Synaptic Pathology in Prefrontal Cortex is Present Only with Severe Dementia in Alzheimer Disease

STEPHEN L. MINGER, PHD, WILLIAM G. HONER, MD, MARGARET M. ESIRI, FRCPATH, BRENDAN MCDONALD, MRCPATH, JANET KEENE, DPHIL, JAMES A. R. NICOLL, MRCPATH, JANET CARTER, TONY HOPE, FRCPsych, AND PAUL T. FRANCIS, PHD

Abstract. Synaptic pathology is proposed to be integral to the clinical expression of Alzheimer disease (AD). Most studies have assessed only the vesicle protein synaptophysin as a measure of synaptic integrity. The interrelationships of synaptophysin, other presynaptic proteins, the cholinergic system, and severity of dementia in AD remain unclear. We studied the presynaptic proteins synaptophysin, syntaxin and SNAP-25, along with choline acetyltransferase (ChAT) activity in prefrontal cortex (BA 46) samples from 18 subjects with AD and 16 controls. Mean values of presynaptic protein immunoreactivities were significantly reduced, by 21%–28%, and ChAT activity was reduced by 41% in the AD groups. Synaptic protein immunoreactivity and ChAT activity were correlated with Mini-Mental State Examination scores obtained 1 yr prior to death. When AD cases were subgrouped into mild/moderate and severe illness at time of death, all differences in presynaptic proteins and ChAT activity were significant between controls and severe cases. However, no significant differences were detected in BA 46 between controls and mild/moderate cases. Considerable synaptic reserve or plasticity remains in BA 46 until the late stages of AD. Synaptophysin and ChAT appear to be more vulnerable in severe AD than are syntaxin or SNAP-25.

Key Words: Alzheimer disease; Choline acetyltransferase; SNAP-25; Synapses; Synaptophysin; Syntaxin.

INTRODUCTION

Synaptic terminals are important sites of pathological change in Alzheimer disease (AD). Studies of synapses in AD using electron microscopy demonstrated synaptic loss, as well as evidence for compensatory changes in the remaining terminals (1–3). Analysis of proteins enriched in presynaptic terminals is a complementary approach. Synaptophysin is an intrinsic protein of the synaptic vesicle membrane and is found in all types of synapses (4–6). Synaptophysin is proposed to function as a regulator of the mechanism of synaptic vesicle priming, docking, fusion, and exocytosis (7). SNAP-25 is located primarily in the presynaptic membrane, where it is a member of the SNARE complex of proteins vital to synaptic neurotransmission (8–10). SNAP-25 appears to be present in most but not all synapses (8, 11, 12). Syntaxin is another member of the SNARE complex, found predominantly at presynaptic membranes (13). One ultrastructural study in rats indicated syntaxin was enriched in asymmetric compared with symmetric synapses (14). Simultaneous investigation of multiple presynaptic proteins in AD indicated reductions in synaptophysin, SNAP-25, and syntaxin in most but not all studies (15–18). Each protein may not be equally affected. In hippocampus, reduced synaptophysin but unchanged SNAP-25 was reported (19). In a study of temporal cortex, synaptophysin was reduced by 30%, SNAP-25 by only 10%, and syntaxin was unchanged (17).

Understanding of the mechanism of synaptic pathology in AD benefits from concurrent study of cytoskeletal markers of disease such as tau protein, or neurochemical correlates such as choline acetyltransferase (ChAT) (20). In AD, reduced synaptophysin mRNA expression was reported in neurons with neurofibrillary tangles (21). Loss of synaptophysin protein was apparent in neocortical regions that were unaffected by neurofibrillary pathology (22). Loss of synaptophysin was detected in the outer molecular layer of the dentate gyrus in cases with the earliest phases of AD-type pathology, while loss in frontal cortex was apparent only in more severe cases (23). An apparently biphasic response in association cortex synaptic proteins in AD was observed in a recent study, with initial increases in synaptophysin, SNAP-25, and syntaxin followed by decreases in late neuropathological stages of illness (24). These observations are consistent with reports of control subjects with high levels of plaques and tangles having either no change or slight elevation in synaptophysin compared with controls that were free of lesions (25, 26).

Studies of synaptophysin immunostaining led to the proposal that synaptic loss is the structural basis of cognitive decline in AD (27). Several studies of AD and other dementias indicated significant correlations between antemortem assessments of cognition and postmortem assays of presynaptic proteins or the coding mRNAs (16, 27–31).
The Oxford study of dementia was designed to provide prospective, longitudinal cognitive assessments of a cohort of elderly demented individuals for correlation with postmortem studies. Brain tissue collected from this cohort and from a control group allows testing of several hypotheses related to synapses and AD. First, we expected the vesicle marker synaptophysin and ChAT activity to be more affected than the presynaptic membrane proteins SNAP-25 and syntaxin. This hypothesis was suggested by the findings of simultaneous study of multiple presynaptic proteins (17, 19). Second, both presynaptic markers and ChAT activity were expected to show correlations with prospective antemortem cognitive assessments. Finally, we hypothesized presynaptic membrane and vesicle proteins would be relatively preserved in patients with mild to moderate severity of dementia, and reduced in patients with severe AD. This hypothesis is based on contradictory results in the literature (24, 27), and warrants examination.

**MATERIALS AND METHODS**

**Subjects and Clinical Evaluation**

The Oxford study of dementia is described in detail elsewhere (32–34). Briefly, at the time of study entry, prospectively ascertained subjects met clinical criteria for dementia and lived at home with a caregiver who could give an accurate account of the subject’s day-to-day behavior. Initial assessments and diagnoses were made using CAMDEX (35), DSM-III-R criteria (36), and NINCDS-ADRDA criteria (37). Inclusion criteria were a diagnosis of AD and/or vascular dementia. Exclusion criteria were a caregiver not able to give an accurate account of the day-to-day behavior of the participant and/or other possible contributory causes for the dementia such as Pick disease.
Parkinson disease, premorbidly heavy alcohol intake, or a serious head injury. Changes in cognitive status were assessed at 4 monthly intervals using the Mini-Mental State Examination (MMSE) (38). At the same time, behavioral and psychiatric assessments were carried out using the Present Behavioral Examination (PBE) (39).

Control subjects were obtained from an autopsy series of cases without any history of neurological or psychiatric illness. The controls were not prospectively assessed cognitively, but they were all cases for which there was circumstantial evidence of cognitive competence such as living independently in the community and dying suddenly of a heart attack. All control cases were examined neuropathologically and none met CERAD criteria for possible, probable, or definite AD.

Preparation of Tissue Samples and Neuropathology

Frozen brain tissue was obtained from 18 cases with the neuropathological diagnosis of AD and 16 controls. For all subjects, consent for postmortem examination and use of tissue for research was obtained from a close relative. The study had full local ethics committee approval.

Bodies were refrigerated shortly after death and remained at 4°C until just before the autopsy was performed. One hemisphere was reserved for histological preparation and neuropathological examination. Paraffin-embedded sections were cut 10 μm in thickness and prepared with methenamine silver and modified Palmgren stains. All cases were analyzed for histopathological hallmarks of AD according to CERAD criteria (40). Sections from BA 46/9 were independently scored by 2 neuropathologists using a semiquantitative 0–3 rating system for neuritic plaques, and for neurofibrillary tangles to provide a regional estimation of the density of these features for each case.

Blocks of frontal cortex (approximating BA 46) were taken from the other hemisphere, then slow frozen by placing at –70°C. Samples remained at this temperature until several hours prior to dissection when they were placed at –20°C. Samples were dissected while still frozen on a glass Petri dish in contact with ice water at 0–4°C. Cortical gray matter (0.5–0.8 g) was dissected free of meninges and white matter then homogenized in approximately 5 ml 0.1 M phosphate buffer (pH 7.4), on ice using a Teflon/glass power-driven homogenizer (8 strokes at low speed). Aliquots were immediately frozen on powdered dry ice and stored at –70°C until processed for use in enzyme-linked immunoadsorbent assays (ELISA) and for ChAT activity determinations. Protein concentration was assessed in triplicate using Coomassie Plus protein assay reagent (Pierce and Warner, Chester, UK) and measuring absorbency at 595 nm. APOE genotype was determined from fresh frozen...
ELISA for Synaptic Proteins

Monoclonal antibodies detecting synaptophysin (EP10), SNAP-25 (SP12), and syntaxin (SP6) were used to quantify synaptic antigen immunoreactivity. These antibodies were characterized in studies using fusion proteins and peptide mapping (42, 43). All antibodies used for ELISA determinations were hybridoma supernatants and ELISA procedures were performed essentially as previously described (43). Briefly, brain homogenates were diluted to 60 µg protein/ml with distilled water. Duplicate samples were dried onto individual wells of a 96-well plate following 8 steps of 1:1 serial dilution (128-fold). Nonspecific binding was inhibited by incubation in 5% nonfat milk in Tris buffered saline (TBS) for 1 h at room temperature. Samples were washed an additional 5 times as above and 50 µl of 2,2’-azino-di-3-ethylbenzthiazoline sulphonate (Bio-Rad, Hercules, CA) were added to each well and incubated for 30 min. The optical density (OD) was then determined at 405 nm. Supernatant from the parental, nonsecreting myeloma cell line was used to determine background reactivity. The OD values from the blanks were subtracted from the OD values from each sample. The mean concentration of protein from each sample required to obtain an OD value of 0.500 was determined, and these normalized values were used for comparisons between individual groups and different antigens.

Choline Acetyltransferase (ChAT) Activity

ChAT enzymatic assays were performed using the technique of Fonnum (44), with minor modification (45). Samples were assayed in triplicate with average blank counts subtracted from sample counts prior to correcting for protein concentration. Data were expressed as nanomoles of acetylcholine (ACh) formed per hour per mg protein.

Statistical Analysis

Linear regression was used to analyze relationships between synaptic protein immunoreactivity or ChAT activity and age, postmortem interval, and storage duration. Mean values of the synaptic markers and ChAT were compared between groups using t-tests. Intercorrelation of neurochemical variables was tested either by Pearson product moment or Spearman rank correlation, as appropriate. Synaptic proteins were correlated with semiquantitative assessments of neuritic plaques and tangles using Spearman rank correlation with Z value correction for ties. Spearman rank correlation was also used for initial studies of the relationships between severity of dementia (MMSE nearest to death, and MMSE 1 yr prior to death) and neurochemical measures. Subjects were also categorized into 3 groups for analyses of illness severity (Table 1): controls; mild/moderate AD (MMSE nearest to death score 5–26, n = 6); and severe AD (MMSE prior to death 0–2, n = 12). Values were compared between these 3 groups using 1-way ANOVA, followed by the LSD test. In all cases the null hypothesis was rejected at p < 0.05.

RESULTS

Demographic and illness related variables for each case appear in Table 1. Mean age differed between the groups (t = 3.06, df = 32, p = 0.005); postmortem time and storage interval did not differ (Table 2). Neuropathologic examination revealed incidental small vessel subcortical disease in 2 of the AD cases. In the controls, synaptic protein immunoreactivity and ChAT activity did not exhibit significant correlations with age, postmortem interval, or storage time. Covariates were therefore not used in comparisons between controls and AD cases. Figure 1 presents ChAT activity and presynaptic protein immunoreactivity relative to postmortem interval. Cases of AD were more likely to have died of pneumonia than controls. To determine if pneumonia as a cause of death (and possible antemortem hypoxia) might influence the immunoreactivity or enzyme activity of the target proteins, this was investigated in the AD patients. No statistically significant differences in synaptic protein immunoreactivity or ChAT activity were observed between AD with pneumonia as a cause of death and AD with other cause of death (t = 0.22–1.11, df = 15, p = 0.28–0.83). Of the 18 AD cases, 5 had a history of treatment at some time with antipsychotic drugs and 5 had a history of treatment at some time with sedative-hypnotic drugs. The synaptic protein immunoreactivity and ChAT activity did not differ between treated and nontreated cases for either class of drug.

**TABLE 2**

| Demographic Values for Groups of Subjects (Mean ± Standard Deviation) |
|--------------------------|--------------------------|--------------------------|--------------------------|
| Controls                 | All AD                   | Mild/Mod AD              | Severe AD                |
| Age (yr)                 | 74.2 (11.2)              | 83.5 (6.1)               | 81.2 (5.6)               | 84.7 (6.3)               |
| Gender                   | 9m, 7f                   | 6m, 12f                  | 4m, 2f                   | 2m, 10f                  |
| Onset age (yr)           | 74.1 (6.2)               | 73.1 (5.6)               | 74.7 (6.7)               |
| Duration (yr)            | 8.5 (3.4)                | 8.1 (2.0)                | 8.7 (4.1)                |
| Postmortem time (h)      | 44.5 (24.8)              | 39.4 (26.6)              | 40.9 (24.5)              | 38.7 (28.6)              |
| Storage interval (months)| 72.2 (13.7)              | 67.7 (12.6)              | 70.3 (8.2)               | 66.3 (14.5)              |

Mild/moderate and severe refer to severity of illness at time of death (see text).
Synaptic Probes in Alzheimer Disease


Fig. 2. Mean values of presynaptic proteins and ChAT in BA 46, all normalized to control (left panel) and neuropathological ratings (right panel). Mild/moderate and severe represent severity of AD at time of death. P values refer to post-hoc LSD tests following an omnibus ANOVA for each measure. Differences between controls and severe dementia were present for all measures. However, in mild/moderate cases presynaptic proteins were relatively preserved. Abbreviations: Synphy, synaptophysin; Synt, syntaxin; NFT, neurofibrillary tangles; NP, neuritic plaques. Error bars represent standard error of the mean.

Synaptic Protein Immunoreactivity, ChAT Activity, and Pathological Markers

Cases with AD had significantly less immunoreactive syntaxin (24%, \( t = 2.54, df = 32, p = 0.02 \)), SNAP-25 (28%, \( t = 3.83, df = 32, p = 0.0006 \)), and synaptophysin (21%, \( t = 2.97, df = 32, p = 0.006 \)) than controls. As expected in AD, ChAT activity was significantly reduced (41%, \( t = 3.35, df = 32, p = 0.002 \)). A repeated measures analysis was used to determine if changes in the synaptic markers were of different magnitude. No statistically significant interaction between diagnosis and synaptic protein immunoreactivity was observed (\( F = 0.22, df = 2, 64, p = 0.81 \)), suggesting that in the overall sample all presynaptic proteins were similarly affected by AD.

To determine whether apolipoprotein E genotype influenced either synaptic protein immunoreactivity or ChAT activity, the AD cases were separated into those with one or more ε4 alleles (n = 14, 1 homozygote) and those lacking an ε4 allele (n = 4). No significant differences in synaptic immunoreactivity or cortical ChAT activity were found in these 2 groups of AD cases. The small number of cases without an ε4 allele largely precludes meaningful comparisons, however description of the frequency of the ε4 allele in the present series provides a context for comparison with other studies.

In controls, synaptophysin immunoreactivity correlated with syntaxin and with SNAP-25 (Table 3), however syntaxin and SNAP-25 immunoreactivities themselves were not significantly correlated. There were no significant correlations between any of the presynaptic markers and ChAT activity. In the overall AD group, each of the 3 presynaptic protein markers correlated with the other two. In contrast to the controls, in AD all presynaptic protein immunoreactivities were correlated with ChAT activity. In AD, presynaptic protein immunoreactivities did not correlate with the pathological markers of neuritic plaques or tangles.

Synaptic Protein Immunoreactivity and Cognitive Severity of Illness

The results of the cognitive assessment closest to death did not show significant correlations with any of the measures. However, the MMSE scores obtained in the assessment 1 yr prior to death were significantly correlated with each of the synaptic protein immunoreactivities, with ChAT activity, and with neurofibrillary tangle score.

Mean values for controls and for cases with mild/moderate and severe AD at the assessment nearest to death are shown in Figure 2. ANOVAs indicated significant differences between the groups for all measures (F range 5.23–37.43, \( df = 2, 31, p < 0.0001–0.05 \)). Two patterns of findings emerged. For neurofibrillary tangles and neuritic plaques, the expected clear differences between controls and subjects with mild/moderate AD were observed. This was not the case for ChAT, synaptophysin, syntaxin or SNAP-25, where mild/moderate cases showed no significant differences in mean values compared with controls. Although there were significant numbers of neuritic plaques and neurofibrillary tangles present in BA 46 in the mild/moderate AD cases, presynaptic and cholinergic markers did not differ from the elderly controls. All 4 markers showed differences between controls and the severe cases. However, comparisons between the mild/
Moderate and severe cases demonstrated further differences. ChAT and synaptophysin mean values differed significantly between mild/moderate and severe cases, the mean ChAT value in particular was reduced by over 50%. Syntaxin and SNAP-25 mean values were not significantly different between mild/moderate and severe cases. These observations may indicate differential vulnerability of ChAT activity and synaptophysin in the more severe stages of AD compared with syntaxin and SNAP-25.

**DISCUSSION**

The present study provides an analysis of the relationships between frontal synaptic pathology, ChAT, and severity of dementia in AD. Comparable effects of illness on each of the 3 presynaptic proteins were observed, however subgroup analysis indicated more effect of AD on synaptophysin in later stages of illness. Impaired cognitive function was correlated with reduced presynaptic protein immunoreactivity, and with reduced ChAT activity. However, when AD cases were separated into 2 groups according to severity of illness, these findings were observed to be largely restricted to the subgroup with the most severe cognitive impairment. In the prefrontal cortex of less severely ill subjects, considerable preservation of presynaptic proteins and ChAT was observed, despite the presence of significant numbers of neuritic plaques and neurofibrillary tangles. In the severely impaired subjects, presynaptic proteins were reduced, although not to the extent observed for ChAT activity.

Increasing evidence suggests that prefrontal synapses in AD are relatively intact until the later stages of the illness (24, 25). Trophic effects may partially compensate for the initial phases of degeneration. As well as pathology within synapses, considerable loss of terminals is likely due to loss of projections from distant tangle-containing neurons (21). This may be an explanation for the poor correlation between local neurofibrillary tangles and synaptic protein immunoreactivity within the same region.

Loss of activity of the cholinergic marker ChAT was most closely related to loss of immunoreactivity of the synaptic vesicle marker synaptophysin. In animal studies, SNAP-25 and syntaxin appeared to exhibit some heterogeneity in distribution between synapses (8, 12, 14), while synaptophysin was ubiquitous (4). Differences in patterns of change in ChAT and synaptophysin compared with SNAP-25 and syntaxin in AD may indicate compensatory mechanisms involving synaptic vesicles are different from those involving presynaptic membranes. Brain-derived neurotrophic factor (BDNF)-related effects might be of interest in this context. In a BDNF knockout mouse model, the synaptic vesicle proteins synaptophysin and VAMP were selectively impaired while SNAP-25 and syntaxin were relatively unaffected (46).

Cognition assessed 1 yr prior to death correlated with the presynaptic protein measures, ChAT, and neurofibrillary tangle severity. These correlations were not significant if the scores closest to death were used, likely because of the compressed range of scores in the assessment nearest to death. Severity of cognitive impairment at this assessment may have been influenced by multiple systemic as well as neurological mechanisms.

There are a number of caveats to the present study. The sample size was limited, particularly for less severe cases. The choice of MMSE scores nearest to death of 5–26 for mild/moderate and 0–2 for severe was necessary to allow at least 1/3 of subjects to be in the mild/moderate group. Interestingly, all the members of the group we defined as mild/moderate scored >10 on the MMSE 1 yr prior to death, while none of the severe group scored in this range. This suggests the grouping method appeared to be meaningful, at least for the present sample. We also carefully reviewed the single subject with an MMSE >
24, this individual had been a highly functional professional and there was no doubt about the clinical diagnosis of dementia.

The presynaptic protein assay was an ELISA, which has good reliability and wide linear range. Another recent report, which showed preservation of presynaptic proteins until the later stages of illness, also used an ELISA approach (24). The ELISA strategy cannot detect possible changes in synaptic proteins in specific cortical layers or on particularly vulnerable neuronal subpopulations, which requires an immunocytochemical assay as used in studies demonstrating reduced presynaptic proteins even in less severe cases (27). Finally, while the present study had the advantage of longitudinal assessments of cognition and behavior, these remain relatively crude clinical measures.

In summary, the present results indicate that synapses in the prefrontal cortex appear to be globally relatively resistant to the pathological processes of AD until advanced stages of the disease. Even in the presence of neurofibrillary tangles, the majority of the neuropil appears to retain synaptic terminal proteins. Understanding of the mechanism of relative resistance might help prevent synapse loss in more vulnerable brain regions such as the entorhinal cortex and hippocampus.

ACKNOWLEDGMENTS

SLM and JC were supported by a project grant from the Wellcome Trust to PTF, MME, and TH. WGH was supported by a Vancouver Hospital Scientist Award and the Canadian Institutes of Health Research (MT 14037). The collection of cognitive and behavioral data was supported by a grant from the Medical Research Council, UK. We thank Dr. Peter Davies for helpful suggestions and discussion of our results. We also thank Catherine White-Horne for assistance with harvesting of brain tissue, Jane Xie for ELISA determinations, and Janice Stewart for assistance with the APOE genotyping. We are also indebted to Kathy Gedling and Sandra Cooper for helping to collect the clinical data, and to the patients and caregivers who took part in the study.

REFERENCES

44. Fonnum F. A rapid radiochemical method for the determination of choline acetyltransferase activity. J Neurochem 1975;24:407–9
45. Minger SL, Davies P. Persistent innervation of the rat neocortex by basal forebrain cholinergic neurons despite the massive reduction of cortical target neurons. II. Neurochemical analysis. Exp Neurol 1992;117:139–50

Received October 17, 2000
Revision received April 19, 2001
Accepted June 18, 2001