E) cases. Numbers identify cortical layers 1 and 2. Neurofibrillary tangles and SP are identified by open arrows and solid black arrows, respectively. In definite AD (A, B) and early AD (C, D) groups, neurofibrillary tangles were always present in the entorhinal cortex, but the apparent abundance and distribution within layer 2 varied. Although less frequent, neurofibrillary tangles were also present in entorhinal cortex of controls (E). Scale bar = 100 μm.

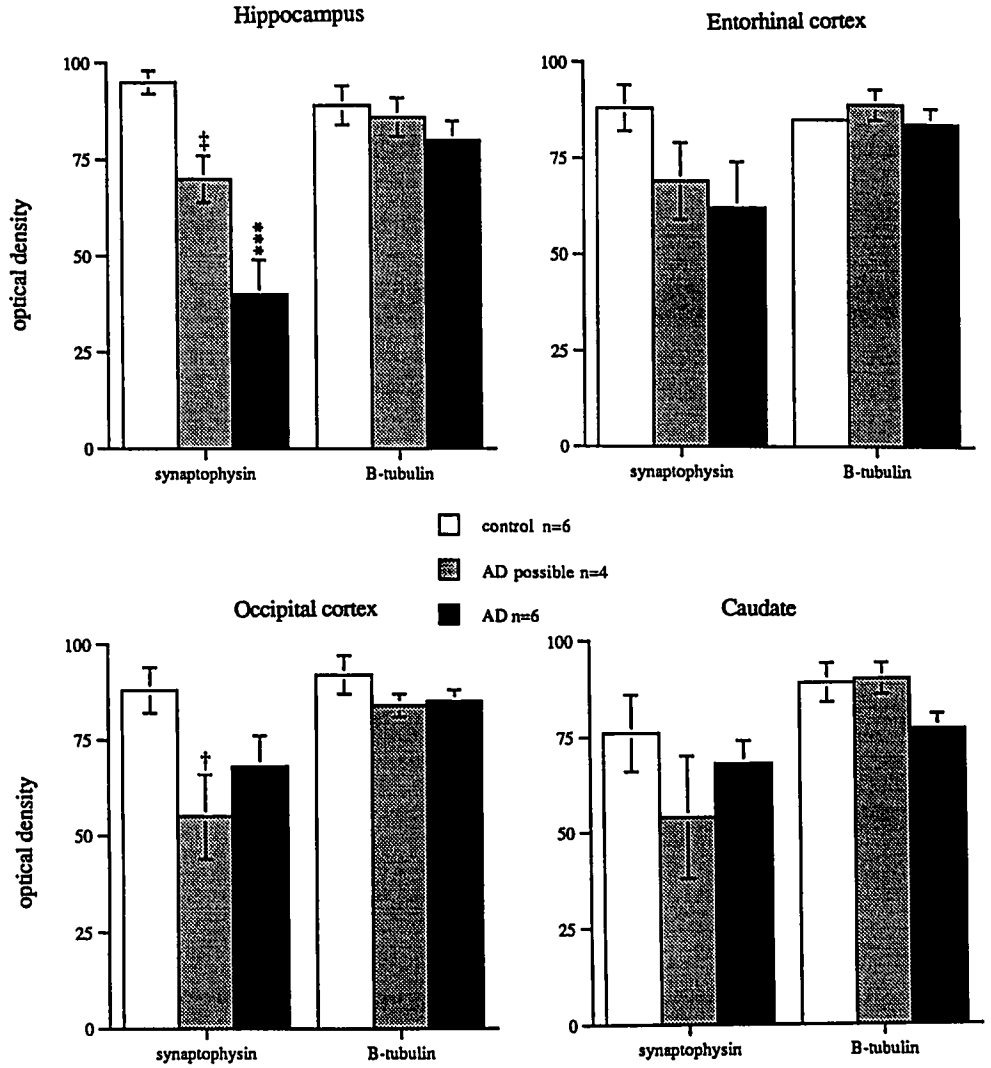

Fig. 5. Histogram summary of quantitative analyses of immunoblots of synaptophysin and β-tubulin levels in hippocampus (primarily CA1), entorhinal cortex, occipital cortex, and caudate from controls and subjects with p-AD (early AD) and definite AD. Values (in relative OD) are mean ± standard error. The level of statistical significance (determined by student *t*-test) for control vs definite AD comparisons is indicated by triple asterisk ($p < 0.0001$). The levels of statistical significance (determined by student *t*-test) for control vs p-AD comparisons are indicated by a double dagger ($p < 0.005$) and a single dagger ($p < 0.05$). Compared with age-matched controls, individuals with definite AD had synaptophysin levels that were reduced by 55% in the hippocampus ($p < 0.0001$). Synaptophysin changes in the entorhinal cortex (26%) and occipital cortex (20%) in cases of definite AD were not significant. Compared with age-matched controls, individuals with p-AD had synaptophysin levels that were reduced by 25% in the hippocampus ($p < 0.005$) and by 33% in the occipital cortex ($p < 0.05$). No significant differences in β-tubulin immunoreactivity were found in hippocampus, entorhinal cortex, occipital cortex, or caudate nucleus. The error bar for β-tubulin in the control entorhinal cortex is not visible because of low variability (SD = 1.4).

of all cases, densities of SP were inversely correlated with levels of synaptophysin in the hippocampus ($r = 0.89$, $p < 0.0001$, Spearman correlation = −0.88) (Fig. 7A). This correlation remained even after excluding control cases ($r = 0.89$, $p < 0.005$, Spearman correlation = −0.83). There was also an inverse correlation between number of SP and levels of synaptophysin in the entorhinal cortex (Figs. 3, 7B), but not in occipital cortex or caudate nucleus (not shown).

No significant correlation between synaptophysin levels and postmortem delay or age was identified in the hippocampus or entorhinal cortex (Fig. 8).

DISCUSSION

The neurobiological mechanisms that influence the progressive impairments in memory and intellectual performance occurring with AD are not understood. The cellular and molecular mechanisms of memory and learning

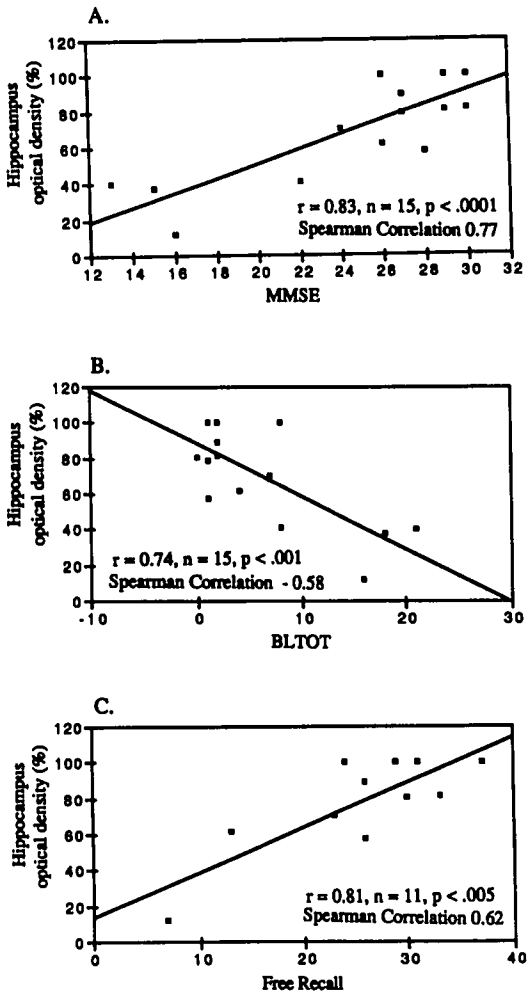

Fig. 6. Regression analysis between synaptophysin levels in the hippocampus, and MMSE (A), Blessed (B), and Free Recall scores (C) in aged controls and subjects with p-AD and definite AD. See Table 1 data set. In A, one control case with an MMSE score of 29 was not included in the regression analysis because this score was derived from the Blessed score; therefore, the number of individuals included in the regression was 15, not 16. In A, only 14 data points are shown because 2 cases of definite AD with MMSE scores of 24 had the same synpatophysin levels in the hippocampus. In B, 2 definite AD cases with Blessed scores of 7 had the same synaptophysin levels in the hippocampus.

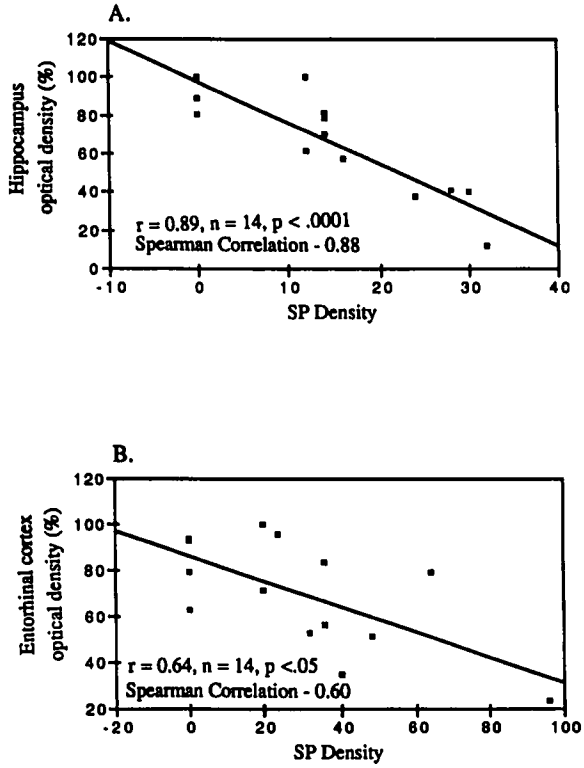

Fig. 7. Regression analysis between synaptophysin levels in the hippocampus (A) and entorhinal cortex (B), and SP (neuritic and diffuse) density in elderly controls and individuals with p-AD and definite AD.

depend on synaptic function in the hippocampus (6, 29). Regulated exocytosis of neurotransmitter-containing vesicles is obligate for normal synaptic function (30–32). Synaptophysin (p38), an integral membrane glycoprotein of small synaptic vesicles in the presynaptic terminal (33, 34), is one protein that functions in regulated exocytosis (32). β-tubulin is the principal constituent of microtubules, the key cytoskeletal components within presynaptic and postsynaptic elements of the synaptic complex (35, 36).

Here, we have identified a reduction in synaptophysin in the hippocampus of individuals with AD who had moderate to severe deficits in memory. This finding is not surprising in view of the vulnerability of the hippocampus in advanced AD (19). A more interesting finding is the loss of synaptic marker in early AD (or p-AD) cases, diagnosed as such based on Braak's staging scheme (26) or CERAD criteria (25), with no detectable cognitive impairment. The severity of the decrement in synaptophysin in hippocampus correlated strongly with the magnitude of memory impairment in individuals with AD. In contrast, no alterations in β-tubulin were observed in hippocampus in individuals with AD, confirming a previous radioimmunoassay study (27) and suggesting that the physical structure of the synaptic complex is unaltered or that compensatory repair or sprouting mechanisms within the neuropil occur concomitantly to maintain β-tubulin levels. We conclude that defects in presynaptic components for neurotransmitter release occur in the hippocampus of individuals with AD and that this defect in subjects with early AD may foreshadow the clinical appearance of memory/cognitive impairment.

Immunoreactivity for synaptophysin or other presynaptic proteins in brain samples of individuals with AD has been quantified previously by densitometric analysis of immunohistochemical sections (10, 11, 14, 37, 38),

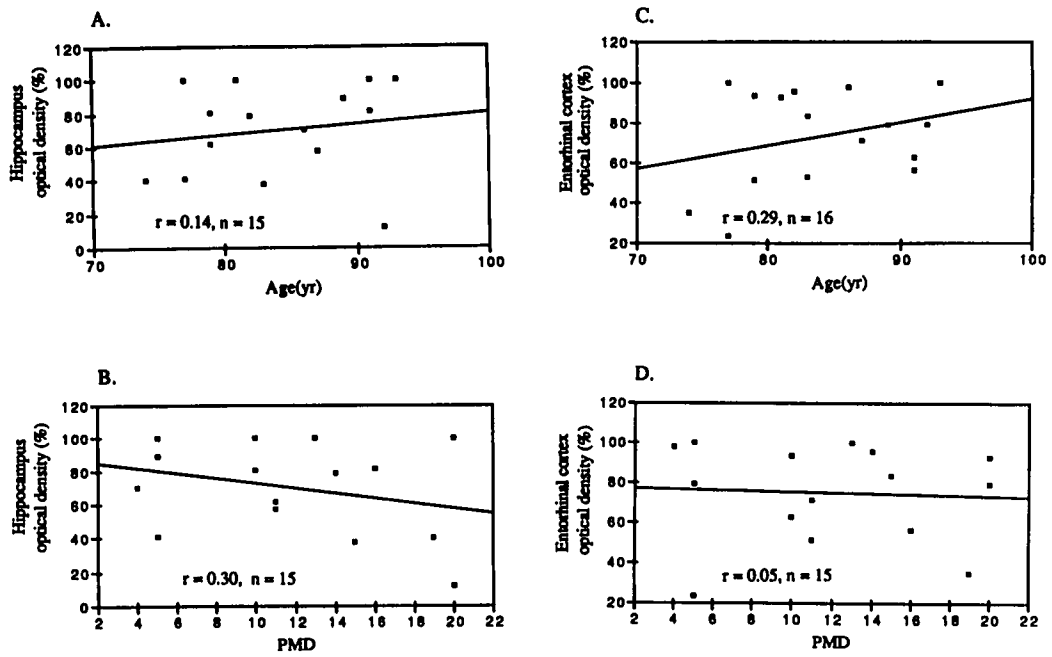

Fig. 8. Regression analysis of synaptophysin immunoreactivity in the hippocampus and entorhinal cortex vs patient age at death (A, C) and postmortem delay (PMD) (B, D).

immunodot blots (39), and ELISA (15). Despite differences in the methods used here and in previous studies, the results are remarkably comparable in light of possible variations in the severity of neuropathology among individuals, tissue sampling differences, and the use of unfixed frozen or fixed postmortem brain samples. By ELISA, reductions in synaptophysin-like immunoreactivity in hippocampus (77%) and temporal cortex (54%), but not in caudate and occipital cortex, have been found in AD (15). By immunohistochemistry, synaptophysin immunoreactivity in frontal, parietal, and temporal neocortices was reduced (45%) in cases of AD compared with controls, and, by immunoblotting, a 60% reduction in synaptophysin was found in the frontal cortex of AD cases (40). By dot immunobinding, the same group (39) reported a 40% decrease in synaptophysin immunoreactivity in the midfrontal cortex of cases of AD compared with controls. These observations indicate that there is widespread loss of synaptic markers in association neocortex and hippocampus in individuals with definite AD with moderate to severe cognitive abnormalities. Our new observations based on individuals with early AD and normal cognition suggest that loss of synaptophysin in hippocampus precedes the appearance of functional impairments detected by detailed neuropsychological testing. Thus, we conclude that presynaptic defects in the hippocampus may be an early, primary change associated with the onset of memory impairment in AD.

Several studies have evaluated levels of synaptophysin immunoreactivity in the brains of subjects with AD, but relatively few have attempted to establish direct correlations between reduced synaptic markers and cognitive performance. One study reported that decreases in synaptophysin immunoreactivity correlate better with the degree of dementia in AD than the classical neuropathological features such as neurofibrillary tangles or SP (14). Other studies indicate that cytoskeletal changes are reliable correlates of AD-related dementia (20, 41). These correlations, however, appear to be regionally and even subregionally specific (20). Previously, we have observed that neurofibrillary tangles are present in the entorhinal cortex of elderly subjects in the BLSA, occurring independently of cognitive status (24). The presence of neurofibrillary tangles in layer 2 of the entorhinal cortex of controls was also shown here. In the present study, we used the Braak staging scheme to evaluate AD-related neurofibrillary changes (26) and found that subjects with early AD (stage III) tended to have better cognitive performance and higher synapse levels than AD cases at Braak stages V and VI. Our experiments demonstrate a strong correlation between subject MMSE, Blessed, and Free Recall scores and synaptophysin levels in hippocampus of individuals with AD. This correlation remained after exclusion of control cases. This result contrasts with findings by another group (37) showing that significant correlation vanished after control cases were excluded. Individuals with early AD (Braak stage III) are more similar cognitively to age-matched normal controls than to individuals with definite AD (Braak stage V and VI). However, subjects with early AD also had synaptophysin

abnormalities in the hippocampus and, to a lesser extent, in the primary visual cortex. We conclude that the elderly control subjects with stable cognitive performance and few SP, and elderly individuals who are normal cognitively but have sufficient numbers of neocortical SP for the neuropathological diagnosis of p-AD are 2 distinct populations that differ in presynaptic functioning. The loss of synaptophysin in the visual cortex of subjects with p-AD further supports this idea. Although we cannot yet explain this difference, this abnormality may be independent of AD because synaptophysin levels in the visual cortex of definite AD cases were not significantly different from controls. These p-AD cases with normal cognitive status, abundant neocortical SP, and synaptic abnormalities may represent early AD, as suggested by the Braak stage III classification, or they may represent a variant of normal aging characterized by visual system and hippocampal abnormalities.

Synaptic alterations in the neuropil may be related to amyloid deposits (42–44) and cytoskeletal abnormalities (44). In aged individuals with minimal cognitive dysfunction, synaptic loss (determined immunocytochemically) in the molecular layer of the dentate gyrus occurs early in the development of AD and is associated with abnormal accumulation of amyloid precursor protein and cytoskeletal proteins in entorhinal cortex (44). Our results are in accordance with this concept. For example, we found a strong inverse correlation ($r = 0.89$) between synaptophysin loss and the density of neuritic and diffuse plaques in the hippocampus. It is not surprising to find an inverse correlation between SP density and synaptophysin immunoreactivity, because synaptic abnormalities in the neuropil are early events in the formation of SP (43).

The full interpretation of changes in synaptic markers in the brains of demented individuals with AD and elderly, cognitively normal subjects with possibly incipient AD relies on several yet to be clarified issues. The relationships between synaptophysin protein levels, the number of neurotransmitter vesicles, and the number of synaptic contacts are unclear. A regionally selective loss of synaptophysin in subjects with AD or p-AD could result from the degeneration of entire presynaptic terminals, loss of neurotransmitter vesicle density, redistribution of vesicles or proteins within the presynaptic terminal, or a combination of these possibilities as a result of primary loss of postsynaptic targets. If reduced immunological detectability of presynaptic vesicle proteins corresponds to physical loss of entire presynaptic terminals, then presynaptic terminal density should be reduced in AD. Although electron microscopic studies of synapse counts in AD hippocampus have not been conducted, loss of synaptic density in subjects with AD occurs in layers III and V of several neocortical regions (9, 12, 13, 45, 46), but not in layers III and V of entorhinal cortex (47). Our measurements of synaptophysin in homogenates of entorhinal cortex of definite AD cases were not significantly different from control cases, consistent with synapse density measurements in this region (47), despite definite AD cases having neurofibrillary changes in entorhinal and transentorhinal regions that ranged from Braak stages III to VI. However, the variability in the entorhinal cortical synaptophysin measurements was high, which likely contributes to the lack of significant change in view of the small group sizes. This lack of significant change in synapses in the entorhinal cortex is surprising in light of the finding that neurons are lost in entorhinal cortex in early and severe AD, with layer 2 being severely affected (48). However, our observation can be partially reconciled in view of the variability in the laminar neurofibrillary pathology (and perhaps subregional pathology) in the entorhinal cortex of definite and early AD cases (Fig. 3) and the small tissue mass sampled relative to the total volume of entorhinal cortex (Fig. 1). Measurements of synaptic markers within a brain region are likely to provide highly sensitive information about the integrity of the neuropil (rather than direct counts of neuronal cell bodies) within a small sample of the total region; thus, large differences in synapses in AD entorhinal cortex compared with age-matched controls may be obscured, possibly by subregional or topographic variations in neuropathology and by normal age-related declines in synaptic markers occurring in elderly controls. To resolve this uncertainty, synapse levels in the entorhinal cortex of young controls should be compared with elderly controls and AD cases with larger group sizes and using a tissue sampling design that encompasses a greater volume of entorhinal cortex.

Loss of presynaptic vesicle protein in hippocampus may be a secondary change resulting from degeneration of postsynaptic structures. Dendritic atrophy, as determined by Golgi studies, occurs in the brains of aged individuals and subjects with AD (49, 50). Alternatively, loss of presynaptic vesicle proteins may be secondary to impairments in the expression or function of other proteins involved in the synaptic vesicle cycle, including proteins that function in neurotransmitter vesicle docking, priming, fusion, and exocytosis/endocytosis (32). Additional studies of the brains of normal individuals and elderly subjects with mild to severe impairments in cognition are necessary to systematically evaluate proteins that function in synaptic physiology to help identify the neurobiological mechanisms for age-related memory loss in elderly humans.

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