Decreased Activity of Hippocampal Neurons in Alzheimer's Disease Is Not Related to the Presence of Neurofibrillary Tangles

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Abstract. Numerous studies have established the key role of the Golgi apparatus (GA) in post-translational processing, transport and targeting of proteins destined for secretion, lysosomes and plasma membranes. Moreover, several studies performed in our laboratories have shown that the size of the immunocytochemically detected neuronal GA is a reliable index of neuronal activity in aging, Alzheimer's disease (AD) and amyotrophic lateral sclerosis. It has been suggested that in AD there is decreased neuronal activity, e.g. in terms of glucose metabolism and protein synthetic capability. To further explore the hypothesis of decreased neuronal activity in AD, in this study the size of the GA was measured in pyramidal neurons of the CA1 area of the hippocampus of non-demented controls and AD patients. The size of the GA was measured separately in neurons with and without neurofibrillary tangles (NFT). Moreover, in order to establish a correlation between the density of NFT and the size of the GA, the density of extraneuronal NFT was determined around each neuron and related to the size of its GA. The results, quantified by image analysis, indicate that there is a significant reduction in GA size in the neurons of the CA1 area of the hippocampus of AD patients. However, there was no significant relationship between the size of the GA and the presence or absence of intracellular NFT. In addition, there was no correlation between the density of extracellular NFT and GA size of adjacent neurons. These findings are consistent with the conclusion that in AD there is evidence of decreased protein processing and secretion in the affected neurons of the CA1 area of the hippocampus. However, we failed to detect a relationship between intracellular or extracellular NFT and neuronal protein synthetic ability. These results justify the hypothesis that in AD a primary lesion is hypoactivity of neurons that is not directly linked with the development of intracellular or extracellular NFT.

Key Words: Alzheimer's disease; Golgi apparatus; Hippocampus; Neurofibrillary tangle.

INTRODUCTION

One of the most prominent neuropathological hallmarks of Alzheimer's disease (AD) is the presence of neurofibrillary tangles (NFT). Microscopically, these cytoplasmic structures are composed of paired helical filaments (PHF), of which the tau protein constitutes the major component. It has been shown that tau, which plays a crucial role in the stability of the neuronal cytoskeleton and axonal transport, is abnormally phosphorylated in AD (1–3). There is a clear relationship between the severity of dementia in AD patients and the number of NFT (4). However, NFT are not specific for AD, since they can also be found in a variety of other neurodegenerative diseases, such as in Parkinson's dementia of Guam (5) and dementia pugilistica (6). In AD patients NFT are preferentially formed in a rather selective population of neurons, e.g. the pyramidal neurons in the CA1 area of the hippocampus, layers II and IV of the entorhinal cor-

tex, layers III and V of the associative cortex, the locus ceruleus, nucleus raphe dorsalis and septal nucleus.

It has been proposed that decreased neuronal activity and the development of AD changes are associated (7). Protein metabolism is indeed affected in AD. The AD brain contains a lower total amount of protein than that of non-demented controls (8). Furthermore, a clear reduction in total messenger RNA (9), nucleolar volume, cytoplasmic RNA content (10–12) and glucose metabolism (13) has been observed in the cortex of AD patients. In the present study we determined whether changes in secretory protein metabolism as judged by the size of the Golgi apparatus (GA) and cell profile area are correlated with the presence or absence of NFT in the same cell. The GA processes newly synthesized proteins and the size of this organelle can be used as a sensitive index of neuronal protein synthetic ability (14–18).

Since it is presumed that NFT impair neuronal function (19, 20), we studied whether there is a relationship between the reduction of protein synthetic ability and the appearance of intra- and extracellular NFT as stained by Bodian. It has been shown that compared with other histochemical methods for NFT staining, Bodian's method visualizes high numbers of NFT in AD brains (21). In earlier studies we showed in AD patients that the pre-tangle stages of cytoskeletal alterations that are not stained by Bodian but stained prominently by Alz-50 which reacts with a variety of proteins, including phosphorylated tau (22) and other antibodies against cytoskeletal alterations (23), do not affect protein synthetic ability of the area (17). The present study aimed to es-

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TABLE 1
Patient Data

Autopsy no.	Sex	Age (years)	PMD (h)	BW (g)	NFT mm ²	GDS*	Cause of death/clinical diagnosis
Controls							
1) 88149	m	58	24	1,800	0.45	—	Brain infarction, myocardial infarction
2) 90183	f	65	4.2	1,270	0.67	—	Lung carcinoma, massive hemorrhage
3) 90202	m	72	4.2	1,330	7.60	—	Myocardial infarction
4) 9190	f	80	36	1,205	0.87	—	Cardiogenic shock
5) 91126	f	82	48	1,100	7.89	—	Myocardial infarction
6) 90203	m	85	4.5	1,050	1.31	—	Myocardial infarction, diabetes mellitus, lung emphysema
Mean $\pm$ SEM		74 $\pm$ 4.4	20.0 $\pm$ 7.7	1,292.5 $\pm$ 110	3.13 $\pm$ 1.4		
Alzheimer cases							
7) 91092	f	54	3.1	1,055	45	6	AD, cachexia
8) 92140	m	62	6.0	1,180	21	6	AD
9) 88252	f	66	3	1,250	85	7	AD, cachexia and sepsis
10) 91118	f	73	4	1,106	28	7	AD, dehydration
11) 90349	m	77	3.5	1,168	52.7	7	AD, pneumonia
12) 87017	f	83	3.3	970	50	nd	AD, lung embolism, cachexia
13) 90345	m	86	4.1	1,303	3.2	7	AD, uremia
14) 91061	m	88	4.4	1,058	27.3	5	AD, decompensatio cordis
Mean $\pm$ SEM		73.62 $\pm$ 4	4.0 $\pm$ 3.5	1,136.25 $\pm$ 38	39.1 $\pm$ 8.8		

* Global deterioration scale (27).

g = gram, h = hour, f = female, m = male, BW = brain weight, PMD = postmortem delay, nd = not determined.

tablish whether there is a relationship between advanced stages of cytoskeletal changes, i.e. NFT, and neuronal protein synthetic activity as judged by the size of the GA. We measured the size of the GA in NFT-bearing neurons in the CA1 area of the human hippocampus. This area is consistently affected in AD by neuropathological changes, i.e. NFT and senile plaques (SP), and a decrease in cell number (24, 25). Possible direct as well as indirect effects of NFT on the protein synthetic ability of the pyramidal neurons in AD were determined by measuring (i) the GA size and cell profile area of AD patients and controls, (ii) the size of the GA and cell profile area of NFT-bearing or non-NFT-bearing neurons of AD patients, and (iii) the density of extraneuronal NFT around every neuron stained for the GA.

MATERIALS AND METHODS

Tissue Preparation

Brains of 14 human subjects were obtained at autopsy (see Table 1 for details and clinico-pathological information). The material consisted of six non-demented 58–85-year-old controls (74 $\pm$ 4 years of age) without any primary neurological or psychiatric disorder and eight Alzheimer patients aged 54–88 years (73 $\pm$ 4 years of age). There was no significant difference in age ($p = 0.10$) or brain weight ($p = 0.08$) between controls and AD patients. Although there was a significant difference in postmortem delay between controls and AD patients ($p = 0.02$), there was no significant relationship between the size of the GA and postmortem delay ($p = 0.07$). Therefore, the difference in postmortem delays did not influence the results. The diagnosis

“probable AD” was established by excluding other possible causes of dementia according to NINCDS-ADRDA criteria (26). All patients had a global deterioration scale of 5–7 for severity of dementia (27) (Table 1) and neuropathological examination showed extensive neocortical and hippocampal SP and NFT. Brains were removed at autopsy and weighed. The hippocampus containing the CA1 area was dissected and fixed at room temperature in 4% buffered formaldehyde for about 1 month. The fixed hippocampus was dehydrated in graded ethanol, embedded in paraffin and cut serially in 6 μ m thin frontal sections. The sections were mounted on chrome-aluminum-sulphate-coated slides, deparaffinized, hydrated and stained either by thionine (0.5%), Bodian silver staining or immunocytochemistry for MG-160, a protein marker of the GA.

A polyclonal antibody has been raised against immunoaffinity purified MG-160, a sialoglycoprotein of medial cisternae of rat neuronal GA. This antibody recognizes the medial cisternae of the GA of neurons and glia in formalin-fixed, paraffin-embedded tissue of the human brain (28). The specificity of the antibody for GA membranes had been earlier established (29). By immunocytochemistry at the electron microscopic level, performed on human temporal lobe excised for epilepsy, the antibody stained exclusively the GA of neurons (28). For optimal retrieval of the MG-160 antigen, we used microwave oven treatment before incubating tissue sections with the first antibody (15). The immunocytochemical procedures have already been described extensively (15, 16). Briefly, the sections were incubated with anti-MG-160 diluted 1:800 for 1 hour at room temperature followed by an overnight incubation at 4°C. Subsequently the sections were washed in Tris-buffered saline and incubated with biotinylated goat anti-rabbit (Vector Laboratories Inc.) at a dilution of 1:500 for 30 minutes and finally with

avidin-biotin coupled to peroxidase (Vector Laboratories Inc.) at a dilution of 1:1,500 for 1 hour. 3',3'-Diaminobenzidine tetrahydrochloride (Sigma) was used as a chromogen. Staining enhancement was obtained by adding ammonium nickel sulphate (2.2 mg/ml) (30). Following the measurement of the size of the cell profile area and the GA in the sections stained with anti-MG-160, the coverslip was removed and the sections stained for NFT and SP using a modified Bodian's method (31).

Morphometry

In order to avoid a possible effect of the silver staining on the measurement of the GA size, the staining and measurement of the GA size were performed prior to the silver staining. Briefly there were three steps in the measurement: 1) Measurement of the GA size and neuronal cell profile by an IBAS-KAT image analysis system (Kontron). Details of the morphometrical and sampling methods have been described earlier (16). Subsequently, in order to visualize the NFT, the coverslip was removed and the section stained with silver. Briefly, step one consists of seven stages: i) outlining of the CA1 area using a 2.5 $\times$ objective, followed by ii) superimposing a grid (indicating the area seen at 40 $\times$) over the CA1 area, iii) random sampling of 30–40 fields by the computer, iv) calculating and saving of the scanning stage coordinates using a 2.5 $\times$ objective, v) loading of the selected fields at a 40 $\times$ objective, vi) making a mask for the stained GA in every cell (the size of the GA per cell consisted of the sum of all cytoplasmic areas stained with anti-MG-160), followed by vii) manually outlining of the neurons and measuring the mask and cell profile area by using a 40 $\times$ objective. Furthermore, at this stage the scanning coordinates of every outlined cell were stored together with the images. 2) Retrieval of every neuron measured in step one to observe whether it contained NFT. 3) Measurement of the density of extraneuronal NFT in a circle with the retrieved neuron in the center and a diameter of 247 μ m (the largest circular area on the screen) around every measured cell. All data were stored in Ascii format and used for statistical evaluation.

Statistical Methods

Using an SPSS program (SPSS Inc., Chicago), a multiple regression method was applied to study the relationship between the different variables. Furthermore, Student's *t*-test was used to compare variables between controls and AD subjects. A $p < 0.05$ was considered to be significant.

RESULTS

Following the Bodian staining we were able to distinguish both intra- and extraneuronal NFT in sections which had previously been stained for the GA. In order to exclude a possible interference of the immunocytochemical staining of the GA on the subsequent silver staining, the NFT density was determined using an image analysis system in ten alternating sections (from two patients #88252 and #87017) that were either stained by silver only or by silver staining following the immunocytochemical staining for the GA. Using one way ANOVA, no significant effect of the immunocytochemical GA staining on the NFT density was observed ($p = 0.71$).

The GA staining appeared as cytoplasmic granular perinuclear structures (Fig. 1A). The GA size was generally smaller in AD patients than in non-demented controls. Bodian silver staining clearly showed the presence of NFT and SP in old controls and all AD patients (Table 1). Microscopically, a large proportion of the neurons in the CA1 area of the hippocampus contained NFT (Fig. 1B). Counting both intra- and extraneuronal NFT in controls and AD patients showed a clear difference ($p = 3.59 \times 10^{-5}$) in the number of NFT in the CA1 area (Table 1). Of the 860 NFT counted, 74.4% were intracellular and 25.6% extracellular.

The Student's *t*-test showed a significant decrease in the GA size of AD patients compared to controls ($p = 0.01$). There was no difference in cell profile area between controls and AD patients ($p = 0.85$) (Table 2). Furthermore, the ratio of the GA size to the cell profile area was significantly smaller in AD patients (7.62%) than controls (12.42%) ($p = 0.011$). The size of the GA in NFT-bearing cells (28 ± 0.27) was generally larger than that of NFT-free neurons (17.80 ± 0.26). However, using multiple regression tests to study the possible correlation between different variables, no significant correlation was found between the presence of intracellular NFT ($p = 0.44$), the density of extraneuronal NFT around that cell ($p = 0.18$), and the size of the GA per cell (Table 3).

DISCUSSION

In previous studies of the GA in normal aging, AD and amyotrophic lateral sclerosis, we showed that the morphometric analysis on the immunocytochemically detected GA is a reliable index of neuronal activity in different regions, including the supraoptic nucleus (14, 15) and nucleus tuberalis lateralis (17) in the hypothalamus, nucleus basalis of Meynert (16) in basal forebrain and of motor neurons in the spinal cord (32, 33). In the present study, using the size of the GA as an index of metabolic activity, we showed that in AD the protein synthetic or secretory function of neurons was decreased in both tangle-bearing and non-tangle-bearing neurons in the CA1 area of the hippocampus. However, the presence or absence of intra- or extracellular NFT did not affect the extent to which the protein synthetic ability of the neurons in this area was reduced.

The present study shows also that although the pyramidal neurons of the CA1 area in AD contain a smaller GA, this change is not reflected in a significant decrease in the cell profile area. This indicates that the size of the GA is a more sensitive parameter for neuronal activity than cell size, an observation which agrees with previous reports (14, 16). Furthermore, a multiple regression analysis of the relationship between the size of the GA and the cell profile area, the presence or absence of intracellular NFT and the density of extraneuronal NFT showed

Fig. 1. Immunocytochemical staining of the GA (arrows) in an Alzheimer patient (a). Silver staining of the same group of neurons in the same section (b). iNFT = intraneuronal neurofibrillary tangle. Bar = 30 μ m.

that the reduced size of the neuronal GA did not correlate with the presence or absence of NFT (Table 3).

The smaller GA in AD is an index of reduced protein synthetic capability, which agrees with the observation that in AD the brain contains less protein (8), smaller nucleoli and less mRNA (12). Furthermore, it is in accordance with the data of Mann (12) which showed a significant decrease in RNA content, nuclear and nucle-

olar volume in both tangle-bearing and non-tangle-bearing cortical neurons in AD.

A number of studies have focused on the possible relationship between the presence of NFT in the neurons

TABLE 2
The Size of the GA and Cell Profile in the CA1 Area of the Hippocampus

	Control	Alzheimer	p*
Golgi apparatus/ μ m ²	31.3 $\pm$ 3.7	19.3 $\pm$ 2.7	0.015
Cell profile area/ μ m ²	252.7 $\pm$ 20	247.5 $\pm$ 17	0.850
Ratio (Golgi/cell) %	12.4 $\pm$ 0.1	7.7 $\pm$ 0.1	0.011

* Student's *t*-test.

TABLE 3
Analysis of the Relationship between the Size of the GA and Cell Profile Area, the Presence or Absence of NFT and the Density of Extraneuronal NFT in a Circle with a Diameter of 247 μ m around each Neuron in AD Patients

Variable	Sig. T*
Cell profile area	0.0000
Ratio (GA size/cell profile area)	0.0000
NFT (presence or absence)	0.4454
Density of extraneuronal NFT	0.1765

* Multiple regression (participation in total significance of correlation which is <0.0000).

of AD patients and the reduction of protein synthetic capability (11, 19, 34–36). Using the size of the nucleolus as an indicator of neuronal metabolic activity, Dayan and Ball (19) reported a clear decrease in the size of the nucleolus in NFT-bearing neurons compared to the adjacent non-NFT-bearing neurons in the temporal cortex. However, Curcio and Kemper (34) did not find such a reduction in nucleolar size in the nucleus raphe dorsalis, where tangle formation is prominent. It has been suggested that one reason for this controversy is the difficulty of accurately measuring the size of the nucleolus (37). An alternative explanation is that the presence of NFT and the reduced metabolism in AD are basically two independent phenomena that may in some areas sometimes be present in the same neurons. The last possibility is in accordance with our present data which show that the decreased size of the GA, which is clearly present in AD, is not related to the presence of intra- or extraneuronal NFT. Furthermore, the possibility that these two phenomena are independent is in accordance with the observation of Gertz et al (35), who showed that the presence of intraneuronal NFT in the CA1 area of the hippocampus is not related to nucleolar or cell size. Taken together, all these data suggest that a reduced protein synthetic ability of neurons is present in AD that does not, however, directly correlate with the formation of NFT.

We have shown earlier that the protein synthetic and secretory ability as determined by the size of the GA remains unchanged in neurons showing only pretangle stages of cytoskeletal alterations (17). This conclusion is based on measurements in the nucleus tuberalis lateralis, an area of the human hypothalamus in which silver-stained NFT are not present, although intense staining with Alz-50 (23, 38) and other antibodies against cytoskeletal alterations in AD, i.e. tau-1 and 3-39 (anti-ubiquitin) (23), are prominently staining cell bodies and dystrophic neurites.

The present paper indicates that the formation of NFT, a major hallmark of AD, and decreased neuronal protein synthetic ability are not directly related. However, since every staining method for NFT has its limitations (21), confirmation of these results with other methods of NFT staining in other brain areas would be important. In addition, we are developing a method to study the relationship between the presence of SP and the GA size in AD.

It has been shown in tissues obtained at autopsy that in AD the major constituent of PHF, i.e. tau, is abnormally phosphorylated (1–3), although this has also been observed in biopsies from control brains (39). Key enzymes for the phosphorylation of tau in PHF are a group of protein kinases, e.g. tau protein kinase-1 and PK40^{erk}. Recently, Blanchard and colleagues (40) have shown that one of the major factors in the determination of PK40^{erk} activity is the level of ATP. Furthermore, it has been shown recently that in AD the level of cytochrome ox-

dase which is involved in ATP production (41) is reduced in both areas which are affected heavily by NFT (e.g. CA1) and in the areas which are relatively spared from these changes (e.g. the molecular layer of the dentate gyrus) (42, 43). It remains to be studied, however, whether within an area like CA1 the reduction of cytochrome oxidase is different between NFT-bearing and NFT-free neurons.

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