

## Two-Dimensional Characterization of Paired Helical Filament-Tau from Alzheimer's Disease: Demonstration of an Additional 74-kDa Component and Age-Related Biochemical Modifications

N. Sergeant, J.-P. David, \*M. Goedert, \*R. Jakes, P. Vermersch,  
L. Buée, D. Lefranc, A. Watzet, and A. Delacourte

*INSERM Unité 422, Lille, France; and \*MRC Laboratory of Molecular Biology, Cambridge, England*

**Abstract:** PHF-tau proteins are the major components of the paired helical filament (PHF) from Alzheimer's disease (AD) neurofibrillary lesions. They differ both qualitatively and quantitatively in their degree of phosphorylation when compared with native tau proteins. However, little is known about the extent and heterogeneity of phosphorylated sites or the isoform composition and the isoelectric variants of PHF-tau. Therefore, we have characterized PHF-tau proteins from cortical brain tissue homogenates of 13 AD patients using two-dimensional gel electrophoresis. Whatever the topographical origin of brain tissue homogenates, PHF-tau proteins shared the same two-dimensional gel electrophoresis profile made of a tau triplet of 55, 64, and 69 kDa. A 74-kDa hyperphosphorylated tau component was detected particularly in the youngest and most severely affected AD patients. This additional component of hyperphosphorylated tau was shown to correspond to the longest brain tau isoform. Furthermore, the isoelectric points of PHF-tau from older AD patients were significantly more basic, indicating a lower degree of phosphorylation. These results show that the severity of neurofibrillary degeneration of AD is modulated by age. **Key Words:** Alzheimer's disease—Aging—Tau proteins—Antibody—Two-dimensional gel electrophoresis—Western blot.  
*J. Neurochem.* **69**, 834–844 (1997).

Alzheimer's disease (AD) is a neurodegenerative disease characterized by severe neuronal loss that progressively leads to dementia. The cerebral cortex in AD shows numerous histopathological features consisting of senile plaques and neurofibrillary tangles (NFTs) (McKhann et al., 1984; Khachaturian, 1985). SPs are extracellular amyloid deposits mainly made of amyloid  $\beta$  peptide ( $A\beta$ ) (Glennner and Wong, 1984), whereas NFTs are intraneuronal fibrous components ultrastructurally formed by the paired helical filament (PHF) (Kidd, 1963). Senile plaques are found

throughout the cerebral cortex in AD (Braak and Braak, 1991). Conversely, NFTs are found predominantly in the hippocampal formation and in association cortical areas (Arnold et al., 1991; Hof et al., 1992). The progression of dementia in AD is related to the number of NFTs and their regional distribution in cortical areas (Bierer et al., 1995), making them of particular interest.

Microtubule-associated protein tau is the major component of the PHF (Brion et al., 1985; Delacourte and Défossez, 1986; Grundke-Iqbal et al., 1986; Ihara et al., 1986; Yen et al., 1987; Goedert et al., 1988; Kondo et al., 1988; Wischik et al., 1988; Lee et al., 1991). Tau is expressed in the central nervous system and is composed of six isoforms in adult human brain. The six tau isoforms are produced by alternative mRNA splicing from a single gene transcript and differ by the presence or absence of three inserts encoded by exons 2, 3, and 10 (Goedert et al., 1989, 1992). They differ by the presence or absence of 29 or 58 amino-acid inserts near the amino-terminus (exons 2 and 3) and contain three or four (exon 10) highly conserved microtubule-binding repeats in the carboxy-terminal half. In vitro studies have shown that normal tau proteins promote the assembly of tubulin and stabilize microtubules (Cleveland et al., 1977; Goode and Feinstein, 1994). This assembly is reduced by phos-

---

Received January 3, 1997; revised manuscript received April 2, 1997; accepted April 2, 1997.

Address correspondence and reprint requests to Dr. A. Delacourte at INSERM Unité 422, 1, place de Verdun, 59045 Lille, France.

**Abbreviations used:** 1-D, one-dimensional gel electrophoresis; 2-D, two-dimensional gel electrophoresis;  $A\beta$ , amyloid  $\beta$  peptide; AD, Alzheimer's disease; NEPHGE, nonequilibrium pH gradient gel electrophoresis; NFT, neurofibrillary tangle; OD, optical density; PAGE, polyacrylamide gel electrophoresis; PHF, paired helical filament; PHF-tau, abnormally hyperphosphorylated tau proteins aggregated into PHF;  $R_s$ , Spearman's rank correlation coefficient; SDS, sodium dodecyl sulfate.

phorylation (Brandt et al., 1994), which is also the most important posttranslational modification of tau proteins (Butler and Shelanski, 1986). A large number of protein kinases can phosphorylate tau proteins in vitro (Steiner et al., 1990; Drewes et al., 1992; Hanger et al., 1992; Lew and Wang, 1995), but little is known about protein kinases phosphorylating tau in vivo (Lovestone et al., 1994; Sperber et al., 1995).

In contrast to tau from normal brain, PHF-tau is glycosylated (Ledesma et al., 1994) and ubiquitinated (Morishima and Ihara, 1994), but its most striking modification is the high level of phosphorylation (Matsuo et al., 1994; Sergeant et al., 1995; Hasegawa et al., 1996). The phosphorylation of PHF-tau has been studied using phospho-dependent anti-tau antibodies (Lee et al., 1991; Greenberg et al., 1992; Mercken et al., 1992; Seubert et al., 1995; Buée-Scherrer et al., 1996a; Hasegawa et al., 1996) and mass spectrometry (Morishima-Kawashima et al., 1995). PHF-tau shows distinct biochemical and physiological properties when compared with normal brain tau. PHF-tau proteins have a lower electrophoretic mobility and run as three major bands of 55, 64, and 69 kDa, referred to as pathological tau proteins (Delacourte et al., 1990) or A68 (Ksiezak-Reding et al., 1990; Lee et al., 1991). The three PHF-tau bands are composed of all six tau isoforms (Goedert et al., 1992), which are unable to bind to microtubules (Bramblett et al., 1993; Yoshida and Ihara, 1993). Phosphorylation of tau proteins may be an important event in the transformation of normal tau into PHF-tau, suggesting a dysregulation in the balance of protein kinase/protein phosphatase activities in the AD brain.

The biochemical analysis of PHF-tau was performed using purified PHF-tau preparations (Greenberg and Davies, 1990; Lee et al., 1991) and was largely qualitative. Conversely, when using whole brain tissue homogenates, variable PHF-tau immunoreactivities are found in the AD brain (Flament et al., 1990; Vermersch et al., 1992). This approach has also been used for quantifying pathological tau proteins from other neurodegenerative diseases (Buée-Scherrer et al., 1995, 1996b; Delacourte et al., 1996; Vermersch et al., 1996). Differences in PHF-tau may be directly related either to its quantity or to a variation in its phosphorylation state. In the present study, the degree of phosphorylation of PHF-tau from brain tissue homogenates of AD patients was analyzed by a two-dimensional western blot approach using phospho-dependent and phospho-independent anti-tau antibodies. Two-dimensional profiles of PHF-tau were analyzed in different brain regions, a 74-kDa PHF-tau band was characterized, and age was shown to be a factor for the heterogeneity of PHF-tau. Moreover, a novel antibody (tau-E10) was produced that is the first antibody that distinguishes between tau isoforms with three and four microtubule-binding repeats.

## MATERIALS AND METHODS

### Patients

The brains of 13 AD patients (A.1–A.13) with a mean age of  $76 \pm 14.3$  years were chosen for the present study (Table 1). All patients met the NINCDS–ADRDA criteria for diagnosis of definite AD (McKhann et al., 1984). The brains were obtained from the Neurology Department of Lille Regional Hospital and from the Geriatrics Department of Limeil Brevannes Hospital, in accordance with the French Caillavet Law no. 76-1181 (December 22, 1976) and the local ethics committee (October 19, 1994). Postmortem delays ranged from 5 to 93 h (mean delay: 23 h). Besides the clinical and neuropathological data, the 13 AD brains were used for multiple biochemical analyses (Table 1), including the systematic mapping of neurofibrillary degeneration (Vermersch et al., 1992) and the quantification of insoluble A $\beta$  by dot-blot immunoassay (Permanne et al., 1995). All brains showed a large neurofibrillary involvement in cerebral cortex, associated with various amounts of insoluble A $\beta$  (Table 1). For the present study, brain tissue samples were dissected from Brodmann area 10 (frontal lobe), Brodmann area 22 (temporal lobe), and hippocampus (Table 1), homogenized in 5% (wt/vol) sodium dodecyl sulfate (SDS)/Laemmli sample buffer (1:10, wt/vol), and heat-treated (Laemmli, 1970). All samples were freshly prepared, protein amounts were determined using the BCA assay protein reagent (Pierce) in microtiter plates, and they were stored at  $-80^{\circ}\text{C}$  until use.

### Immunological probes

AD2 is a protein A-purified monoclonal antibody raised against a crude PHF preparation obtained from AD brain. It recognizes NFTs by light microscopy (Buée-Scherrer et al., 1995), PHFs by electron microscopy (Reig et al., 1995), and PHF-tau on immunoblots (Buée-Scherrer et al., 1996a). It is phospho-dependent and specifically recognizes phosphorylated serines 396 and 404 (numbering according to the longest human brain tau isoform) in the carboxy-terminal part of tau (Buée-Scherrer et al., 1996a). AD2 was used at 0.2  $\mu\text{g}/\text{ml}$  in Tris-buffered saline containing 0.05% (wt/vol) Tween-20.

M19G is a polyclonal antibody raised against a synthetic peptide corresponding to the first 19 amino acids of tau (Buée-Scherrer et al., 1996a,b). Polyclonal antibodies 189 and 304 are isoform-specific. Polyclonal antibody 189 was raised against the amino-acid sequence encoded by exon 3 and only recognizes the two longest tau isoforms. Polyclonal antibody 304 was raised against the 29 amino-acid insert (exon 2) that is found in four of the six human brain tau isoforms (Goedert et al., 1992). These phospho-independent polyclonal antibodies recognize both normal tau and PHF-tau. M19G, 189, and 304 were used in Tris-buffered saline with 0.05% (wt/vol) Tween-20 at a final dilution of 1:5,000, 1:400, and 1:1,000 (vol/vol), respectively. A novel polyclonal antibody developed in our laboratory was also used. It was obtained by immunizing a rabbit with a synthetic peptide (Neosystem, France) corresponding to the first 10 amino acids encoded by exon 10 of tau proteins. The specificity of this antibody (called tau-E10) for tau isoforms with four microtubule-binding repeats was established using recombinant tau isoforms (see Results), using the same approach as for antibodies 189 and 304 (Goedert et al., 1992).

TABLE 1. Summary of characteristics of AD patients

AD patients	Sex	Age (years)	Brain tissue homogenates analyzed	PHF-tau cortical mapping <sup>a</sup>	Insoluble A $\beta$ peptide <sup>b</sup>
A.1	F	37	Tp	++	+++
A.2	M	64	Hip, Tp	++	+
A.3	M	64	Fr, Tp	++	+++
A.4	F	65	Tp	+, BA4	+++
A.5	F	70	Fr	++	++
A.6	M	79	Fr	++	+++
A.7	M	79	Tp	+, BA17	++++
A.8	F	80	Tp	++	++++
A.9	F	83	Hip, Tp	+, BA17/BA18	+++
A.10	F	88	Tp	+, BA17	+
A.11	F	90	Fr <sup>c</sup>	++	+
A.12	F	90	Fr	++	+++
A.13	F	90	Hip, Tp	++	+

Hip, hippocampus; Fr, frontal lobe; Tp, temporal lobe; BA, Brodmann area.

<sup>a</sup> Semiquantitative analysis of PHF-tau immunolabeled by AD2 and neurofibrillary degeneration mapping, performed as described by Vermersch et al. (1992). The ++ sign indicates that all cortical areas of an AD patient are affected by neurofibrillary degeneration, whereas the + sign and adjacent Brodmann areas indicate the nonaffected cortical areas.

<sup>b</sup> Total amount of A $\beta$  (Permanne et al., 1995) is given in arbitrary units (+ = 0–50 nmol/g; ++ = 50–100 nmol/g; +++ = 100–200 nmol/g; ++++ > 200 nmol/g of brain tissue).

<sup>c</sup> Two brain tissue samples from AD patient A.11 were dissected from the frontal lobe (Brodmann area 9) and from the medial frontal gyrus (Brodmann area 10).

### One-(1-D) and two-dimensional gel electrophoresis (2-D)

For 1-D slab gels, 90  $\mu$ g of proteins from each AD brain homogenate was loaded onto 15 wells of 10–20% SDS-polyacrylamide electrophoresis gels (PAGE), according to Laemmli (1970). 2-D gels were performed according to modification of the method of O'Farrell (1975). Isoelectric focusing gels contained 4% (wt/vol) acrylamide and 2.5% (wt/vol) bis-acrylamide as the cross-linker, 9.5 mol/L urea, 2% (vol/vol) Triton X-100, 4% (vol/vol) pH 3–10 Pharmalytes, and 1% (vol/vol) pH 4–6.5 Pharmalytes (Pharmacia). Total brain tissue homogenates were heat-treated (100°C for 5 min) and centrifuged (10,000 *g* for 10 min), and 130  $\mu$ g of brain tissue protein was used. The supernatant was added to 1 volume of a solution containing 8 mol/L urea and 4% (vol/vol) Triton X-100. Isoelectric focusing was performed in 12-cm-long and 3-mm-i.d. capillary tubes and run with a total of 10,500 V-h. Isoelectric focusing capillary gels were then extruded and equilibrated for 5 min in an equilibration buffer containing 3% (wt/vol) SDS, 0.25 mol/L Tris-HCl (pH 6.8), and 0.5% (wt/vol) dithiothreitol. The second dimension was performed as for 1-D slab gels, on 10–20% gradient SDS-PAGE. The 2-D pH gradient was calibrated using the Carbamylite calibration kit (Pharmacia). Carbamylated creatine phosphokinase was always loaded with each brain sample homogenate, in accordance with the manufacturer's instructions.

### Western blot analysis

Proteins resolved by 1-D and 2-D gels were transferred onto nitrocellulose membranes (0.45- $\mu$ m pore size, Schleicher and Schuell) for 90 min (current: 0.8 mA/cm<sup>2</sup>) using an LKB Multiphor II Nova Blot (Pharmacia). Proteins were stained with Ponceau Red (2 mg/ml) to check the

quality of resolution for the 1-D and 2-D and to visualize the carbamylated creatine phosphokinase. Membranes were scanned or photographed, and isoelectric points (pI) were calculated based on the theoretical pI given by the manufacturer for the carbamylated creatine phosphokinase (MW = 45,000; pI = 4.9–7.1). Blocking was carried out with Tris-buffered saline containing 5% (wt/vol) dry milk and 0.05% (wt/vol) Tween-20. Monoclonal and polyclonal antibodies were revealed with horseradish peroxidase-labeled sheep anti-mouse and anti-rabbit immunoglobulins (Sigma Immunochemicals), respectively. These antibodies are adsorbed with human serum proteins and do not cross-react with human brain proteins (data not shown). All were detected by chemiluminescence with the ECL western blotting system (Amersham).

### Quantitative and statistical analyses

For each AD brain tissue sample, AD2 immunoblots from 1-D gels were digitized on a Prolinea Compaq PC engine with a film scan unit Sharp JX-325 and assessed for densitometric quantitative analyses. The scanner was calibrated for optical densities (OD) using the photographic calibration step tablet no. 3 (Kodak) with the ImageMaster V1.2 1-D software (Pharmacia). The resulting images of AD2 immunoblots were processed with the ImageMaster V1.2 1-D software. The quantification of the PHF-tau components (tau 55, 64, and 69) was performed using a preestablished calibration curve, and data were collected in the linear range of OD. The curve was calibrated using increasing Laemmli sample volumes (e.g., 1–20  $\mu$ l) from the temporal brain region of a well characterized AD patient. Statistical assessments were performed by calculating the Spearman's rank correlation coefficient ( $R_s$ ) and the mean differences by a Student's *t* test.

## RESULTS

## PHF-tau 1-D analysis

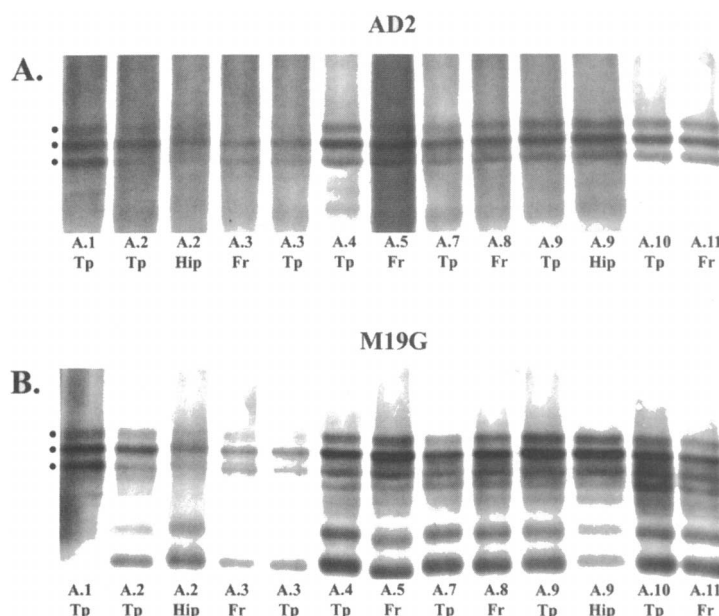
A semiquantitative analysis of PHF-tau within multiple brain areas (Table 1) was performed previously (Vermersch et al., 1992) and enabled us to select a sample of AD brain with an overall neurofibrillary cortical involvement. 1-D western blot analysis with monoclonal antibody AD2 showed the typical PHF-tau triplet in all brain homogenates (Fig. 1A). The PHF-tau bands consist of the 55-, 64-, and 69-kDa components (Delacourte et al., 1990). A large smear along each migrating track was always associated with the PHF-tau triplet (Fig. 1A). The AD2 immunoreactivity of the smears and the PHF-tau components were of different intensities, according to the samples studied. Thus, the PHF-tau triplet appeared either with a faintly detected smear (Fig. 1A, A.10 Tp) or almost completely masked by the smear (Fig. 1A, A.3 Tp).

After AD2 immunolabeling, the polyclonal antibody M19G was incubated with the same nitrocellulose replicas (Fig. 1B). The PHF-tau triplet was also observed. In contrast to the AD2 immunolabeling, the smear was only faintly detected by antibody M19G and low-molecular-weight amino-terminal catabolic tau protein products were visible. We performed a semiquantitative analysis of the AD2 signal for each PHF-tau component, without subtracting the background. This background corresponds to the AD2-immunolabeled smear, composed of partially aggregated amino-truncated PHF-tau (Morishima and Ihara, 1994; Buée-Scherrer et al., 1996a). Conversely, the smear extending toward lower molecular weight than the PHF-tau components may correspond to amino-terminal truncated phosphor-

ylated tau proteins that are partially solubilized from PHF in Laemmli sample buffer after heat treatment. It was also detected by carboxy-terminal anti-tau antibodies, like BR134 and -135 (Goedert et al., 1992) or S28T (Buée-Scherrer et al., 1996a) (data not shown), but not with M19G (Fig. 1B). The smears are totally absent from autopsy-derived brain tissue homogenates from controls (Buée-Scherrer et al., 1996a) and from rapidly processed biopsy-derived control brain tissue homogenates (Sergeant et al., 1995). The OD values of the various PHF-tau components are given as the means  $\pm$  SD in Table 2. PHF-tau components have been compared between each brain region studied. No significant differences of OD were observed in our population (Mann-Whitney *U* test,  $p > 0.1$ ). Therefore, the mean  $\pm$  SD was calculated for all the samples studied, independently of the topographical origin of the brain tissues. The 64-kDa component was by far the most intensely labeled PHF-tau component (Table 2, OD lane), followed by the 55- and 69-kDa PHF-tau components.

## AD PHF-tau 2-D analysis

AD brain tissue proteins were separated by isoelectrofocusing in the first dimension and SDS-PAGE in the second. 2-D blots were immunolabeled first with monoclonal antibody AD2, followed by polyclonal antibody M19G. PHF-tau proteins were specifically detected by AD2 (Fig. 2A-C), whereas both PHF-tau and normal tau proteins were detected by M19G (Fig. 2A'). Yet normal tau proteins were easily distinguishable from PHF-tau proteins, because they were resolved toward a more basic position and also showed a lower molecular weight (Fig. 2A and A'). A vertical



**FIG. 1.** 1-D western blot analysis of PHF-tau proteins from AD brain tissue homogenates. AD cortical brain tissue proteins, homogenized from the frontal (Fr), temporal (Tp), or hippocampal (Hip) gray matter are resolved by 10–20% SDS-PAGE. The same nitrocellulose replica was used for AD2 immunolabeling (A), stripped, and followed by M19G immunolabeling (B). A: AD2 immunostained the PHF-tau triplet (tau 69, tau 64, and tau 55; marked by dots) in each brain tissue homogenate from AD cases (A.1–A.11). Note that the PHF-tau triplet is always detected with a background smear. B: M19G immunolabeling of the same brain tissue homogenates also reveals the PHF-tau triplet. Note that the background smear is almost absent.

**TABLE 2.** Quantitative (OD) and qualitative (acidic pI) values of PHF-tau proteins determined by 1-D and 2-D immunoblot analyses

PHF-tau components	n	OD	n	AD2 acidic pI	M19G acidic pI
Tau 74	—	—	12	6.18 ± 0.27	6.15 ± 0.26 <sup>a</sup>
Tau 69	18	0.510 ± 0.444	18	6.06 ± 0.26	5.96 ± 0.21 <sup>b</sup>
Tau 64	18	0.772 ± 0.557	18	5.83 ± 0.17	5.76 ± 0.16 <sup>c</sup>
Tau 55	18	0.613 ± 0.539	18	5.87 ± 0.21	5.81 ± 0.18 <sup>d</sup>

OD and acidic pI values of PHF-tau components (tau 69, 64, and 55) are expressed as the means ± SD, calculated for the whole AD brain tissue homogenates studied. Note that 74-kDa OD is not reported, because this component is almost indistinguishable from the AD2 smears, whereas it was detected easily on 2-D immunoblots (tau 74, acidic pI). Acidic pI values of PHF-tau proteins determined after M19G immunolabeling (M19G acidic pI column) are also indicated.

The Student's *t* test shows significant higher acidity of PHF-tau isoelectric variants when detected by M19G (<sup>b</sup>*p* < 0.009; <sup>c</sup>*p* < 0.004; <sup>d</sup>*p* < 0.01). Conversely, no significant difference is observed for the 74-kDa component (<sup>a</sup>*p* = 0.107).

smear immunostained by AD2 was located at the basic part of PHF-tau. A large number of low-molecular-weight catabolic tau products were detected by M19G over the whole pH gradient. Most of them, especially the more acidic ones, were not detected by AD2 (Fig. 2A'). They correspond to the amino-terminal truncated catabolic products of tau proteins. Additional PHF-tau isoelectric variants were immunostained by antibody M19G. These were significantly more acidic (Student's *t* test: tau 69, *p* < 0.009; tau 64, *p* < 0.004; tau 55, *p* < 0.01) than the PHF-tau components detected by AD2.

2-D profiles of PHF-tau from hippocampus and from frontal and temporal cortex tissue homogenates were similar (Fig. 2A–C), without any additional isoelectric tau variants in relation to one of the brain regions studied. PHF-tau components (tau 55, 64, and 69) showed distinct acidic pI values (Table 2). Tau 64 was the most acidic component, followed by tau 55 and tau 69.

Tau 64 was the largest AD2-immunoreactive component on 1-D immunoblots and was also the most acidic component (Table 2) by 2-D analysis. A comparison of OD values with matched acidic pI values (Fig. 3) shows that AD2-immunoreactive PHF-tau proteins were more intensively stained, as they were more acidic (Fig. 3A).

In aged AD patients, PHF-tau proteins were more basic than in young cases (Fig. 3B). 1-D analysis indicated a slight decrease of tau 55 immunoreactivity with aging ( $R_s = -0.55$ , *p* < 0.05). Tau 64 and 69 variations showed the same tendencies, but were not significantly correlated with aging (tau 69:  $R_s = -0.454$ , *p* = 0.061; tau 64:  $R_s = -0.417$ , *p* = 0.085). It is interesting that, using the global score of PHF-tau (tau 69 + tau 64 + tau 55), decreased immunoreactivity was significantly correlated with aging (PHF-tau:  $R_s = -0.528$ , *p* < 0.03). In contrast, 2-D analysis showed a basic shift of whole PHF-tau components with aging (Fig. 3B). This basic shift of PHF-tau was confirmed

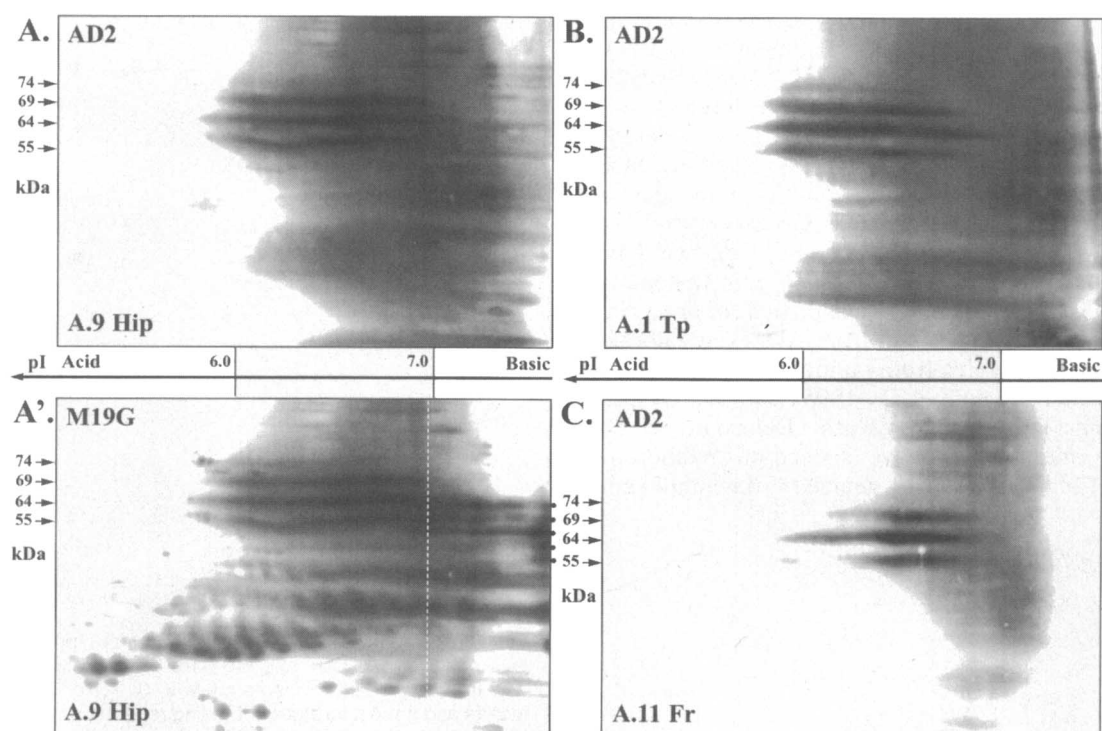
further for the 55- and 69-kDa PHF-tau components immunostained by polyclonal antibody M19G.

#### Characterization of antibody tau-E10

Each of the six recombinant tau isoforms was loaded on SDS-PAGE and immunoblotted either with antiserum BR134 (Goedert et al., 1992) or with antiserum tau-E10. As shown in Fig. 4A, BR134 recognized all six tau isoforms. Conversely, tau-E10 recognized the recombinant tau isoforms with four microtubule-binding repeats, but failed to stain tau isoforms with three microtubule-binding repeats (Fig. 4B).

#### A 74-kDa tau component in AD

Besides the typical PHF-tau triplet, a 74-kDa AD2-immunoreactive component was also detected (Fig. 2A and B). The 74-kDa spot was absent from brain tissue of AD case A.11 (Fig. 2C), as in three other AD patients (A.13, A.6, and A.7). As for PHF-tau proteins, this higher-molecular-weight component was less acidic in older AD patients (Fig. 3B). It was resolved as a large spot with the same pI ranges as for the PHF-tau triplet (Table 2). The only difference for the 74-kDa was a reduced acidity that correlated significantly with postmortem delays ( $R_s = 0.917$ , *p* < 0.03). The 74-kDa component was labeled by the polyclonal antibody M19G (Fig. 2A'). Additional analysis of the 74 kDa component was performed with antibodies 304, 189, and tau E-10 (Fig. 5) specific for exons 2, 3, and 10 of tau (see Materials and Methods). The 74-kDa band was immunostained by antibodies 304 and 189, as well as by tau-E10 (Fig. 5). Of the PHF-tau components, tau 69 was immunostained by antibodies 304, 189, and tau-E10. Tau 64 was detected by 304 and Tau-E10, whereas tau 55 was not detected by antibodies 304, 189, or tau-E10 (Fig. 5, lanes 304, 189, and tau-E10). Antibody 304 stained a band at 55 kDa that was shown to correspond to a normal tau protein by 2-D analysis (data not shown).



**FIG. 2.** Comparative 2-D profiles of PHF-tau proteins from the frontal (Fr), temporal (Tp), or hippocampal (Hip) brain tissue homogenates of three AD cases. **A, B,** and **C:** AD2 immunoblot analysis of PHF-tau proteins from the hippocampus (A) and the temporal (B) and frontal (C) gray matter (AD cases A.9, A.1, and A.11). PHF-tau proteins are specifically immunolabeled by the monoclonal antibody AD2. The PHF-tau triplet made of the 55-, 64-, and 69-kDa components (marked by arrows) shows similar isovariant distribution among each of the three brain regions analyzed. Note that the 74-kDa component shifts toward the vertical AD2-immunoreactive smears (A and B) and is absent in patient A.11 (C). **A':** M19G immunoblot analysis of PHF-tau proteins (indicated by arrows) and normal tau proteins (indicated by dots) from the hippocampus brain tissue homogenate of AD case A.9. The same nitrocellulose replica as in A was used for M19G immunolabeling. The vertical dotted line represents the limit between the basic origin of AD2-immunoreactive PHF-tau components and normal basic tau proteins. Note the numerous low-molecular-weight tau catabolic products that cover the whole pH gradient. The 74-kDa band is clearly visible, because the vertical smears are less immunoreactive with M19G. For each of the 2-D immunoblots, the isoelectrofocusing orientation is indicated by the horizontal arrows, and the basic origin and the acidic ending, as well as the isoelectric points 6.0 and 7.0, are indicated.

## DISCUSSION

In the present study, we used the well characterized monoclonal antibody AD2 (Buée-Scherrer et al., 1995, 1996a,b; Sergeant et al., 1995; Delacourte et al., 1996; Vermersch et al., 1996) and a panel of tau antisera, including a novel antibody (called tau-E10) specifically recognizing tau isoforms with four microtubule-binding repeats, to analyze AD PHF-tau proteins using a combined 1-D and 2-D western blot approach. Five conclusions regarding AD PHF-tau proteins can be given here: (a) 2-D analysis is a useful technique for studying PHF-tau proteins from brain tissue homogenates; (b) similar 2-D profiles of PHF-tau proteins are present in AD brain tissue homogenates from at least three different cortical regions; (c) AD PHF-tau proteins are heterogeneously phosphorylated; (d) a hyperphosphorylated tau component of 74 kDa is observed particularly in young AD patients with a severe and global neurofibrillary degeneration cortical involve-

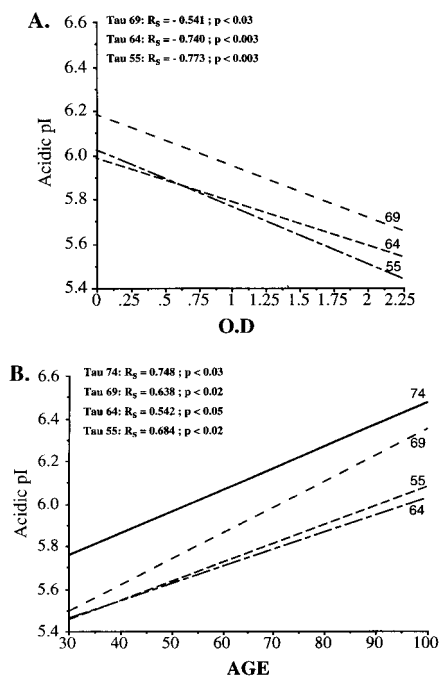
ment; and (e) the severity of neurofibrillary degeneration of AD is modulated by age.

### 2-D analysis: all SDS-soluble PHF-tau can be analyzed

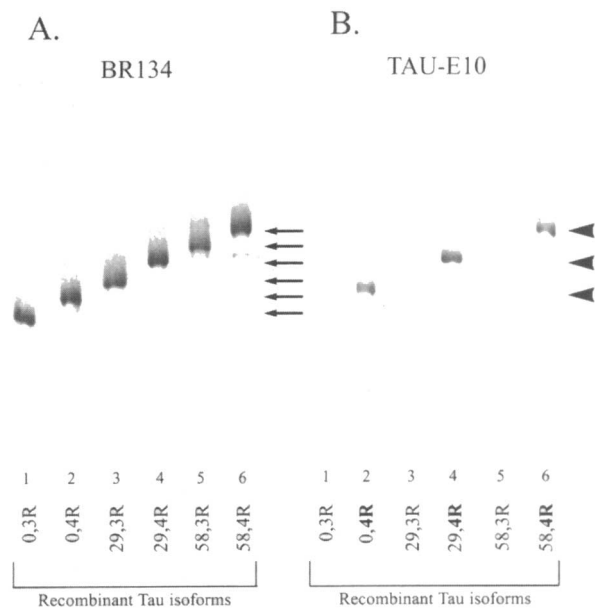
2-D analysis has already been used for the study of both normal tau and PHF-tau. Nonequilibrium pH gradient gel electrophoresis (NEPHGE) (O'Farrell et al., 1977) was used preferentially to analyze the phosphorylation state of tau proteins (Butler and Shelanski, 1986; Litsky and Johnson, 1992). When tau proteins were shown to be the major subunits of the PHF, hyperphosphorylation of AD PHF-tau was also shown to enhance the acidity of tau proteins and to reduce the number of isoelectric variants of PHF-tau by NEPHGE (Greenberg and Davies, 1990; Ksiezak-Reding et al., 1990).

In fact, the most inconvenient characteristic of PHF-tau is its insolubility in nondenaturing solutions (Kondo et al., 1988). Almost all aggregated PHF-tau

proteins can be solubilized using high concentrations of SDS, sonication, and/or heat treatment (Iqbal et al., 1984; Brion et al., 1993; Wang et al., 1995). Because SDS is an anionic detergent, it must be almost totally excluded when using the NEPHGE method (O'Farrell, 1977; Klose and Kobalz, 1995). As a consequence, NEPHGE only allows the most soluble components of purified PHF-tau to be analyzed (Greenberg and Davies, 1990). To overcome this problem, we have adapted the 2-D method (O'Farrell, 1975). In contrast to NEPHGE, 2-D is a powerful approach for analyzing precisely the whole PHF-tau from AD, as already described for tau proteins from rapidly processed human brain tissues (Sergeant et al., 1995) and pathological tau proteins from Pick's disease (Delacourte et al., 1996). Furthermore, we have adapted this method for the use of brain tissue homogenized in Laemmli sam-



**FIG. 3.** Statistical analysis of PHF-tau protein biochemical characteristics and age-related isoelectric variations. **A:** A nonparametric Spearman's correlation test was used to compare the OD values of PHF-tau components and the matched acidic pI values. The Spearman's test shows a significant negative correlation (tau 69:  $R_s = -0.541$ ,  $p < 0.03$ ; tau 64:  $R_s = -0.740$ ,  $p < 0.003$ ; tau 55:  $R_s = -0.773$ ,  $p < 0.003$ ) between the enhanced acidity and the increasing immunoreactivity of PHF-tau proteins detected by AD2. **B:** The ages of death of AD patients are compared with the acidic corresponding pI values of each neocortical PHF-tau component, including the 74-kDa component. The Spearman's correlation test shows a positive correlation (tau 69:  $R_s = 0.638$ ,  $p < 0.02$ ; tau 64:  $R_s = 0.542$ ,  $p < 0.05$ ; tau 55:  $R_s = 0.684$ ,  $p < 0.02$ ) between a decreased acidity of the PHF-tau triplet and increasing age. Note that the Spearman's correlation value of the 69-kDa component differs from those of the 64- and 55-kDa components. The 74-kDa component is also subject to a decreased acidity with increasing age (74-kDa polypeptide:  $R_s = 0.748$ ,  $p < 0.03$ ).

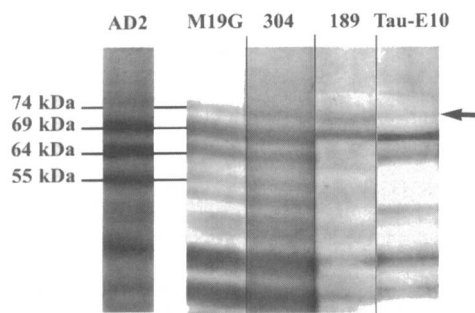


**FIG. 4.** Immunolabeling of recombinant tau protein isoforms with antibodies BR134 and tau-E10. On migrating tracks 1, 3, and 5, recombinant tau isoforms with 0, 29, or 58 amino-acid inserts and three microtubule-binding repeats (referred to as 3R) were loaded. Tau isoforms with four microtubule-binding repeats (referred to as 4R) and 0, 29, or 58 amino-acid inserts were loaded on migrating tracks 2, 4, and 6. **A:** BR134 immunolabels all the recombinant tau isoforms, indicated by arrows. **B:** Tau-E10 strongly stains the recombinant tau isoforms with four microtubule-binding repeats (4R; indicated by arrowheads) and fails to detect recombinant tau isoforms with three microtubule-binding repeats (3R).

ple buffer. We show that high SDS concentrations do not affect the resolving power or the reproducibility of 2-D method (Garrels, 1979) and do not interfere with the immunoreactivity. Working on brain tissue homogenates is also more convenient. It limits the steps of purification that are necessary to prepare PHF-tau proteins and the artefactual changes that can be introduced by these methods (e.g., dephosphorylation and proteolysis). To the best of our knowledge, this article shows for the first time 2-D analyses of PHF-tau proteins from AD brain tissue homogenates.

## 2-D analysis of PHF-tau in AD

Monoclonal antibody AD2 is a highly specific and sensitive immunological probe for PHF-tau from autopsy-derived AD brain tissue homogenates (Buée-Scherrer et al., 1996a). It was used for detecting PHF-tau on 2-D western blots. Each AD2-immunoreactive PHF-tau component was separated as multiple isoelectric variants that were resolved as large spots. This characteristic of PHF-tau was already observed using NEPHGE (Ksiezak-Reding et al., 1988, 1990) and was argued to be the result of hyperphosphorylation (Ksiezak-Reding et al., 1990). Our results are in good agreement with these observations. The fact that PHF-tau



**FIG. 5.** Immunoblots of PHF-tau proteins with monoclonal antibody AD2, polyclonal antibody M19G, and isoform-specific anti-tau antibodies 304, 189, and tau-E10. Fifteen microliters of brain tissue homogenate from the temporal gray matter of the AD case A.4 was resolved on 10–20% gradient SDS-PAGE. AD2 immunolabeling indicates precisely the PHF-tau components (tau 55, 64, and 69). The 74-kDa component is also visualized after a longer exposure. M19G immunolabeling shows the whole tau-immunoreactive polypeptides, including the 74-kDa component. Note that 304 immunostaining is very similar to that obtained with M19G. Conversely, antiserum 189 stains the 69-kDa PHF-tau component and the 74-kDa component (marked by arrow). The upper band stained by antiserum 189 is considered to be nonspecific to tau proteins, because it is not detected by the other tau antibodies. The last lane shows the 1-D profile of PHF-tau components immunostained by antibody tau-E10. The 69-kDa PHF-tau component is the most intensively detected component, followed by the 64-kDa and 74-kDa components.

proteins are not resolved as individual spots might be specific to these proteins, because other proteins in the same range of pI were resolved as spots, including catabolic products of tau. In addition, each PHF-tau band is composed of at least one tau protein isoform (Goedert et al., 1992; Mulot et al., 1994). Altogether, it is therefore not surprising that PHF-tau components are resolved as large spots rather than as multiple isolated spots. PHF-tau from frontal and temporal cerebral cortex or hippocampus tissue homogenates was shown to possess a similar 2-D profile. No specific isoelectric PHF-tau variants and no typical isoelectric modifications (e.g., enhanced acidity of a given PHF-tau component or a specific catabolic product) were observed.

#### PHF-tau proteins are heterogeneously phosphorylated

Many protein kinases can phosphorylate tau. They can be divided into proline-directed protein kinases specific for seryl-proline and threonyl-proline motifs and nonproline-directed protein kinases that phosphorylate tau proteins at serine or threonine residues (for review, see Goedert et al., 1994). In AD, PHF-tau phosphorylation is considered to result from a dysregulation of protein kinase and/or protein phosphatase activities (for reviews, see Iqbal et al., 1994; Trojanowski and Lee, 1995). Herein, using the monoclonal antibody AD2, the phosphorylated serine residues 396 and 404 (Buée-Scherrer et al., 1996a) were specifically analyzed. Using 1-D analysis, all PHF-tau pro-

teins were detected by AD2. In contrast, by 2-D analysis, AD2 PHF-tau profiles did not coincide completely with the profiles obtained after M19G immunolabeling. Furthermore, the PHF-tau proteins detected by M19G were shown to be significantly more acidic. These results support the view that in AD PHF-tau is heterogeneously phosphorylated.

#### A fourth 74-kDa tau component involved in neurofibrillary degeneration

Besides the typical PHF-tau triplet, a component with an apparent molecular mass of 74 kDa was labeled by monoclonal antibody AD2 and polyclonal antibody M19G in nine of the 13 AD brains analyzed. This component shares many biochemical and immunological common properties with PHF-tau (e.g., slow electrophoretic migration, resolution as a large spot, range of pI). The 74-kDa component was immunolabeled by all anti-tau antibodies, including the isoform-specific antibodies 189, 304, and tau-E10. The 74-kDa component corresponds to the longest human brain tau isoform, is hyperphosphorylated, and represents a fourth tau component in PHF-tau. In contrast to the PHF-tau triplet, the 74-kDa band loses its acidity during the postmortem delay, suggesting that it may be more sensitive to dephosphorylation than the PHF-tau triplet. A higher-molecular-mass tau of 72 kDa was already described (Brion et al., 1991; Goedert et al., 1992) and was considered to be a minor additional band (Mulot et al., 1994). Our results are in good agreement with the latter results, but the hyperphosphorylated 74-kDa tau component may be of particular interest, because it is observed especially in young AD patients, who generally show the higher PHF-tau immunoreactivity, making the 74-kDa band a potential marker for the severity of neurofibrillary degeneration.

Using isoform-specific anti-tau antibodies, Goedert et al. (1992) first proposed the composition of PHF-tau proteins in tau isoforms. This distribution was discussed later when *in vitro* phosphorylated recombinant tau isoforms were shown to give an AD PHF-tau-like profile (Mulot et al., 1994). Herein, using AD brain tissue homogenates, we show that the *in vivo* tau isoform distribution in the three PHF-tau bands is very similar to that described by Mulot et al. (1994) using recombinant tau isoforms phosphorylated by glycogen synthase kinase-3 $\beta$ . The smallest tau isoform (3R, 0; according to the nomenclature of Goedert et al., 1994) makes up tau 55; tau isoforms 3R, 29 and 4R, 0 compose tau 64; tau isoforms 3R, 58 and 4R, 29 compose tau 69; and tau 74 is made of the tau isoform 4R, 58.

#### PHF-tau and aging

Herein we show that AD2 immunoreactivity depends on the acidic character of PHF-tau proteins. Semiquantitative analysis of PHF-tau from brain tissue homogenates of different brain areas was shown to be a useful method for the cerebral mapping of neurofibrillary degeneration in AD, as well as in other neuro-

degenerative diseases (Vermersch et al., 1992; Buée-Scherrer et al., 1995, 1996b; Delacourte et al., 1996). Our results give additional evidence that using phospho-dependent antibodies such as AD2, 1-D analysis of PHF-tau is also useful for determining the severity of the degenerative process. By 2-D analysis, we observed that neocortical PHF-tau components, as well as the 74-kDa component, are less acidic in the oldest AD patients. This modulation is also observed by 1-D analysis, especially for the 55-kDa PHF-tau component or when considering the whole PHF-tau (tau 69 + tau 64 + tau 55). These results suppose a similar tendency of PHF-tau to be less immunoreactive and less acidic with aging. However, differences exist between young and old AD patients in our population, suggesting a less phosphorylated state of PHF-tau in old AD patients. These results are further confirmed for the 55- and 69-kDa PHF-tau components after M19G immunolabeling, showing that PHF-tau is heterogeneously modified.

In conclusion, the present study, as well as previous studies from our laboratory (Delacourte et al., 1996; Vermersch et al., 1996), shows that 2-D analysis, coupled with the use of phospho-dependent anti-tau antibodies, is a powerful approach for studying PHF-tau from AD brain, as well as pathological tau proteins from other neurodegenerative diseases. Our results show that PHF-tau proteins are heterogeneously phosphorylated in AD and establish the tau isoform composition of the three PHF-tau bands, with a complete panel of isoform-specific anti-tau antibodies. A fourth PHF-tau band of 74 kDa was characterized and shown to correspond to the longest human brain tau isoform in a hyperphosphorylated state. In addition, PHF-tau proteins were shown to be less phosphorylated in old AD patients. This is the first time that the phosphorylation of PHF-tau proteins is shown to be modified by age.

**Acknowledgment:** This work was supported by INSERM and CNRS. N.S. is a recipient of the fellowship Association France Alzheimer. D.L. is a recipient of the fellowship CHR&U of Lille and Region Nord-Pas-de-Calais. The monoclonal antibody AD2 was developed through a collaborative work with UMR9921 (Prof. B. Pau and Dr. C. Mourton-Gilles, Université de Montpellier), SANOFI/Diagnostic Pasteur, and INSERM. We are grateful to Prof. C. DiMenza (Geriatric Department of Limeil Brevannes Hospital).

## REFERENCES

- Arnold S. E., Hyman B. T., Flory J., Damasio A. R., and Van Hoesen G. W. (1991) The topographical and neuroanatomical distribution of neurofibrillary tangles and neuritic plaques in the cerebral cortex of patients with Alzheimer's disease. *Cereb. Cortex* **1**, 103–116.
- Bierer L. M., Hof P. R., Purohit D. P., Carlin L., Schmeidler J., Davis K. L., and Perl D. P. (1995) Neocortical neurofibrillary tangles correlate with dementia severity in Alzheimer's disease. *Arch. Neurol.* **52**, 81–88.
- Braak H. and Braak E. (1991) Neuropathological staging of Alzheimer-related changes. *Acta Neuropathol.* **82**, 239–259.
- Bramblett G. T., Goedert M., Jakes R., Merrick S. E., Trojanowski J. Q., and Lee V. M.-Y. (1993) Abnormal tau phosphorylation at Ser<sup>396</sup> in Alzheimer's disease recapitulates development and contributes to reduced microtubule binding. *Neuron* **10**, 1089–1099.
- Brandt R., Lee G., Teplow D. B., Shalloway D., and Abdelghany M. (1994) Differential effect of phosphorylation and substrate modulation on tau ability to promote microtubule growth and nucleation. *J. Biol. Chem.* **269**, 11776–11782.
- Brion J.-P., Passareiro H., Nunez J., and Flament-Durand J. (1985) Immunological detection of tau protein in neurofibrillary tangles of Alzheimer's disease. *Arch. Biol.* **95**, 229–235.
- Brion J.-P., Hanger D. P., Couck A. M., and Anderton B. H. (1991) A68 proteins in Alzheimer's disease are composed of several tau isoforms in a phosphorylated state which affects their electrophoretic mobilities. *Biochem. J.* **279**, 831–836.
- Brion J.-P., Couck A.-M., Robertson J., Loviny T. L. F., and Anderton B. H. (1993) Neurofilament monoclonal antibodies RT97 and 8D8 recognize different modified epitopes in paired helical filament- $\tau$  in Alzheimer's disease. *J. Neurochem.* **60**, 1372–1382.
- Buée-Scherrer V., Buée L., Hof P. R., Leveugle B., Gilles C., Loerzel A. J., Perl D. P., and Delacourte A. (1995) Neurofibrillary degeneration in amyotrophic lateral sclerosis/parkinsonism-dementia complex of Guam—immunochemical characterization of tau proteins. *Am. J. Pathol.* **146**, 924–932.
- Buée-Scherrer V., Condamines O., Mourton-Gilles C., Jakes R., Goedert M., Pau B., and Delacourte A. (1996a) AD2, a phosphorylation-dependent monoclonal antibody directed against tau proteins found in Alzheimer's disease. *Mol. Brain Res.* **39**, 79–88.
- Buée-Scherrer V., Hof P. R., Buée L., Leveugle B., Vermersch P., Perl D. P., Olanow C. W., and Delacourte A. (1996b) Hyperphosphorylated tau proteins differentiate corticobasal degeneration and Pick's disease. *Acta Neuropathol.* **91**, 351–359.
- Butler M. and Shelanski M. L. (1986) Microheterogeneity of microtubule-associated  $\tau$  proteins is due to differences in phosphorylation. *J. Neurochem.* **47**, 1517–1522.
- Cleveland D. W., Hwo S. Y., and Kirschner M. W. (1977) Physical and chemical properties of purified tau factor and the role of tau in microtubule assembly. *J. Mol. Biol.* **116**, 227–247.
- Delacourte A. and Défossez 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.
- Delacourte A., Flament S., Dibe E. M., Hublau P., Sablonnière B., Hémon B., Scherrer V., and Défossez A. (1990) Pathological proteins tau 64 and 69 are specifically expressed in the somatodendritic domain of the degenerating cortical neurons during Alzheimer's disease: demonstration with a panel of antibodies against tau proteins. *Acta Neuropathol.* **80**, 111–117.
- Delacourte A., Robitaille Y., Sergeant N., Buée L., Hof P. R., Watzé A., Laroche-Chollette A., Mathieu J., Chagnon P., and Gauvreau D. (1996) Specific pathological tau protein variants characterize Pick's disease. *J. Neuropathol. Exp. Neurol.* **55**, 159–168.
- Drewes G., Lichtenbergkraag B., Doring F., Mandelkow E. M., Biernat J., and Gori J. (1992) Mitogen activated protein (MAP) kinase transforms tau-protein into an Alzheimer-like state. *EMBO J.* **11**, 2131–2138.
- Flament S., Delacourte A., Delaère P., Duyckaerts C., and Hauw J. J. (1990) Correlation between microscopical changes and tau 64 and 69 biochemical detection in senile dementia of the Alzheimer type. Tau 64 and 69 are reliable markers of the neurofibrillary degeneration. *Acta Neuropathol.* **80**, 212–215.
- Garrels J. I. (1979) Two-dimensional gel electrophoresis and computer analysis of proteins synthesized by clonal cell lines. *J. Biol. Chem.* **254**, 7961–7977.
- Glenner G. and Wong C. (1984) Alzheimer's disease: initial report

- of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **120**, 885.
- Goedert M., Wischik C. M., Crowther R. A., Walker J. A., and Klug A. (1988) Cloning and sequencing of the cDNA encoding a core protein of the paired helical filament of Alzheimer disease: identification as the microtubule-associated tau. *Proc. Natl. Acad. Sci. USA* **85**, 4051–4055.
- Goedert M., Spillantini M. G., Jakes R., Rutherford D., and Crowther R. A. (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. *Neuron* **3**, 519–526.
- Goedert M., Spillantini M. G., Cairns N. J., and Crowther R. A. (1992) Tau-proteins of Alzheimer paired helical filaments—abnormal phosphorylation of all six brain isoforms. *Neuron* **8**, 159–168.
- Goedert M., Jakes R., Spillantini M. G., and Crowther R. A. (1994) Tau protein and Alzheimer's disease, in *Microtubules* (Hyams J. S. and Lloyd C. W., eds), pp. 183–200. Wiley-Liss, New York.
- Goode B. L. and Feinstein S. C. (1994) Identification of a novel microtubule binding and assembly domain in the developmentally regulated inter-repeat region of tau. *J. Cell Biol.* **124**, 769–782.
- Greenberg S. G. and Davies P. (1990) A preparation of Alzheimer paired helical filaments that displays distinct  $\tau$  proteins by polyacrylamide gel electrophoresis. *Proc. Natl. Acad. Sci. USA* **87**, 5827–5831.
- Greenberg S. G., Davies P., Schein J. D., and Binder L. I. (1992) Hydrofluoric acid-treated tauPHF proteins display the same biochemical properties as normal tau. *J. Biol. Chem.* **267**, 564–569.
- Grundke-Iqbal I., Iqbal K., and Tung Y. C. (1986) Abnormal phosphorylation of the MAP TAU in Alzheimer cytoskeletal pathology. *Proc. Natl. Acad. Sci. USA* **83**, 4913–4917.
- Hanger D. P., Hughes K., Woodgett J. R., Brion J. P., and Anderton B. H. (1992) Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localisation of the kinase. *Neurosci. Lett.* **147**, 58–62.
- Hasegawa M., Jakes R., Crowther R. A., Lee V. M.-Y., Ihara Y., and Goedert M. (1996) Characterization of mAb AP422, a novel phosphorylation-dependent monoclonal antibody against tau protein. *FEBS Lett.* **384**, 25–30.
- Hof P. R., Bierer L. M., Perl D. P., Delacourte A., Buée L., Bouras C., and Morrison J. H. (1992) Evidence for early vulnerability of the medial and inferior aspects of the temporal lobe in an 82-year-old patient with preclinical signs of dementia—regional and laminar distribution of neurofibrillary tangles and senile plaques. *Arch. Neurol.* **49**, 946–953.
- Ihara Y., Nukina N., Miura R., and Ogawara M. (1986) Phosphorylated tau protein is integrated into paired helical filaments in Alzheimer's disease. *J. Biochem. (Tokyo)* **99**, 1807–1810.
- Iqbal K., Zaidi T., Thompson C. H., Merz P. A., and Wisniewski H. M. (1984) Alzheimer paired helical filaments: bulk isolation, solubility and protein composition. *Acta Neuropathol.* **62**, 167–177.
- Iqbal K., Alonso A. D., Gong C. X., Khatoon S., Singh T. J., and Grundke-Iqbal I. (1994) Mechanism of neurofibrillary degeneration in Alzheimer's disease. *Mol. Neurobiol.* **9**, 119–123.
- Khachaturian Z. S. (1985) Diagnosis of Alzheimer's disease. *Arch. Neurol.* **4211**, 1097.
- Kidd M. (1963) Paired helical filaments in electron microscopy of Alzheimer's disease. *Nature* **197**, 192–193.
- Klose J. and Kobalz U. (1995) Two-dimensional electrophoresis of proteins: an updated protocol and implications for a functional analysis of the genome. *Electrophoresis* **16**, 1034–1059.
- Kondo J., Honda T., Mori H., Hamada Y., Miura R., Ogawara M., and Ihara Y. (1988) The carboxyl third of tau is tightly bound to paired helical filaments. *Neuron* **1**, 827–834.
- Ksiezak-Reding H., Binder L. I., and Yen S.-H. (1988) Immunohistochemical and biochemical characterization of tau proteins in normal and Alzheimer's disease brains with Alz 50 and Tau-1. *J. Biol. Chem.* **263**, 7947–7953.
- Ksiezak-Reding H., Binder L. I., and Yen S. H. (1990) Alzheimer disease proteins (A68) share epitopes with tau but show distinct biochemical properties. *J. Neurosci. Res.* **25**, 420–430.
- Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature* **227**, 680–685.
- Ledesma M. D., Bonay P., Colaco C., and Avila J. (1994) Analysis of microtubule-associated protein tau glycation in paired helical filaments. *J. Biol. Chem.* **269**, 21614–21619.
- Lee V. M.-Y., Balin B., Otvos L. Jr., and Trojanowski J. Q. (1991) A68: a major subunit of paired helical filaments and derivatized forms of normal tau. *Science* **251**, 675–678.
- Lew J. and Wang J. H. (1995) Neuronal cdc2-like kinase. *Trends Biochem. Sci.* **20**, 33–37.
- Litersky J. M. and Johnson G. V. W. (1992) Phosphorylation by cAMP-dependent protein kinase inhibits the degradation of tau by calpain. *J. Biol. Chem.* **267**, 1563–1568.
- Lovestone S., Reynolds C. H., and Latimer D. (1994) Alzheimer's disease-like phosphorylation of the microtubule-associated protein tau by glycogen synthase kinase-3 in transfected mammalian cells. *Curr. Biol.* **4**, 1077–1086.
- Matsuo E. S., Shin R. W., Billingsley M. L., Vandevoorde A., O'Connor M., Trojanowski J. Q., and Lee V. M.-Y. (1994) Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau. *Neuron* **13**, 989–1002.
- McKhann G. et al. (1984) Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA work group under the auspices of Department of Health and Human Services task force on Alzheimer's disease. *Neurology* **34**, 939–944.
- Mercken M., Vandermeeren M., Lübke U., Six J., Boons J., Vanmechelen E., Van de Voorde A., and Gheuens J. (1992) Affinity purification of human  $\tau$  proteins and the construction of a sensitive sandwich enzyme-linked immunosorbent assay for human  $\tau$  detection. *J. Neurochem.* **58**, 548–553.
- Morishima M. and Ihara Y. (1994) Posttranslational modifications of tau in paired helical filaments. *Dementia* **5**, 282–288.
- Morishima-Kawashima M., Hasegawa M., Takio K., Suzuki M., Yoshida H., Titani K., and Ihara Y. (1995) Proline-directed and non-proline-directed phosphorylation of PHF-tau. *J. Biol. Chem.* **270**, 823–829.
- Mulot S. F. C., Hughes K., Woodgett J. R., Anderton B. H., and Hanger D. P. (1994) PHF-tau from Alzheimer's brain comprises four species on SDS-PAGE which can be mimicked by in vitro phosphorylation of human brain tau by glycogen synthase kinase-3 $\beta$ . *FEBS Lett.* **349**, 359–364.
- O'Farrell P. (1975) High resolution two dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007–4021.
- O'Farrell P. (1977) High resolution two dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**, 1133–1142.
- Permanne B., Buée L., David J. P., Fallet-Bianco C., DiMenza C., and Delacourte A. (1995) Quantitation of Alzheimer's amyloid peptide and identification of related amyloid proteins by dot-blot immunoassay. *Brain Res.* **685**, 154–162.
- Reig S., Buée-Scherrer V., Mourton-Gilles C., Défossez A., Delacourte A., Beauvillain J. C., and Mazzuca M. (1995) Immunogold labelling of paired helical filaments and amyloid fibrils by specific monoclonal and polyclonal antibodies. *Acta Neuropathol.* **90**, 441–447.
- Sergeant N., Bussièrre T., Vermersch P., Lejeune J. P., and Delacourte A. (1995) Isoelectric point differentiates PHF-tau from biopsy-derived human brain tau proteins. *Neuroreport* **6**, 2217–2220.
- Seubert P., Mawaladewan M., Barbour R., Jakes R., Goedert M., Johnson G. V. W., Litersky J. M., Shenk D., Lieberburg I., Trojanowski J. Q., and Lee V. M.-Y. (1995) Detection of phos-

- phorylated Ser(262) in fetal tau, adult tau, and paired helical filament tau. *J. Biol. Chem.* **270**, 18917–18922.
- Sperber B. R., Leight S., Goedert M., and Lee V. M.-Y. (1995) Glycogen synthase kinase-3 $\beta$  phosphorylates tau protein at multiple sites in intact cells. *Neurosci. Lett.* **197**, 149–153.
- Steiner B., Mandelkow E. M., Biernat J., Gustke N., Meyer H. E., Schmidt B., Mieskes G., and Soling H. D. (1990) Phosphorylation of microtubule-associated protein-tau—identification of the site for Ca<sup>2+</sup>-calmodulin dependent kinase and relationship with tau-phosphorylation in Alzheimer tangles. *EMBO J.* **9**, 3539–3544.
- Trojanowski J. Q. and Lee V. M.-Y. (1995) Phosphorylation of paired helical filament tau in Alzheimer's disease neurofibrillary lesions: focusing on phosphatases. *FASEB J.* **9**, 1570–1576.
- Vermersch P., Frigard B., and Delacourte A. (1992) Mapping of neurofibrillary degeneration in Alzheimer's disease—evaluation of heterogeneity using the quantification of abnormal tau proteins. *Acta Neuropathol.* **85**, 48–54.
- Vermersch P., Sergeant N., Ruchoux M. M., Hofmann-Radvanyi H., Watez A., Petit H., Dewailly P., and Delacourte A. (1996) Specific tau variants in the brain from patients with myotonic dystrophy. *Neurology* **47**, 711–717.
- Wang J. Z., Gong C. X., Zaidi T., Grundke-Iqbal I., and Iqbal K. (1995) Dephosphorylation of Alzheimer paired helical filaments by protein phosphatase-2A and -2B. *J. Biol. Chem.* **270**, 4854–4860.
- Wischik C. M., Novak M., Edwards P. C., Klug A., Tichelaar W., and Crowther R. A. (1988) Structural characterization of the core of the paired helical filament of Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **85**, 4884–4888.
- Yen S. H., Dickson D. W., Crowe A., Butler M., and Shelanski M. L. (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.* **126**, 81–91.
- Yoshida H. and Ihara Y. (1993)  $\tau$  in paired helical filaments is functionally distinct from fetal  $\tau$ : assembly incompetence of paired helical filament- $\tau$ . *J. Neurochem.* **61**, 1183–1186.