

Microtubule Reduction in Alzheimer's Disease and Aging Is Independent of τ Filament Formation

Adam D. Cash,* Gjumrakch Aliev,[†]
Sandra L. Siedlak,* Akihiko Nunomura,[‡]
Hisashi Fujioka,* Xiongwei Zhu,* Arun K. Raina,*
Harry V. Vinters,[§] Massimo Tabaton,[¶]
Anne B. Johnson,^{||} Manuel Paula-Barbosa,**
Jesus Avila,^{††} Paul K. Jones,^{‡‡}
Rudy J. Castellani,*^{§§} Mark A. Smith,* and
George Perry*

From the Institute of Pathology* and the Departments of Anatomy[†] and Epidemiology and Biostatistics,^{‡‡} Case Western Reserve University, Cleveland, Ohio; the Department of Psychiatry and Neurology,[‡] Asabikawa Medical College, Asabikawa, Japan; the Department of Pathology and Laboratory Medicine,[§] University of California, Los Angeles, California; the Department of Neuroscience,[¶] University of Genoa, Genoa, Italy; the Department of Pathology,^{||} Albert Einstein College of Medicine, Bronx, New York; the Department of Anatomy,^{**} Porto Medical School, Porto, Portugal; the Centro de Biología Molecular,^{††} Universidad Autónoma de Madrid, Madrid, Spain; and the Clinical Center,^{§§} Michigan State University, East Lansing, Michigan

Biochemical studies show that phosphorylated τ , like that found in paired helical filaments (PHFs), does not promote microtubule assembly leading to the view that PHF formation leads to microtubule deficiency in Alzheimer's disease (AD). However, although this issue is one of the most important aspects to further understanding the cell biology of AD, no quantitative examination of microtubule diminution in AD and its relationship with PHFs has been performed. To examine this issue directly, we undertook a morphometric study of brain biopsy specimens from AD and control cases. Ultrastructural analysis of neurons was performed to compare the microtubule assembly state in neurons of diseased and control cases and to examine the effect of PHF accumulation. We found that both number and total length of microtubules were significantly and selectively reduced in pyramidal neurons from AD in comparison to control cases ($P = 0.000004$) but that this decrement in microtubule density was surprisingly unrelated to PHFs ($P = 0.8$). Further, we found a significant age-dependent decrease in microtubule density with aging in the control cases ($P = 0.016$). These findings suggest that reduction in microtubule assembly is not dependent on τ abnormalities of AD and aging. (*Am J Pathol* 2003, 162:1623–1627)

The accumulation of paired helical filaments (PHFs) in Alzheimer's disease (AD) is one of the most striking features of neuronal cytoskeletal pathology in AD and one noted in the earliest description of the disease.¹ The finding that PHFs derive from τ ,² a major microtubule-stabilizing protein, and that the form of τ found in PHFs is highly phosphorylated and inactive in microtubule assembly³ suggested that formation of PHFs may directly underlie the abnormalities in microtubule-based transport thought to occur in AD.^{4,5} In this regard, the observations of increased mitochondrial components in lysosomes,⁶ synaptic vesicles failing to arrive at terminals,^{7,8} and vesicle accumulation in cell bodies⁵ suggest that microtubule-dependent transport of organelles is hindered in AD.⁴ The clinical importance of these findings is that microtubule reduction may underlie the loss of neuronal connectivity suggested as the basis of cognitive loss in AD.⁹ In this study, we sought not only to quantitatively determine whether microtubules are reduced early in the course of AD but also whether the reduction is mechanistically linked to τ phosphorylation by examining neurons with and without PHFs.

Materials and Methods

Biopsy

Tissue was taken for diagnostic procedures from the frontal or parietal cortex of six patients with dementia (52 to 63 years of age), most of which had been included in other clinicopathological studies,^{5,6,10} and with a definite history (duration 3 to 11 years), and clinical presentation of dementia, fulfilling the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Associations (NINCDS-ADRDA) working group criteria for probable AD.¹¹ Thirteen cases of cognitively normal individuals were examined ultrastructurally and four patients (62 to 80 years of age) were selected for this study based on the presence of normal brain tissue. Of the control cases selected,

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Address reprint requests to George Perry, Ph.D., Institute of Pathology, Case Western Reserve University, 2085 Adelbert Rd., Cleveland, OH 44106. E-mail: gxp7@po.cwru.edu.

Table 1. Demographic Data of Patients Used in this Study

Case no.	Diagnosis	Age (yr)	Gender	Pyramidal neurons (n)	Nonpyramidal neurons (n)	Cortical area
1	AD	52	F	1	1	Parietal
2	AD	53	M	0	2	Prefrontal
3	AD	55	M	4	3	Prefrontal
4	AD	56	F	2	1	Frontal
5	AD	59	M	2	0	Prefrontal
6	AD	64	M	1	1	Prefrontal
7	Hydrocephalus	62	M	4	0	Frontal
8	Hydrocephalus	69	M	4	0	Frontal
9	Hydrocephalus	74	F	3	1	Frontal
10	Brain tumor	80	M	2	4	Frontal

Abbreviations: AD, Alzheimer's Disease; F, female; M, male.

three suffered from hydrocephalus and one from a brain tumor, in which no sign of tumor infiltration or other abnormalities were evident in the tissue examined (Table 1). Tissue was fixed in 1.5% glutaraldehyde in cacodylate buffer for 1 hour immediately on removal from the brain and postfixed in 1% osmium tetroxide for 1 hour. After dehydration in graded ethanol and propylene oxide, the tissue was embedded in Epon 812, sectioned at silver interference color, electron contrasted with uranyl acetate and lead citrate, and grids were viewed at 80 KV by using a JEOL 100CX electron microscope. Unfortunately, there is no information on whether an autopsy was performed on these cases to confirm the original diagnosis or to know the progression of pathological changes.

Morphometry

Nonbiased sampling was assured by adherence to a protocol established before microscopy. Micrographs of all neurons containing the nucleolus were made from a single ultrathin section analyzed for each case. Neurons of the third cortical layer identified by the dominance of pyramidal neurons were studied. Micrographs of section planes containing the nucleolus at magnifications of $\times 5000$ were used for identification, and micrographs of $\times 20,000$ were also taken so that a coded montage including the entire cytoplasm could be made to obtain measurements of microtubule number and total length. Four neuropathologists independently assigned cellular identity and only those cells with unanimous agreement of identity were analyzed. Between two to seven neurons met these criteria and were analyzed for each case. Micrographs were examined with a stereomicroscope at $\times 10$ to $\times 20$ (Carl Zeiss Inc., Thornwood, NY) and perikaryal microtubules were identified as 25-nm electron dense tubules with cross-sectional lumen diameter of 10 nm^{12,13} and counted for each neuron. Both the number of microtubules and total length per unit area of microtubules were determined with a micrometer (Bausch & Lomb, Rochester, NY), making necessary conversions according to magnification. For length determination, each microtubule cross-sectional length was included in the data as 25 nm (diameter), while the microtubule density measurement consisted of both number of longitudinal and cross-sectioned microtubules. In no case did the

micrographs show a definitive axon attached to the neuronal cell body, therefore, these processes were not analyzed. The cytoplasmic area (excluding the nucleus and regions of PHF) of each neuron was measured by using NIH Image J program, version 1.06a (<http://rsp.info.nih.gov/ij/>).

The sample population was classified as either pyramidal or nonpyramidal neurons. The individual morphometric data for both length and density were analyzed and means \pm SEM calculated. The nonparametric Student's *t*-test, hierarchical/nested analysis of variance and regression analysis were used for statistics. Observations of $P < 0.05$ were considered significant. We took into account the number of neurons and from which subject they came from when we performed the statistical analysis. The number of neurons ($n = 36$) studied provided sufficient statistical power to render significance (eg, $P = 0.000004$) for our findings.

We quantified vesicles present at, or near, the presynaptic side in the cases examined. Synapses were visually identified as disk-shaped thickenings of the membrane having at least three synaptic vesicles of 40 nm in diameter adjacent to them as previously described.^{14,15} The number of vesicles 0 to 500 nm from the synaptic cleft was determined.

Paraffin-embedded biopsy tissue was available for two control cases, which were immunostained with 4G8, a monoclonal antibody to amyloid β . For immunocytochemistry, after deparaffinization with xylene, sections were hydrated through graded ethanol. Endogenous peroxidase activity in the tissue was eliminated by a 30-minute incubation with 3% H₂O₂ in methanol, and nonspecific binding sites were blocked in a 30-minute incubation with 10% normal goat serum in Tris-buffered saline (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.6). The immunostaining was as previously described¹⁶ for the peroxidase-anti-peroxidase procedure by using 3,3'-diaminobenzidine as co-substrate.¹⁷ The sections were dehydrated through ethanol and xylene solutions and then mounted in Permount (Fisher, Pittsburgh, PA).

To measure the levels of amyloid deposition, sections immunostained for amyloid (4G8) were analyzed by using an Axiocam digital camera and KS300 image analysis software (Carl Zeiss, Inc.). The percent area covered by amyloid, by the immunocytochemical method, was mea-

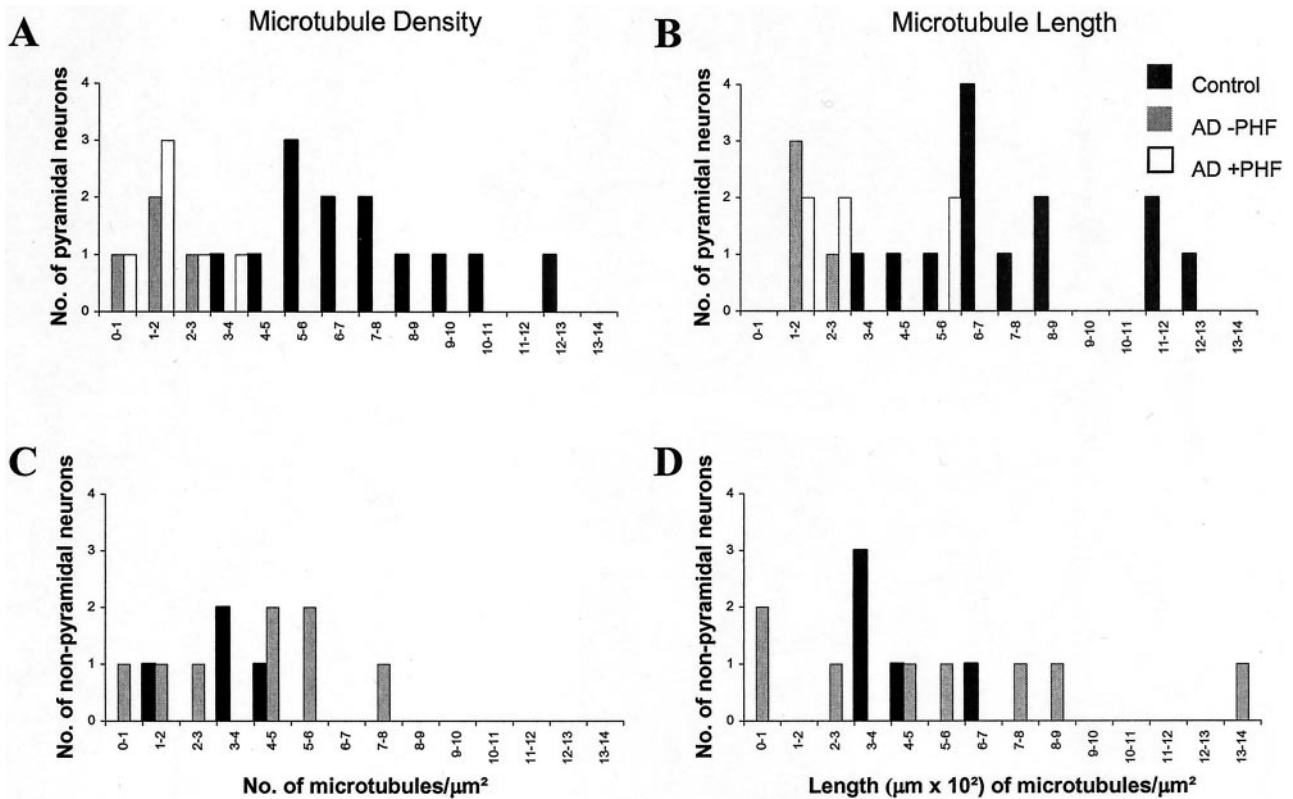


Figure 1. Microtubule density (A, C) and length (B, D) both show striking reductions for pyramidal neurons in AD when compared to controls ($P = 0.000004$, density; $P = 0.00004$, length), whereas no reduction was seen for adjacent nonpyramidal neurons ($P = 0.90$, density; $P = 0.55$, length) in the same section. Surprisingly, there was no difference of microtubule density ($P = 0.80$) or length ($P = 0.15$) dependent on the presence of PHFs.

sured in five fields totaling 1 mm² in each case. The values for each case were averaged and expressed as amyloid density or amyloid burden.

Results

Electron micrographs showed neurofibrillary tangles and amyloid β deposits for AD cases, consistent with the pathological assessment at the time of biopsy. Some of the neurons (33.3%) in the AD cases had neurofibrillary tangles with $16.4 \pm 6.5\%$ of the cytoplasm infiltrated with PHFs. Quantitative measurement of one control case immunostained with 4G8 showed no amyloid β and the other revealed a few sparse deposits (amyloid burden = 0.79%) consistent with normal aging.¹⁷

A comparison of the microtubule density of pyramidal and nonpyramidal neurons was performed. The two groups were distinguished in the micrographs by the observations of four independent experienced observers based on morphological criteria. Two-thirds of the neurons in the experimental population were pyramidal, while the remaining one-third were nonpyramidal. Within the pyramidal classification (total, $n = 23$; AD, $n = 13$; control, $n = 10$), AD neurons showed a significant reduction in microtubule density (1.2 ± 0.8 microtubules/ μm^2) when compared to controls (7.1 ± 2.6 microtubules/ μm^2) ($P = 0.000004$) (Figure 1A). The total length of microtubules for individual AD neurons was also reduced (170.8 ± 54.4 μm of microtubules/ μm^2) when compared

to controls (768.9 ± 77.2 μm of microtubules/ μm^2) ($P = 0.00004$) (Figure 1B). Within the nonpyramidal classification (total, $n = 13$; AD, $n = 8$; control, $n = 5$), there was no difference for microtubule density ($P = 0.90$) (Figure 1C) or length ($P = 0.55$) (Figure 1D). The greater change in microtubule density for pyramidal neurons versus nonpyramidal neurons acts as an internal control for alterations brought about by tissue handling and fixation. Although one might think the pyramidal-specific decrement in microtubules was related to PHF formation, surprisingly, neither microtubule density ($P = 0.80$) nor length ($P = 0.15$) was related to PHFs, and microtubules were often seen in close juxtaposition to PHFs (Figure 2).

When we further analyzed the microtubule density in the control cases, we found an age-dependent decrease in microtubule density among pyramidal neurons in the control individuals ($P = 0.016$; regression analysis, $r^2 = 0.9841$) (Figure 3). Analysis of covariance was performed comparing neurons from patients with AD versus controls and by using age as a covariate. The mean age was 56.1 years ($n = 18$ neurons) for patients with AD and 72.2 years ($n = 18$ neurons) for controls (Table 2). Age adjustment of mean was made to the overall sample mean of 64.1 years ($n = 36$ neurons).

The age-adjusted means for patients with AD for microtubule length per unit area and density were lower than the corresponding means for controls. However, difference in mean microtubule length was significant for pyramidal neurons ($t = -4.37$, $P < 0.00001$), but not for

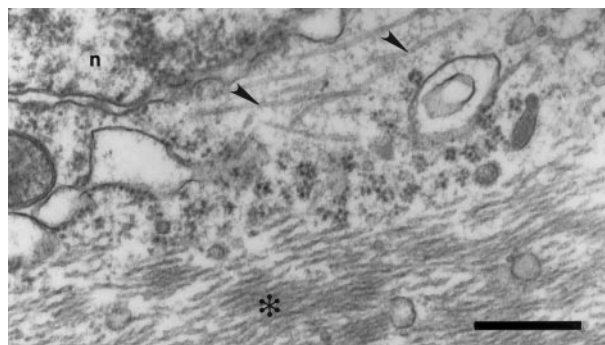


Figure 2. Abundant microtubules were often seen in close juxtaposition to PHFs. Electron microscopic examinations of microtubules for both AD and control cases were performed in specimens preserved from biopsy fixed in 1.5% glutaraldehyde and 1% osmium tetroxide. Microtubules (arrowhead) could often be seen in close juxtaposition to PHFs (asterisk) in AD. n, nucleus. Scale bar, 250 nm.

nonpyramidal neurons ($t = -1.13$, $P = 0.3$). The overall mean length of microtubules for neurons in AD was less than that for control neurons ($t = -2.61$, $P = 0.01$).

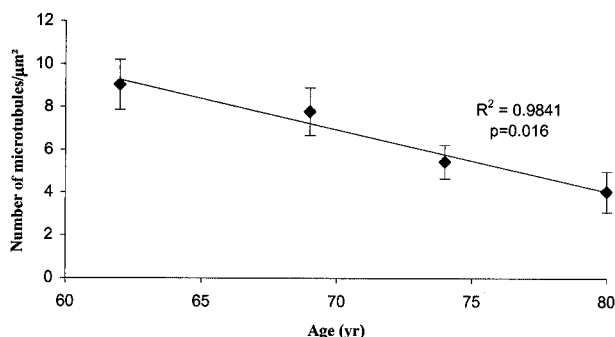


Figure 3. There is a notable age-dependent decrease in microtubule density in pyramidal neurons among control individuals ($P = 0.016$; regression analysis, $r^2 = 0.9841$). Error bars represent SEM.

Difference in age-adjusted mean microtubule density was significant for both pyramidal neurons ($t = -7.32$, $P < 0.0001$) and for nonpyramidal neurons ($t = -2.87$, $P = 0.01$). Also, the overall mean microtubule density for neurons in AD was less than that for control neurons ($t = -5.03$, $P < 0.0001$). No gender differences in microtubule diminution were found.

Both pyramidal and nonpyramidal neurons in AD cases show a reduction in synaptic vesicle number in presynapses juxtaposed to them when compared to controls, although statistical significance was not met.

Discussion

In this study, we documented a clear microtubule deficit in pyramidal neurons in AD, one seen even in the absence of PHFs, and also with aging among normal individuals. The findings presented here are consistent with microtubule changes being concurrent with the mitochondrial abnormalities and increased oxidative damage and these changes occurring before any detectable changes in τ .^{5,18-20} The findings are also consistent with previous work by one of the authors (MP-B) noting a microtubule decrease of near 50% in dendrites but that did not correlate the data with PHFs or age.²¹ Our result showing a 55% reduction in microtubule density in normal patients from age 62 to age 80 years is consistent with a biochemical study reporting a 90% decrease in tubulin concentrations in normal human cerebral cortex from infants to age 90 years,²² further buttressing the case that microtubule diminution is independent of τ abnormalities and closely linked to aging.

The finding of a small decrease in synaptic vesicle number in AD cases is consistent with past studies quantitating reduction of the vesicle-docking protein synaptophysin.^{15,23} Based on these studies, Terry^{7,24} proposed

Table 2. Analysis of Covariance for Microtubule Length and Density with Age as a Covariate

Group	n	Unadjusted mean	Student's t test	Adjusted mean	Analysis of covariance with age — T value*
Length					
Nonpyramidal					
AD	8	549	$P = 0.55$	382	-1.13
Control	5	424		705	$P = 0.2663$
Pyramidal					
AD	10	249	$P < 0.0001$	105	-4.37
Control	13	769		875	$P < 0.0001$
Combined					
AD	18	383	$P = 0.01$	228	-2.61
Control	18	673		828	$P = 0.0139$
Density					
Nonpyramidal					
AD	8	4.12	$P = 0.90$	2.07	-2.87
Control	5	3.94		7.37	$P = 0.0073$
Pyramidal					
AD	10	1.76	$P < 0.0001$	0.01	-7.32
Control	13	7.06		8.36	$P < 0.0001$
Combined					
AD	18	2.81	$P = 0.0002$	0.92	-5.03
Control	18	6.20		8.08	$P < 0.0001$

*The T values and the corresponding P values are calculated on the age-adjusted differences in means ie, Alzheimer's mean versus control mean, both adjusted to the overall mean age of 64.1 years.

that reduced axoplasmic flow and resulting loss of synaptic connectivity underlie the cognitive deficits of AD. Further findings of Golgi disruption²⁵ and altered mitochondrial turnover⁶ in vulnerable neurons in AD, organelles completely dependent on microtubules, are consistent with microtubule reduction. Our findings, in sum, suggest microtubule reduction in AD is marked, specifically limited to vulnerable pyramidal neurons, and not dependent on abnormalities in τ .

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