Eur Arch Psychiatr Neurol Sci (1990) 239:314–319

European Archives of Psychiatry and Neurological Sciences

Ultrastructural Evidence That Insoluble Microtubules Are Components of the Neurofibrillary Tangle*

Miguel A. Pappolla[1], Julia Alzofon[2], James McMahon[3], and Theodor J. Theodoropoulos[4]

[1]Department of Pathology, Veterans Administration Medical Center, Montrose, New York and Department of Pathology and Neuropathology, New York Medical College, New York, USA
[2]Department of Pathology, Veterans Administration Medical Center, Montrose, New York, USA
[3]Electron Microscopy Services, Cleveland Clinic Foundation, Cleveland, Ohio, USA
[4]Veterans Administration Medical Center, Montrose, New York and Department of Medicine, New York Medical College, New York, USA

Received May 12, 1989

Summary. The ultrastructure of Alzheimer's neurofibrillary tangles is heterogeneous and includes abnormal paired helical filaments (PHF) and various other insoluble structures. Insoluble non-PHF components isolated from neurofibrillary tangles were examined by electron microscopy. Comparison of these fractions with normal assembled neurofilaments and normal brain microtubules revealed scattered profiles which were morphologically (not chemically) identical to structures present in the microtubule, but not in the neurofilament preparations. These results support the notion that insoluble microtubules contribute to the make up of the neurofibrillary tangle. Based on these findings, preliminary experiments were conducted which suggest that non-enzymatic glycosylation may be a pathway leading to insolubility of the microtubules.

Key words: Alzheimer's disease – Aging – Cytoskeleton – Glycosylation – Neuropathology

Introduction

Alzheimer's disease (AD) is characterized by global and progressive deterioration of memory and intellectual functions (Terry et al. 1983). Patients with this disease develop intraneuronal filamentous accumulations called neurofibrillary tangles (NFT) and discrete foci of degeneration of the neurophil frequently accompanied by deposition of amyloid (senile "neuritic" plaques) (Tomlinson et al. 1975). Paired helical filaments (PHF) are the most distinctive constituents of the tangles (Kidd 1963a), although straight filaments, membranous profiles and granular material contribute to their heterogeneous make up (Hirano et al. 1968; Kidd 1963a, b). The insoluble quality of some NFT components has limited their chemical analysis; however, substantial information has been gained by immunochemical methods (Anderton et al. 1982; Delacourte et al. 1986; Grundke-Iqbal et al. 1986a; Ishii et al. 1979; Kosik et al. 1986; Wood et al. 1986; Yen et al. 1987). Currently, there is extensive evidence that some NFT components derive from the neuronal cytoskeleton.

This study focused on the ultrastructural analysis of non-PHF insoluble structures isolated from hippocampal neurons of AD brains. We found insoluble elements which were morphologically identical to profiles present in preparations of normal (soluble) microtubules. In an attempt to reproduce the findings in vitro, we explored a possible pathway leading to protein insolubility. Data from these experiments suggest that non-enzymatic glycosylation may be a mechanism contributing to insolubility of the cytoskeleton.

Materials and Methods

Insoluble tangle fractions were obtained from pooled homogenates of hippocampal cortex from AD brains as described by Selkoe et al. (1984). Two batches of tissue (weight 7 g each) enriched in NFTs were prepared from two groups of AD brains. All cases of AD obtained for these studies met the National Institutes of Health consensus criteria for the autopsy diagnosis of the disorder (Khachaturian 1985). The tissue was minced

*This work has been supported in part by a grant from the Veterans Administration to M.A.P.

Offprint requests to: M. A. Pappolla

and incubated for 2 h at 25°C in 35 ml of a buffer containing 50 m*M* trishydroxyethylaminomethane (TRIS), 0.1 MB-mercaptoethanol (BME) and 2% sodium dodecyl sulfate (SDS). The suspensions were homogenized in a Dounce, heated to 100°C for 5 min, sieved through a 110-μm nylon mesh (TETKO, Elmsford, NY) and allowed to cool. The filtrates were centrifuged at 100000 *g* for 30 min at 25°C and the pellet resuspended in a buffer containing 50 m*M* TRIS, 1% SDS at pH 7.6; sucrose was added to bring the molarity to 0.4 *M*. After ultracentrifugation in a sucrose gradient (0.4 *M*–1.0 *M*–1.2 *M*–1.4 *M*–2 *M*) at 243000 *g* for 2 h at 25°C, NFTs were collected at the 1.4 *M*–2.0 *M* interface. NFTs were identified by examining smears of this interface stained with congo red. The NFTs were sonicated and spun at 200 *g* for 10 min. The presence of insoluble structures in the supernatant was demonstrated by electron microscopy as depicted in the Results section (note that the *g* forces used at this step are not sufficient to pellet the insoluble material). Microtubules were prepared from fresh calf brains obtained from a local slaughter house by the polymerization-depolymerization method of Borisy et al. (1975). Bovine brain tissue was placed on ice immediately upon collection. The cortices were dissected, minced and homogenized in a buffer containing 100 m*M* piperazinebisethanesulfonic acid (PIPES), 2 m*M* ethyleneglycolbis-(B-aminoethyl ether) N,N,N,N,-tetreacetic acid (EGTA), 1 m*M* MgSO4 and 0.1 *M* guanosine 5-triphosphate (GTP). The homogenate was cleared by centrifugation at 10000 *g* for 30 min at 0°C. GTP was added to the supernatant to bring the concentration to 2.5 m*M* and this extract was centrifuged at 100000 *g* for 1 h at 0–5°C. The first polymerization cycle was initiated by incubation in a 30°C water bath for 1 h. Thereafter, the sample was centrifuged at 40000 *g* for 30 min at 30°C and the pellet resuspended in a buffer containing 100 m*M* PIPES, 1 m*M* EGTA, 1 m*M* MgSO4, 1 m*M* GTP at pH 6.64 (other species require different pHs). Depolymerization was accomplished on ice (30 min) and the suspension was centrifuged at 40000 *g* for30 min at 30°C to remove non-microtubule aggregates. The pellet was resuspended in the polymerization buffer and run through two additional assembly-disassembly cycles. Neurofilaments were isolated from rat spinal cords by the axonal flotation method (Iqbal et al. 1979; Wisniewski et al. 1981; Merz et al. 1981). Rat spinal cords were homogenized in a medium containing 0.9 *M* sucrose, 100 m*M* NaCl, 20 m*M* KCl and 2 m*M* ethylenediaminetetraacetic acid (EDTA) at pH 6.8. After centrifugation at 9500 *g* for 50 min, a "floating pad" was obtained. This pad was again recentrifuged twice as above and a third time at 100000 *g* for 15 min in the same medium. A final floating pad was osmotically shocked in a solution containing 0.32 *M* sucrose, 100 m*M* *NaCl, 20 mM* KCl and 2 m*M* EDTA, and extracted for 1 h at 0–4°C (This step rids the preparation of myelin lipids.) The suspension was centrifuged at 100000 *g* for 1 h at 0–4°C and a pellet containing the neurofilaments was obtained. All preparations were electrophoresed in polyacrylamide gels (Laemmli 1970) to confirm the nature of the main protein components. Identification of beta tubulin as one of the major protein components of the microtubular preparation was carried out by the Western Blot (Towbin et al. 1979) and the "dot" blot methods (Nibbering et al. 1985) using a specific monoclonal antibody. Electron microscopic examination of each preparation was carried in Formvarcoated copper grids after staining with 2% phosphotungstic acid.

Preliminary experiments were conducted to examine a possible pathway leading to insolubility of the microtubules. Polymerized microtubules were incubated in 100 m*M* glucose-6-phosphate (G-6-P) at 37°C in microwells for 1, 3, 7 and 14 days. G-6-P is a readily available intracytoplasmic metabolite, known to induce rapid nonenzymatic glycosylation of proteins with formation of insoluble Amadori products (Monnier et al. 1981). Generation of insoluble moieties was monitored by high-speed ultracentrifugation, ultraviolet spectroscopy (Monnier et al. 1981) and electron microscopy.

Results

Polyacrylamide gel electrophoresis showed the neurofilament triplet as a component of assembled neurofilaments (Fig. 1). Beta tubulin was identified in Western blots of microtubular fractions as one of the major protein constituents (Fig. 1). Electron microscopy of insoluble preparations from AD brain cortex showed various insoluble structures. These included PHF, 10- to 15-nm straight filaments, and insoluble

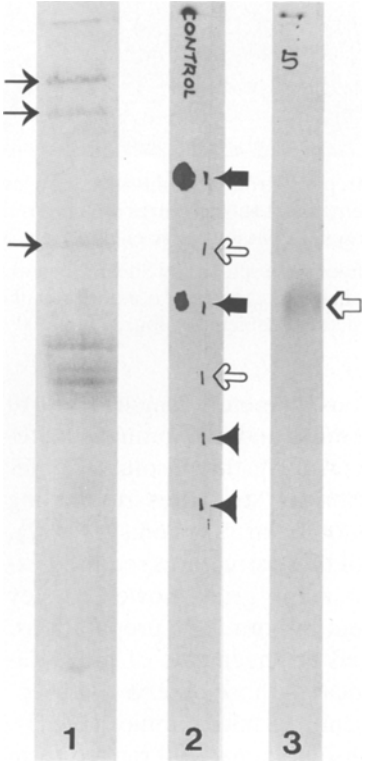

Fig. 1. *1* 10% Polyacrylamide gel electrophoresis of assembled neurofilaments showing the neurofilament triplet *(arrows)*, Coomassie blue stain. *2* "Dot blots" of microtubules *(solid arrows)* immunostained with a monoclonal antibody directed against beta tubulin; albumin dots *(open arrows)* and cytochrome C *(solid arrowheads)* did not immunostain. *3* Western blot experiment following electrophoresis of a microtubule preparation. A band at the beta tubulin position was detected with the same monoclonal antibody as used in strip number 2 *(open arrow)* and represented one of the major protein components of the microtubule preparations. Immunostaining of nitrocellulose blots was done using alkaline phosphatase-conjugated goat anti-rabbit IgG included in a commercial package (Biorad, Richmond, Calif.)

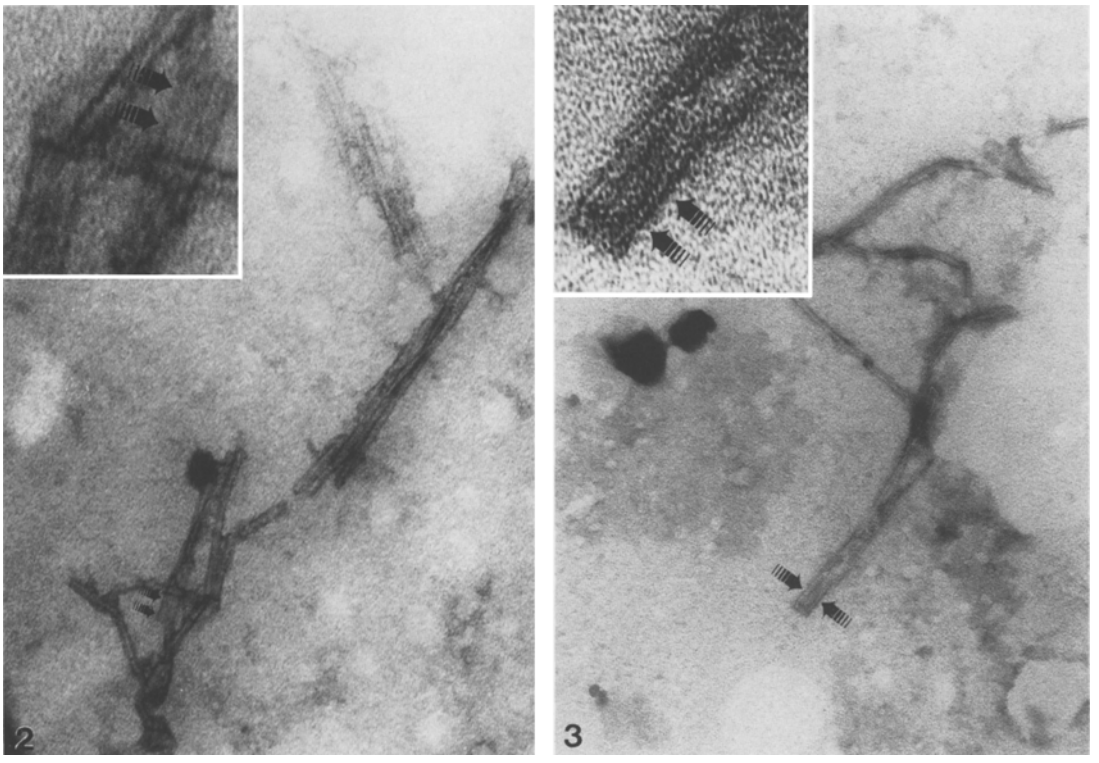

Fig. 2. Insoluble material prepared from neurofibrillary tangles. Although the harsh SDS treatment hampered sharp ultrastructural resolution, parallel arrangements of substructures are discernible (perpendicular to the major axis of *arrows*). Note the "rigid" appearance of the profiles. *Inset* is a photographic enlargement of the area pointed by the *small arrows*. × 80000

Fig. 3. Microtubules assembled as described by Shelanski et al. (1973). The *arrows* indicate parallel arrangements of substructures, each measuring 3–5 nm in diameter. The *inset* is a photographic enlargement of the area indicated by the *arrows* in the field. Note identical appearance to structures depicted in Fig. 2. × 80000

tubular profiles of various diameters ranging from 10 to 35 nm; lipofuscin granules and membranous material were also present. Among this insoluble material, we found characteristic structures resembling normal (soluble) microtubular segments (Fig. 2). Positive identification of these structures required extensive examination of many grids; however, they were consistently present in both NFT preparations. This component showed arrangements of protofilaments in patterns identical to those observed in preparations of normal assembled microtubules (Fig. 3). Protofilaments in the insoluble material ranged from 3 to 5 nm in diameter (Fig. 2). Normal calf microtubules were visualized as straight profiles which ranged from 8 to 22 nm in diameter and showed occasional branching. The variation in diameter observed in these preparations was attributed to different stages of assembly. Profiles identical to those observed in the insoluble fractions from AD were frequently present in preparations of microtubules (Fig. 3). In contrast, extensive examination of several preparations of assembled neurofilaments did not show the structures described in the AD or the microtubular fractions above. Instead, these preparations showed the expected non-branching and "flexible" appearance of neurofilaments (Fig. 4).

Insoluble pellets were obtained by ultracentrifugation (100000 *g*) of glycosylated microtubules after the 7th day of incubation with 100 m*M* G-6-P. Protein cross-linking ("browning") was evidenced by formation of characteristic fluorescent moieties with emission maxima at 440 nm (excitation at 360 nm). Electron microscopy of these insoluble pellets showed mostly amorphous clumps of electron-dense material (Fig. 5). Profiles such as those depicted in Fig. 1 and 3 were not found in the glycosylated fractions. However, singlets and pairs of insoluble, poorly formed filaments measuring 3–5 nm in diameter were present (Fig. 5). These latter profiles were not found in preparations of glycosylated bovine serum albumin used as control for morphology.

Discussion

Neurofilaments and microtubules are major cytoskeletal components of the neuron. The remarkable similarity between some of the insoluble tangle material

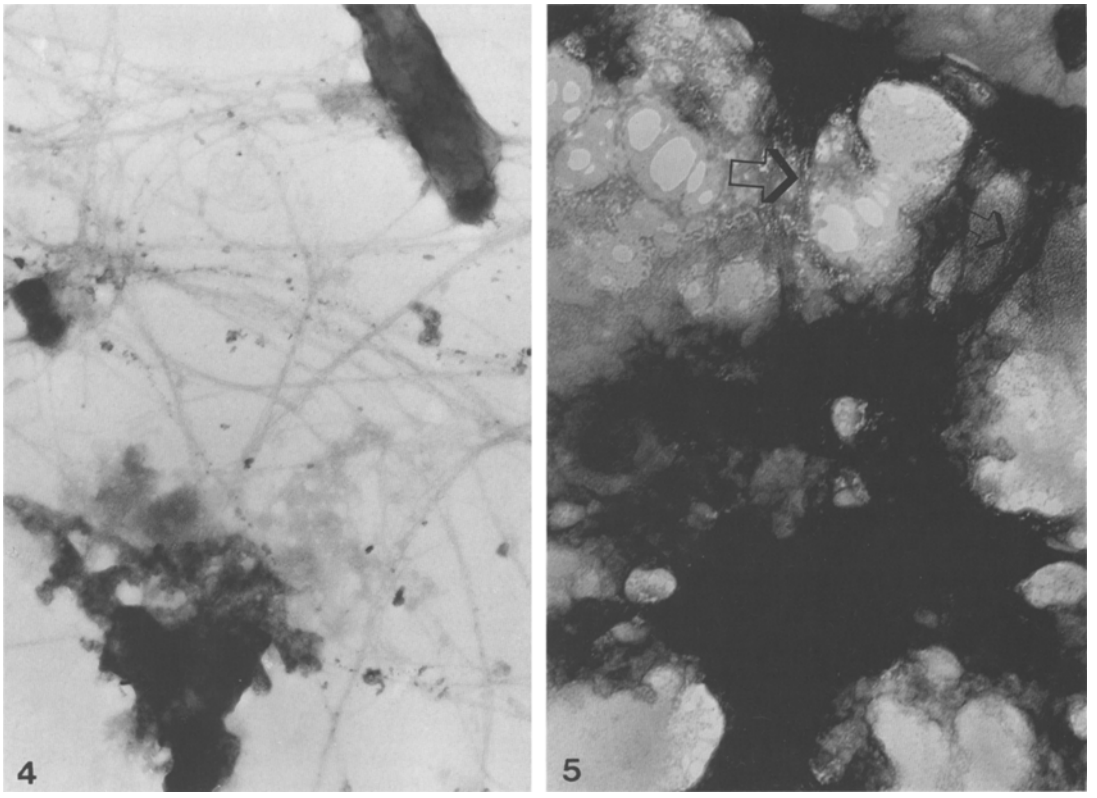

Fig. 4. Electron microphotograph of assembled neurofilaments. This illustration (× 20000) intends to show the general pattern displayed by assembled neurofilaments. Note their "flexible" appearance and "indefinite" lengths. Structures such as those depicted in Figs. 2 or 3 were not found

Fig. 5. Electron microphotograph of glycosylated microtubules. These preparations consisted mostly of clumps of insoluble amorphous material. However, poorly formed filamentous profiles could be identified *(arrows)*. × 80000

and the profiles depicted in the normal microtubular fractions (but not in the neurofilament preparations) is evidence which suggests that insoluble microtubules are present in NFT.

Immunochemical studies have offered most (but not all) of the information available regarding the composition of the NFT (Anderton et al. 1982; Delacourte et al. 1986; Grundke-Iqbal et al. 1986a; Ishii et al. 1979; Kosik et al. 1986; Wood et al. 1986; Yen et al. 1987). Cytoskeletal elements identified within NFT have included several microtubule associated proteins (MAPs) (particularly the TAU family) and various neurofilament subunits. The heat shock protein ubiquitin has also been detected as a component of the NFT and the PHF (Cole et al. 1987; Mori et al. 1987). The presence of neurofilaments in the PHF has recently been questioned because of antigenic cross-reactivity between phosphorylated forms of TAU and various anti-neurofilament antibodies (Selkoe 1987). This is relevant in view of the fact that PHF-associated TAU is phosphorylated (Grundke-Iqbal et al. 1986b; Nukina et al. 1986). Moreover, cross-reactivity between phosphorylated MAP 1B, MAP 1A and the high and middle molecular weight neurofilament subunits was reported with a monoclonal antibody (Luca et al. 1986). Although the weight of the current arguments favors the presence of neurofilaments in PHF (Gambetti et al. 1986), it is apparent that the role of other cytoskeletal components needs further clarification. The major argument against a microtubular component is that NFTs do not react with specific anti-tubulin antibodies. However, alpha and beta tubulin are very labile proteins (Selkoe 1987; Pappolla, personal observations) and the same processes which lead to their insolubility may also affect their antigenicity.

The morphologic similarity between normal microtubules and some of the insoluble tangle elements illustrated in this study was not limited to the arrangement of substructures. The "rigid" and straight pattern displayed by normal microtubules was closely mirrored by the insoluble material. On the other hand, assembled neurofilaments showed a diverging appearance, as already described (Fig. 3).

The relationship between the protofilamentous components of microtubules, neurofilaments and

PHF is not clear. While some workers propose that PHF protofilaments are different from microtubule or neurofilament protofilaments (Wang et al. 1985; Wisniewski et al. 1984), other investigators believe that protofilaments in PHF and neurofilaments may be related (Brion et al. 1984). We are not yet ready to comment on the similarities or differences between PHF and neurofilament-associated protofilaments because the methods used in this study did not allow clear visualization of protofilaments in neurofilaments.

The mechanisms leading to insolubility of various cytoskeletal components are also unclear. Many long-lived proteins undergo age-related changes characterized by non-enzymatic glycosylation (Maillard reaction) and cross-linking (Amadori rearrangements) (Monnier et al. 1981; Monnier and Cerami 1983). With aging, low turnover proteins such as collagen, elastin, glycosaminoglycans and some nucleoproteins become increasingly glycosylated, cross-linked and insoluble (Kohn et al. 1984). We theorized that intracellularly accumulated proteins, such as those present in the NFT, might become glycosylated and cross-linked via G-6-P. G-6-P is known to glycosylate proteins more effectively than glucose (Kohn et al. 1984; Monnier, personal communication). Since intraneuronal accumulations of cytoskeletal proteins may follow diverse types of injury, non-enzymatic glycosylation may be an important pathway leading to protein insolubility within the neuronal perykaryon. Our results show that G-6-P induces rapid insolubility of proteins contained in the microtubular fractions. Insoluble profiles identical to those shown in AD could not be reproduced in vitro, probably because of the great difficulty in maintaining microtubules polymerized for several days, even after periodic additions of various phosphonucleotides. However, the rapid formation of insoluble filamentous forms after glycosylation may be significant and could be interpreted as preliminary evidence that glycosylation may play some role in the formation of the abnormally insoluble cytoskeleton. It will be important to determine, in future studies, whether Amadori products can be detected as intrinsic components of the NFT.

Acknowledgements. We are grateful to V. Monnier, M.D., Ph.D., for his advise on the spectroscopic technique and glycosylation experiments and to D. Maldonado for preparing the manuscript.

References

Anderton BH, Breinburgh D, Downes MJ, Green PJ, Tomlinson BE, Ulrich J, Wood JN, Kahn J (1982) Monoclonal antibodies show that neurofibrillary tangles and neurofilaments share antigenic determinants. Nature 298:84–86

Borisy GG, Marcum JM, Olmsted JB, Murphy DB, Johnson KA (1975) Purification of tubulin and associated high molecular weight proteins from porcine brain and characterization of microtubule assembly in vitro. Ann NY Acad Sci 253:107–132

Brion JP, Couck AM, Flament-Durand J (1984) Ultrastructural study of enriched fractions of 'tangles' from human patients with senile dementia of the Alzheimer's type. Acta Neuropathol (Berl) 64:148–152

Cole GM, Timiras PS (1987) Ubiquitin-protein conjugates in Alzheimer's lesions. Neurosci Lett 79:207–212

Delacourte A, Defossez A (1986) Alzheimer's disease: TAU proteins, the promoting factors of microtubule assembly, are major components of paired helical filaments. J Neurol Sci 76:173–186

Gambetti P, Perry G, Autillo-Gambetti L (1986) Paired helical filaments: do they contain neurofilament epitopes? Neurobiol Aging 7:451

Grundke-Iqbal I, Iqbal K, Quinlan M, Tung K, Zaida MS, Wisniewski HM (1986a) Microtubule-associated protein TAU. A component of Alzheimer paired helical filaments. J Biol Chem 261:6084–6089

Grundke-Iqbal I, Iqbal K, Tung K, Quinlan M, Wisniewski HM, Binder LI (1986b) Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. Proc Natl Acad Sci USA 83:4913–4917

Hirano A, Dembitzer HM, Kurland LT, Zimmerman HM (1968) The fine structure of some intraganglionic alterations. Neurofibrillary tangles, granulovacuolar bodies and "rod-like" structures as seen in Guam Amyotrophic Lateral Sclerosis and Parkinsonism-dementia Complex. J Neuropathol Exp Neurol 27:167–182

Iqbal K, Wisniewski HM (1979) Neurofilament proteins. Effect of ionic strength and a calcium chelator. Trans Am Soc Neurochem 10:168

Ishii T, Haga S, Tokutake S (1979) Presence of neurofilament protein in Alzheimer's neurofibrillary tangles (ANT). An immunofluorescent study. Acta Neuropathol (Berl) 48:105–112

Khachaturian ZS (1985) Diagnosis of Alzheimer's disease. Arch Neurol 42:1097–1105

Kidd M (1963a) Paired helical filaments in electron microscopy of Alzheimer's disease. Nature 197:192–194

Kidd M (1963b) Alzheimer's disease – an electron microscopical study. Brain 87:7307–7320

Kohn RR, Cerami A, Monnier VM (1984) Collagen aging in vitro by nonenzymatic glycosylation and browning. Diabetes 33:57–59

Kosik KS, Joachin CL, Selkoe DJ (1986) The microtubule-associated TAU is a major antigenic component of paired helical filaments in Alzheimer's disease. Proc Natl Acad Sci USA 83:4044–4048

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Luca FC, Bloom GS, Vallee RB (1986) A monoclonal antibody that cross-reacts with phosphorylated epitopes on two microtubule-associated proteins and two neurofilament polypeptides. Proc Natl Acad Sci USA 83:1006–1010

Merz PA, Somerville RA, Wisniewski HM, Iqbal K (1981) Abnormal fibrils from scrapie infected brain. Acta Neuropathol (Berl) 54:63–74

Monnier VM, Cerami A (1981) Nonenzymatic browning in vivo: possible process for aging of long lived proteins. Science 30:491–493

Monnier VM, Cerami A (1983) Detection of nonenzymatic browning products in the human lens. Biochim Biophys Acta 760:97–103

Monnier VM, Stevens VJ, Cerami A (1981) Maillard reactions involving proteins and carbohydrates in vivo: relevance to diabetes mellitus and aging. Prog Food Nutr Sci 5:315–327

Mori J, Kondo J, Ihara Y (1987) Ubiquitin is a component of paired helical filaments in Alzheimer's disease. Science 235:1641–1644

Nibbering PH, Leijh PCJ, Van Furth R (1985) Quantitation of monoclonal antibody binding to individual cells by cytophotometry. In: Bullock GR, Petrusz P (eds) Techniques in immunocytochemistry, vol 3. Academic Press, Orlando, pp 97–113

Nukina N, Ihara Y (1986) One of the antigenic determinants of paired helical filaments is related to TAU protein. J Biochem 99:1541–1544

Selkoe DJ (1987) Deciphering Alzheimer's disease: the pace quickens. Trends Neurosci 10:181–184

Selkoe DJ, Ihara Y, Salazar F (1984) Alzheimer's disease: insolubility of purified paired helical filaments in sodium dodecyl sulfate and urea. Science 215:1243–1245

Terry RD, Katzman R (1983) Senile dementia of the Alzheimer's type. Ann Neurol 14:497–506

Tomlinson BE, Corsellis JAN (1975) Ageing and the dementias. In: Adams JH, Corsellis JAN, Duchen LW (eds) Greenfield's neuropathology. Wiley, New York, pp 951–1025

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Wang E, Fischman D, Liem RKH (1985) Intermediate filaments. Intermediate filament associated proteins. Ann NY Acad Sci 455:32–56

Wisniewski HM, Czosnek H, Mack K, Iqbal K, Wisniewski K (1981) Cytoskeletal protein degenerative disorders of axons. Transactions of the American Society for Neurochemistry Program. 12 Annual Meeting Richmond, VA, vol 12, 134 pp. Abstract no 330

Wisniewski HM, Merz PA, Iqbal K (1984) Ultrastructure of paired helical filaments of Alzheimer's neurofibrillary tangle. J Neuropathol Exp Neurol 6:643–656

Wood JG, Mirra SS, Pollock NJ, Binder LI (1986) Neurofibrillary tangles of Alzheimer's disease share antigenic determinants with the axonal microtubule-associated protein TAU (tau). Proc Natl Acad Sci USA 83:4040–4043

Yen SH, Dickson DW, Crowe A, Butler M, Shelanski ML (1987) Alzheimer's neurofibrillary tangles contain unique epitopes and epitopes in common with the heat-stable microtubule associated proteins TAU and MAP2. Am J Pathol 1:81–91