Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease

Article abstract—The expression levels of three synaptic proteins (synaptophysin, synaptotagmin, and growth-associated protein 43 [GAP43]) in AD cases clinically classified by Clinical Dementia Rating (CDR) score were analyzed. Compared with control subjects (CDR = 0), mild (early) AD (CDR = 0.5 to 1) cases had a 25% loss of synaptophysin immunoreactivity. Levels of synaptotagmin and GAP43 were unchanged in mild AD, but cases with CDR of >1 had a progressive decrement in these synaptic proteins. Thus, synaptic injury in frontal cortex is an early event in AD.

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The cognitive alterations in patients with AD are closely associated with synaptic loss[1] and neurofibrillary pathology[2] in the limbic system and neocortex. Understanding of the time course of and relationship between neuropathologic and behavioral alteration in AD has been greatly enhanced in recent years by the development of more sensitive neuropsychologic and neuroanatomic tools that can detect subtle cognitive and structural alterations in patients with early AD. Among them, the Clinical Dementia Rating (CDR) scoring system and the Braak staging system[3] have been especially useful. Based on these criteria, some studies have proposed that diffuse amyloid deposits might contribute to neurodegeneration in early stages of the disease,[4] and others have suggested that synaptic and neurofibrillary pathology might be an early/earlier event preceding extracellular amyloid deposition.[5] This indicates that alterations in synaptic functioning might occur early in AD and that molecular biomarkers of active synapses, such as synaptotagmin (p65),[6] could be good indicators of early synaptic damage. Synaptotagmin is a 65-kd calcium sensor protein found in the synaptic vesicles that is modulated during synaptic activation. The hypothesis is that changes in synaptotagmin might be the most sensitive indicator of synaptic changes in mild (early) AD. In this context, the main objective of the present study was to analyze expression levels of three synaptic proteins, namely, synaptophysin (general synaptic marker), synaptotagmin (marker of synaptic activity),[7] and growth-associated protein 43 (GAP43; marker of synaptic sprouting) in the brains of patients with mild (early), moderate, and severe AD.

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Materials and methods. This study was performed with autopsy material from 42 patients (table) studied neurologically and psychometrically during life at the AD Research Center/University of California, San Diego, and at the Washington University School of Medicine, St. Louis, MO. The CDR score was assigned during life following previously published criteria; this scoring has demonstrated interrater reliability.[8] Furthermore, to assess the cognitive status just before death, a retrospective CDR score was assigned based on family interviews. Therefore, the CDR score reflects cognitive status right before death. The CDR scale ranged from 0 to 3 (0, 0.5, 1, 2, and 3). Paraffin sections from 4% buffered formalin-fixed neocortical, limbic system, and subcortical material were used for routine neuropathologic examination and Braak staging.[3] Based on the clinical and pathologic findings, cases were subdivided into four groups: A) nondemented aged control subjects (CDR = 0, Braak stage = 0); B) mild (early) AD (CDR = 0.5 to1, Braak stage = I); C) moderate AD (CDR = 2, Braak stage = II to IV); and D) severe AD (CDR = 3, Braak stage = V to VI). Results of previous studies support the view that mild AD might represent an early (or incipient) stage of the disease.[3]

For analysis of expression of synaptic proteins, frozen tissue from the frontal cortex was homogenized and processed into particulate and cytosolic fractions. Particulate fractions were utilized for quantitative dot-blot assays with mouse monoclonal antibodies against synaptophysin (0.1 μg/ml; Chemical, Temecula, CA), synaptotagmin (43 μ/ml; StressGen Biotechnologies Corp., Victoria, BC, Canada), and GAP43 (1 μg/ml; Sigma Chemical Co., St. Louis, MO). In brief, 2 μg of protein per case was applied to nitrocellulose paper placed in a blotting manifold. Blots were blocked in 0.1% Tween 20 in phosphate-buffered saline (pH 7.4) for 2 hours at 4 °C and incubated overnight with the primary antibody, followed by rabbit antimouse immunoglobulin G and ^{125}I-protein A (0.1 μCi/mL). Blots were scanned with the PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and determinations of average immunoreactivity were performed with the ImageQuant software and expressed as arbitrary units per 1 μg of protein. All experiments were repeated at least once to ensure the reproducibility of the results. Similar to synaptophysin, preliminary studies showed that levels of GAP43 and synaptotagmin immunoreactivity by confocal microscopy correlated with dot-blot levels.

All samples were blind-coded and run in triplicate. Once analysis was finalized, the code was broken and cases were

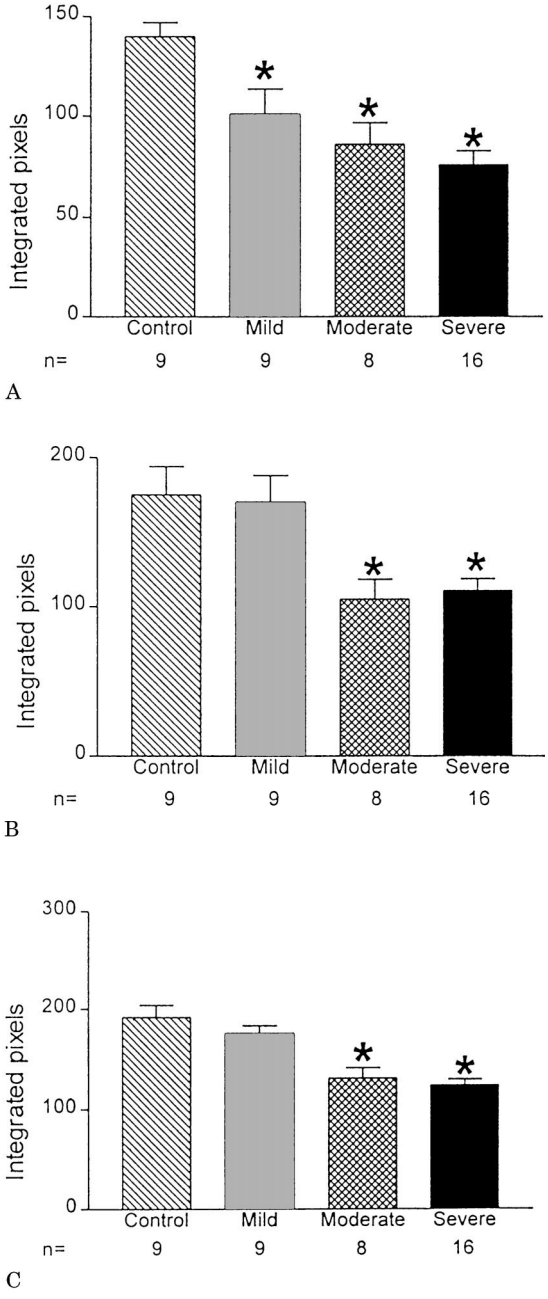

*Figure. Dot-blot analysis of the control and AD frontal cortex. (A) Compared with control cases, mild AD cases showed a 25% loss in synaptophysin immunoreactivity. Levels of synaptophysin were further decreased in both moderate and severe cases. (B) Whereas there was no difference in levels of synaptotagmin between control and mild AD cases, there was a significant reduction in levels of immunoreactivity in moderate and severe AD. (C) Levels of growth-associated protein 43 (GAP43) were unchanged in mild AD cases compared with control subjects. In contrast, mild and moderate cases showed a significant and progressive reduction in levels of GAP43 immunoreactivity. *Indicates* $p < 0.05$ *(one-way analysis of variance with post hoc Dunnet's test).*

assigned to specific groups. Statistical comparisons among the changes in relative levels of synaptic proteins in control and AD cases at the various stages were performed using analysis of variance (one-factor, with post hoc Dunnet's or Tukey–Kramer test; SuperANOVA program; Abacus Concepts, Berkeley, CA). Results were expressed as means ± SEM.

Table *Summary of clinicopathologic characteristics*

Group	n	Age, y	PMT, mean ± SD	CDR	Braak
Control	9	80 ± 3.6	6 ± 1	0	0
Mild	9	82 ± 3.5	7 ± 1	0.5–1	1
Moderate	8	83 ± 2.0	9 ± 1	2	II–IV
Severe	16	79 ± 1.7	7 ± 1	3	V–VI

CDR = clinical dementia rating; PMT = postmortem time.

Results. Because previous studies have shown that synaptic loss in the frontal cortex correlates with cognitive performance[1] and that this area is relatively devoid of plaques and tangles in early AD, study of expression of synaptic proteins was focused on this brain region. Synaptophysin is considered to be a reliable general synaptic marker, GAP43 is a marker of sprouting, and synaptotagmin has been proposed as a potential marker of activated synapses.[7] Dot-blot analysis showed that in the frontal cortex of mild (early) AD cases (CDR = 0.5 to 1), there was a 25% loss of synaptophysin immunoreactivity compared with control samples (CDR = 0) (figure, A), and levels of synaptophysin were further decreased in moderate and severe cases (see the figure, A). Whereas levels of synaptotagmin (see the figure, B) and GAP43 (see the figure, C) were unchanged in mild AD cases, in more advanced cases (CDR > 1), there was a significant and progressive decrement in these synaptic proteins (see the figure, B and C) comparable with reduction in levels of synaptophysin. These results support the contention that synaptic injury is an early event in AD. Additional analysis of patterns of altered expression of synaptic proteins in mild AD was performed by estimating the relative ratio of synaptotagmin to synaptophysin (p65/syn). This study showed that in control cases, the p65/syn ratio was 1.3, whereas in mild AD cases, the ratio was 2.0, suggesting that a relative increase of synaptotagmin per synapse at early disease stages might represent a compensatory mechanism. In contrast, at later stages of AD, the p65/syn ratio decreased to 1.2, indicating that the loss of synaptotagmin parallels that of synaptophysin.

Discussion. This study revealed that synaptic injury in the frontal cortex is an early event in patients with AD. Previous studies have shown early loss of synaptophysin immunoreactivity in the hippocampus and to a lesser extent in the neocortex.[5] We found a more extensive loss of synaptophysin in early AD than previously reported. This might be attributed, at least in part, to the greater number of cases analyzed and that they were clinically characterized by utilizing the CDR score. The CDR scale was developed as a staging system for AD and has been especially sensitive at identifying cases with early AD.

Another finding in this study was that synaptophysin loss preceded the reduction in synaptotagmin

and GAP43 immunoreactivity. This was surprising because synaptotagmin has been proposed as a marker of synaptic activity,[7] suggesting that this calcium-sensitive synaptic protein should be affected earlier. This might indicate that despite the fact that synapses are being lost very early (as indicated by synaptophysin loss), some compensatory mechanisms are still in place, which allow the relative upregulation of other molecules such as synaptotagmin and GAP43. Such a possibility is further supported by our data showing that the p65/syn ratio is nearly double in early AD cases and decreases in later stages. This is consistent with previous studies where other regulated synaptic proteins such as α-synuclein have been shown to be initially upregulated, followed by a decline.[9]

Synaptotagmin, a Ca^{2+} binding protein that is essential for Ca^{2+}-triggered release, plays a critical role in vesicle fusion to the synaptic membrane. In the human brain, synaptotagmin is abundant in the synapses and can be found free in the CSF, suggesting that it can be used as a surrogate marker of synapses in vivo.[6] In this regard, previous studies have shown that synaptotagmin levels are reduced in the CSF, neocortex, and hippocampus in AD[6]; these latter findings are consistent with our study. The mechanisms of synapse loss in AD are presently under close scrutiny. Although several possibilities are being considered, an especially attractive hypothesis is that early accumulation of intracellular β-amyloid might trigger neuronal injury and synaptic damage. This is supported by human brain studies showing that levels of soluble β-amyloid 1–42 correlate with synaptophysin.[10] Thus, it is possible that in early AD, intracellular accumulation of β-amyloid might lead to synaptic damage in the absence of plaque formation, which might explain the apparent discrepancy between synapse loss, plaque formation, and cognitive impairment. Another factor that needs to be considered in the pathogenesis of synaptic damage and cognitive deficits is the presence of neurofibrillary tangles.

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