Tau Proteins in the Temporal and Frontal Cortices in Patients With Vascular Dementia

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Abstract
We previously reported that, in the brains of older patients with vascular dementia (VaD), there is a distinctive accumulation of detergent-extractable soluble amyloid-β, with a predominance of Aβ42 species. It is unclear, however, if tau proteins also accumulate in the brains of older VaD subjects. Using antibody-specific immunoassays, we assessed concentrations of total tau (t-tau) and phosphorylated tau protein, measured at 3 phosphorylated sites (i.e. Thr181, Ser202/Thr205, and Ser262), as well as synaptophysin in the temporal and frontal cortices of 18 VaD, 16 Alzheimer disease (AD), and 16 normal age-matched control subjects. There was selective loss of t-tau protein in VaD compared with controls and AD subjects (p < 0.01 and p < 0.001, respectively). In contrast, phosphorylated tau levels were similar to controls in VaD in both regions, but they were increased in the temporal lobes of patients with AD (p < 0.01 and p < 0.0001 for Ser202/Thr205 and Ser262 phosphorylated sites, respectively). The reduced t-tau in the VaD group was unrelated to any low-level neurofibrillary or amyloid pathology or age at death. These findings suggest that breaches of microvascular or microstructural tissue integrity subsequent to ischemic injury in older age may modify tau protein metabolism or phosphorylation and have effects on the burden of neurofibrillary pathology characteristic of AD.

Key Words: Aging, Alzheimer disease, Ischemia, Microvascular, Stroke, Tau, Vascular dementia.

INTRODUCTION
Alzheimer disease (AD) is characterized by the accumulation of amyloid-β (Aβ) protein in senile plaques and hyperphosphorylated tau in neurofibrillary tangles; together these are considered the substrates of dementia in AD patients. The amyloid cascade hypothesis has perpetuated the search for a link between accumulation of the 2 proteins (1); however, there are several caveats, one of which is the distinct and independent anatomic patterns in the distribution of the 2 pathologic accumulations (2). Moreover, the 2 molecular signature proteins of AD also accumulate in older patients in whom may be no evidence of dementia (3).

Our previous work demonstrated that Aβ protein accumulation, although prominent in AD and present in other aging-related dementias, also occurs in older patients with vascular dementia (VaD) (4). The Aβ42 species was the predominant form of extractable amyloid peptide, representing more than 70% of the amyloid protein found in the brain parenchyma. Remarkably, VaD subjects older than 80 years have almost equal concentrations of soluble Aβ42 as AD subjects (4), suggesting that increasing age is a risk factor for the development of Aβ-like neuropathology. Vascular dementia is diagnosed in the absence of insufficient neurofibrillary pathology, as defined by low Braak stages (5). Consistent with this, a recent large pathologic study linked coincident cerebrovascular disease with lower neurofibrillary pathology, corresponding to Braak stages I to IV, and subjects with frontotemporal lobar dementia had a more than 60% lower prevalence rate of vascular pathology than that seen in Aβ (6). These findings suggest that vascular changes might impede or interfere with age-related progression of neurofibrillary degeneration. It is plausible that vascular changes may influence the pathophysiologic mechanisms underlying the development of neurofibrillary pathology, including phosphorylation (7) and truncation (8) of tau proteins. Despite the fact that all of the molecular ingredients of the pathologies are present in the aging brain, the events that lead to distinct accumulation patterns of the proteins that define the dementia syndromes (e.g. AD or VaD) need further evaluation. Thus, a critical question arises: what befalls the microtubule-associated tau protein that could explain the lack of characteristic neurofibrillary pathology in patients with VaD?

Given that soluble tau, but not PHF-tau, decreases in older age and is nearly equally distributed between gray and white matter (including axons) (9–12), we assessed concentrations of
total tau (t-tau) and phosphorylated tau (p-tau) proteins by immunoassays in the gray matter from the frontal and temporal lobes of patients with VaD and AD and aging control subjects. The tau proteins were measured in the same samples previously used to determine Aβ species (4). It is also possible that blood-brain barrier (BBB) permeability abnormalities in patients with VaD or vascular cognitive impairment (13) may be another factor that changes the intracellular distribution or metabolism of key structural proteins such as tau.

**MATERIALS AND METHODS**

**Patients and Brain Samples**

Brain samples were obtained from the frontal and temporal lobes (BA9 and BA21, respectively) from control, VaD, and AD patients (Table 1). Gray matter without any trace of white matter was dissected from each sample at −20°C in the glove box. We also ensured that the sampled areas did not have any visible infarcts or vascular lesions. All postmortem tissues were retrieved from the Newcastle Brain Tissue Resource Centre at the Institute of Neuroscience, Newcastle University. The local research ethics committee of the Newcastle Hospital Foundation Trusts, Newcastle upon Tyne, United Kingdom, approved use of tissue for this research.

Table 1 shows the demographic details of the subjects and the standard neuropathologic findings. In the majority of the subjects, bronchopneumonia was recorded as the cause of death. Four subjects died because of heart failure. Autopsies were performed within 12 to 36 hours after death. The postmortem intervals between death and tissue retrievals were not different for the 3 groups. Similarly, all analyzed groups had similar age at death.

All subjects were part of longitudinal studies of dementia and VaD and had undergone extensive and comprehensive clinical and cognitive assessments, including the Cambridge Neuropathologic Examination for Mental Disorders in the Elderly (14) and Mini-Mental State Examination (15). The diagnosis of AD was in accordance with the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer’s Disease (16). Clinical diagnosis of VaD was consistent with the proposed Harmonization Guidelines for vascular cognitive impairment (17). The control groups consisted of subjects with no evidence of dementia, as determined by retrospective assessments in the absence of neurologic or psychiatric disorder.

**Neuropathologic Examination**

As part of the neuropathologic routine diagnostic assessment within the Newcastle Brain Tissue Resource Centre, all deceased subjects underwent standard gross brain examination of both cerebral hemispheres. Microscopic examination of the right cerebral hemisphere was performed to assess Consortium to Establish a Registry for Alzheimer’s Disease scores for the density of neuritic plaques (18), Braak staging for neurofibrillary tangles (19), mean neocortical tangle densities (20), cortical Lewy Body staging for grading of α-synuclein pathology (21), and macroscopic and microscopic vascular disease (5).

Standard neuropathologic lesions were visualized by silver impregnation methods and conventional stains, as previously described (4). Briefly, Bielschowsky silver preparation was used to visualize amyloid plaques; Palmgren for neurofibrillary tangles; Loyez or Luxol fast blue stain for white matter myelin loss; and hematoxylin and eosin to visualize infarcts, vascular changes, and rarefaction. The mean density of plaque and tangle counts was determined by manual counting. Additional immunohistochemistry was performed on sections adjacent to the sections stained for routine histopathology. Amyloid plaques and neurofibrillary pathology were visualized with monoclonal antibodies 4G8 and AT-8, respectively.

The pathologic diagnosis of VaD was defined by the presence of multiple or cystic infarcts involving both cortical and subcortical areas.

### TABLE 1. Patient Demographic and Neuropathologic Data

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 16)</th>
<th>VaD (n = 18)</th>
<th>AD (n = 16)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at death, years</td>
<td>79.00 ± 2.97</td>
<td>81.44 ± 2.67</td>
<td>80.06 ± 2.59</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>9/7</td>
<td>10/8</td>
<td>10/6</td>
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<tr>
<td>Braak staging</td>
<td>3.25 ± 0.49</td>
<td>2.86 ± 0.48</td>
<td>5.47 ± 0.13</td>
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</tr>
<tr>
<td>Mean plaques/mm²</td>
<td>2.71 ± 1.13</td>
<td>8.85 ± 2.44</td>
<td>28.40 ± 4.98</td>
<td></td>
</tr>
<tr>
<td>Mean tangles, mm²</td>
<td>0 ± 0</td>
<td>2.04 ± 0.99</td>
<td>18.05 ± 3.11</td>
<td></td>
</tr>
<tr>
<td>Infarcts</td>
<td>2</td>
<td>6</td>
<td>0</td>
<td></td>
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<tr>
<td>Microinfarcts</td>
<td>0</td>
<td>9</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>White matter changes</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td></td>
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<tr>
<td>Vascular lesions</td>
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<td></td>
</tr>
<tr>
<td>CAA</td>
<td>0</td>
<td>2</td>
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</table>

*Numbers represent mean ± SE. Significance: differences in means determined by Mann-Whitney U test. The mean densities of plaques and tangles were determined from the four cortical lobes and hippocampal formation. Braak staging: mean Braak scores were computed from individual scores obtained from each hippocampus and neocortex. CAA was graded (scores 1–4), as previously described (14). Vascular lesions, which included large infarcts, lacunae, and microinfarcts (5), were graded from 0 to 3 at 3 different coronal levels. Bonferroni-adjusted p values are used. AD, Alzheimer disease; CAA, cerebral amyloid angiopathy; CAMCOG, Cambridge Assessment Mental Disorders in the Elderly; F, female; M, male; NS, no significant difference; VaD, vascular dementia.*
subcortical regions, border zone infarcts, lacunes (<15 mm), microinfarcts (visible by microscopy only), and small-vessel disease in subcortical structures in the general absence of neurofibriillary pathology. None of the VaD cases had Braak stage above III or the double diagnosis of AD or mixed type of dementia (5).

**Tissue Preparation**

Tissue samples solely containing gray matter (0.2–0.7 g wet weight) were homogenized in 10 volumes of cold (4°C) 5 mol/L guanidinium hydrochloride (Gdn HCl; Sigma-Aldrich) buffer containing 5-mmol/L HEPES (pH 7.3; Sigma-Aldrich) and protease inhibitors. Formaldehyde (25% v/v) and 10% glycerol 0.01% (wt/vol) 4% (wt/vol) SDS (Sigma-Aldrich), 0.1 mol/L dithiothreitol were added and the homogenate was mixed by vortexing. The samples were centrifuged at 15,000 g for 30 minutes in a refrigerated swing-out rotor at 4°C. The supernatant was discarded, and the remaining pellet was resuspended in 1 mL of 0.5% SDS buffer (Sigma-Aldrich), and protease inhibitors.

**Ethanol Precipitation of Guanidine Samples**

For sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), Gdn HCl-treated samples were precipitated in ethanol (22). Briefly, 100 μL of the Gdn HCl-treated sample was incubated with 9 volumes of cold ethanol 100%, mixed, and left overnight at −20°C. The samples were centrifuged at 15,000 × g for 15 minutes at 4°C; the supernatant was discarded, and the remaining pellet was washed in 90% cold (−20°C) ethanol. The samples were resuspended in 1× SDS mix plus dithiothreitol (125 mmol/L Tris-HCl, pH 6.8; 4% [wt/vol] SDS [Sigma-Aldrich], 0.1 mol/L dithiothreitol [Thermo Scientific Pierce, UK], 10% glycerol 0.01% [wt/vol] bromophenol blue).

**SDS-PAGE and Immunoblotting**

The ethanol-precipitated samples were resolved in 10% acrylamide gels in a vertical gel tank (OminiPAGE Mini Wide electrophoresis module; Scientific Laboratory Supplies, Nottingham, UK) at constant 125 V for 80 minutes in 2× SDS buffer (0.025 mol/L Tris, 0.192 mol/L glycine, 0.1% [wt/vol] SDS). After separation, proteins were transferred to a nitrocellulose membrane (pore size, 0.45 μm; Thermo Scientific Pierce, UK) using a Transblot cell with plate electrodes (Bio-Rad Laboratories, Hertfordshire, UK) at constant current of 500 mA for 2 hours. Membranes were then blocked in blocking buffer 5% wt/vol dried nonfat milk in Tris-buffered saline plus detergent (20 mmol/L Tris HCl pH 7.5, 150 mmol/L NaCl, 0.1% [wt/vol] Tween 20).

Antibodies were diluted in blocking buffer, and the membrane was incubated in primary antibody solution overnight at 4°C (monoclonal antibody [mAb] EP10 at 1:100; mAb 7.51, 1:100; mAb AT8, 1:1000 [Phospho-PHF-tau pSer202/Thr205 Antibody from Thermo Scientific Pierce Antibodies, Rockford, IL]; polyclonal antibody [pAb] Ser262, 1:1000 [A01099-200 GenScript, Piscataway, NJ]; pAb Thr181, 1:1000 [Tau pT181 Antibody A7244 Assay Biotech Inc, San Francisco, CA]; and pAb αβ-tubulin [Cell Signal antibody 21488S; New England BioLabs Inc, Ipswich, MA], 1:1000). After incubation in primary antibody, membranes were washed in 6 changes of wash buffer (1% wt/vol dried nonfat milk in Tris-buffered saline plus detergent) before incubation with horseradish peroxidase–conjugated secondary antibodies (anti-mouse Cell Signal Ab 7076S or anti-rabbit Cell Signal Ab 7074S; both at 1:2000) for 2 hours in blocking buffer. Membranes were washed in 6 changes of buffer before chemiluminescence detection according to manufacturer’s instructions (SuperSignal West Pico Chemiluminescent Substrate, Pierce, Thermo Fisher Scientific, UK) using a digital imager (ImageQuant LAS 4000; GE Healthcare, Buckinghamshire, UK).

**Immunoassays**

Indirect ELISA was used to determine the protein amounts: t-tau protein (mAb 7.51, which recognizes the microtubule-binding region of tau protein, used in 1:500 dilution; gift from Prof. C.M. Wischik, Aberdeen University, UK), pAb Thr181 (1:1000); Tau pT181 Antibody A7244 Assay Biotech Inc), and pAb Ser262 (1:1000; A01099-200 GenScript), as described previously (23–25). In parallel assays, we also determined synaptophysin (mAb EP10, 1:10 dilution) concentrations. Briefly, triplicates of double dilutions of the antigen over 6 wells were coated overnight at 4°C using carbonate-bicarbonate buffer, washed in 0.05% Tween, blocked with 1% dried skimmed milk, and incubated for 1 hour at 37°C. Plates were again washed for 1 hour at 37°C and incubated with the primary antibodies to the antigen of interest, diluted in 0.05% Tween in PBS (pH 6.8) for 1 hour. After another wash in 0.05% Tween in PBS, plates were incubated with secondary antibodies conjugated to horseradish peroxidase and incubated again for 1 hour at 37°C. Colorimetric analysis of reaction with 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich) was performed, and the reaction was quenched after 10 minutes with 2N H2SO4. Plates were read with Vmax plate reader (Molecular Devices, Sunnyvale, CA), and assay curves were plotted using SOFTmaxPro (version 4.7.1; Molecular Devices). All values were normalized for 0.3-mL fraction from 0.3 to 0.5 g brain tissue and expressed as relative arbitrary units of immunoreactivity (23).

Amyloid β assays were performed as previously described (4) using homogeneous time-resolved fluorescence immunoassay developed for the detection of Aβ peptides (26). The homogeneous time-resolved fluorescence assay consists of capturing of the peptides of interest by a double sandwich and detection with the Discovery microplate analyzer (PerkinElmer LAS, Ltd, Beaconsfield, UK), which provides simultaneous fluorescence measurement at 665 nm and 620 nm for the calculation of signal ratios that is used to extrapolate the brain extract Aβ concentration off the standard curve established using synthetic Aβ1–40 and Aβ1–42 peptides. The assay used mAb G2-10 and mAb G2-11 antibodies in combination with mAb 4G8 as a capture antibody to detect amyloid peptides cleaved at residues 40 and 42, respectively. As in all our previously reported ELISAs (23), the interrater reliability of the assays was greater than 90%.

**Dot Blot Analysis**

Because indirect ELISA was not able to detect the p-tau concentrations, we used dot blot analysis to determine the
protein level of p-tau202/205 protein using mAb AT8, as previously described (27). Briefly, triplicates of 1 μL of homogenate was applied on the nitrocellulose membrane and allowed to air-dry for 30 minutes at room temperature. Nonspecific binding was blocked with 3% bovine serum albumin/PBS Tris (PBST) for 1 hour at room temperature. The membranes were rinsed in 2 washes of PBS and once in PBST (5-minute washing cycles each) and incubated with mAb AT8, diluted 1:500 in 0.05% Tween in PBS (pH 6.8) for 1 hour at room temperature. After another washing cycle, the blots were incubated with secondary antibody conjugated to horseradish peroxidase (1:1,000 dilution; Dako, Glostrup, Denmark). After another washing cycle of PBS/PBST washes, the immunoprobe was visualized with 3,3’-diaminobenzidine (10 mg/mL) as the chromogen for 2 minutes, rinsed in distilled water, and left to dry at room temperature.

Protein quantification from the dot blots was achieved by measuring the dot intensity when scanned and comparing the grayscale values corrected for the background staining. Measurements were carried out blind to neuropathologic diagnosis. In brief, the stained blots were photographed using an AutoChemiSystem Digital camera, and the optical density of the images was recorded using LabWorks image analyzer software. Averages of triplicate values were calculated, and this value represented the mean optical density for the analyzed sample. An internal control was used to calibrate the software.

Statistical Analysis

Standard statistical analysis using nonparametric techniques and linear regression analysis were appropriate for the data analysis. All statistical analyses were performed using SPSS v19 software. Comparisons between categories and numeric variables (e.g. age at death, biochemical and neuro-pathologic measures of tau, and amyloid concentrations and/or densities) were assessed by Mann-Whitney U test, using Bonferroni correction to adjust the probability, and χ² analysis. Statistical associations between numeric variables were determined by Spearman rank correlation analysis, and significance was determined at values of p < 0.05.

RESULTS

Neurofibrillary and Amyloid Pathology

As expected, AD subjects had substantially higher Braak stages compared with both the controls and VaD subjects (Table 1). The mean tangle density of AD subjects was nearly 9-fold higher than that in VaD. Similarly, the mean plaque density was also substantially higher in the AD patients than that present in both VaD and control subjects. In contrast, the control and VaD groups had similar negligible neurofibrillary pathology by immunohistochemistry (Figs. 1, 2). The VaD group exhibited marginally higher tangle and plaque content than the control subjects (Table 1). These differences between VaD and controls were not significant (p > 0.05); the levels were substantially lower than those in the AD subjects (Table 1). The VaD group also characteristically exhibited higher frequencies of in-farcts (p = 0.0271) and microinfarcts (p = 0.0011) than the control and AD subjects.

Tau Protein Analysis

Tau protein analysis by indirect ELISA and immunoblotting indicated that the distribution of t-tau and p-tau had distinct profiles in the temporal and frontal cortices (Table 2; Fig. 3). Although the relative amounts of p-tau phosphorylated at Thr181, Ser202/Thr205, and Ser262 were comparable in both regions, the concentrations of t-tau were significantly higher in the temporal cortex compared with the frontal cortex for all groups (1.9- to 4.3-fold higher; p < 0.0001). Alzheimer disease subject samples had the highest amounts of t-tau protein in the frontal lobe compared with both the control and VaD groups (p = 0.021 and p = 0.009), with VaD and control subjects exhibiting very similar levels of t-tau (p = 0.997). By contrast, t-tau protein immunoreactivity in the temporal lobe showed a different pattern, with the VaD group having 1.5- and 1.8-fold reduced t-tau protein in comparison with both control (p = 0.021) and AD (p < 0.001) group patients, respectively (Table 2).

The observed changes in t-tau protein distribution were also confirmed in the analysis of p-tau protein. In the frontal lobe, p-tau Ser202/Thr205 but not p-tau Thr181 and Ser262 immunoreactivity was significantly higher in the AD group in relation to controls (p < 0.0001) and the VaD (p = 0.004) subjects; the VaD and control subjects had similar levels of p-tau for Ser202/Thr205 and Ser262 measures (p = 0.538 and p = 0.524, respectively). Similar patterns of p-tau distribution were also observed for the temporal lobe, with the AD group having significantly higher amounts of p-tau, both Ser202/Thr205 and Ser262, but not Thr181 measures, in comparison with controls (p < 0.0001) and VaD (p = 0.006 for Ser202/Thr205 and p < 0.001 for Ser262 measures). There were no significant differences in p-tau in the temporal cortex in VaD and control subjects (Table 2). The t/p-tau was not altered between the groups, with the exception of the t/p-tau Thr181 in the temporal cortex, which was significantly lower in the VaD cases versus the AD group (p = 0.045) (Table 2).

Immunoblotting confirmed the expected sizes of the protein bands and showed that t-tau protein was markedly greater in the temporal cortex compared with the frontal cortex in AD (Fig. 3). There were no apparent differences in synaptophysin or αβ-tubulin in subjects with and without dementia and between the frontal and temporal cortices (Fig. 3). In contrast, the p-tau Ser262 levels were highest in the AD temporal cortex, whereas the p-tau Thr181 levels, although similar between the cases in both the frontal and the temporal lobe, had somewhat weaker staining of the 68-kDa band in the VaD case in both brain regions (Fig. 3).

In addition, we performed correlational analyses to determine whether the selective loss of t-tau in the temporal lobe in the VaD cases was attributable to underlying neuropathology within the same brain area or whether it was influenced by other factors such as distal pathology (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A695 and Table, Supplemental Digital Content 2, http://links.lww.com/NEN/ A696). None of the measures of neurofibrillary pathology, senile plaques, and biochemical measures of amyloid was associated with the t-tau or p-tau protein reduction in VaD. The
only other correlation of interest was the tendency for t-tau and p-tau to be associated with markers of AD pathology.

**DISCUSSION**

As expected, our findings showed that VaD subjects had a rather negligible amount of neurofibrillary pathology (tangles) in both cortices compared with that evident in AD. We also confirm our previous findings of AD and control subjects having substantial amounts of t-tau protein in neocortical areas, as well as the differential regional distribution of tau protein, with substantially higher levels in the temporal neocortex (11). There were specific increases in both p-tau Ser202/Thr205 and Ser262, tau epitopes associated with paired helical filaments in AD. However, we report the selective reduction of t-tau protein by about 2-fold in the temporal cortex of VaD subjects (5). The specificity of this observation is supported by comparable amounts of tubulin in all 3 groups. The apparent loss or dissipation of t-tau from the gray matter occurred in the absence of overt synaptic loss or changes in p-tau content and was not associated with the extent of neurofibrillary pathology (23), soluble amyloid burden, and/or the age at death.

The reduction of t-tau protein in VaD appears to parallel the previously reported age-related loss in normal controls, with an average loss of 14% of soluble tau per decade after the age of 20 years (9), but the disposition of tau protein in cerebrovascular disease remains poorly understood. We propose some explanations on the neurobiological mechanisms underlying reduced t-tau protein levels in VaD. It is plausible that t-tau protein is rapidly metabolized or eliminated after neuronal cytoskeletal changes early during and after remote ischemic injury (28). Substantial proportions of t-tau could then be dissipated from microtubule lattices because of parenchymal tissue changes in ionic strength and pH (29) that result directly from infarction or local oligemia. The released tau protein as a result of neuronal changes or shrinkage may then aggregate into oligomers or be sequestered by glia (30). There could also be increased exosomal secretion of both whole length and p-tau in VaD cases. Exosomal release is a

**FIGURE 1.** (A–I) Neurofibrillary pathology in the frontal cortex of patients with Alzheimer disease (C, F, I) compared with patients with vascular dementia (B, E, H) and control (A, D, G) subjects. In contrast to the AD case with abundant neurofibrillary pathology, there is absence of tau pathology in VaD and the control case, showing only diffuse intraneuronal C-terminal end and p-tau protein immunoreactivities. (A–C) Monoclonal antibody 11.57; (D–F) polyclonal anti-Ser262; (G–I) polyclonal anti-Thr181. Scale bar = 100 μm.
widely characterized mechanism that mediates unconventional secretion of other aggregation-prone proteins (e.g. α-synuclein, prion protein, and Aβ) in neurodegenerative diseases (30, 31).

Another more plausible explanation is that white matter changes in VaD (32), which are relatively less abundant in aging controls, influence the disposition of t-tau. In addition to overt ischemic injury, the presence of subcortical tissue changes within the white matter (5) might account for this loss. Axonal changes or injury may disrupt tau transport and turnover along projections from the cell bodies. However, given the presence of characteristic white matter disintegrality in VaD, this may be the key driver of differential mobilization of t-tau, which needs to be elucidated.

Studies in transgenic mice genetically engineered to express AD pathology show that transient global ischemic injury significantly decreased amounts of t-tau, soluble tau, and p-tau with the coincident activation of macroautophagy and ubiquitin-proteosome pathways (33, 34). The pattern of decreased t-tau, altered p-tau, and increased Aβ appears to persist for several weeks after transient cerebral hypoperfusion injury in these models (34). In contrast to this rodent study, however, in a canine ischemic model, ischemia resulted in dephosphorylation at Thr181 site and a weaker 68-kDa tau protein band (35), similar to what we describe in the current study. This argues that ischemia per se may have a distinct effect on phosphorylation of tau protein isoforms depending on the animal model studied.

A recent study drew the attention to the genetic link between the MAPT gene and VaD (36). Thus, MAPT single-nucleotide polymorphism rs1467967 is linked to VaD, with the G allele in particular, doubling the risk for developing VaD (36). The G allele is also associated with lower cerebrospinal fluid (CSF) t-tau levels (37) and appears not to influence the phosphorylation of tau at Thr181 (38). Because the G allele is common in both white and Chinese populations (57% and 84%, respectively) (36, 37), it is plausible that the allele may be over-represented in VaD and thus linked to the downregulation of t-tau protein in VaD, thus influencing the further development of neurodegenerative changes in the aging brain.

FIGURE 2. Neurofibrillary pathology in the temporal cortex of patients with Alzheimer disease (C, F, I) compared with patients with vascular dementia (B, E, H) and control (A, D, G) subjects. In contrast to the AD case with abundant neurofibrillary pathology, there is absence of tau pathology in VaD and the control case, showing only diffuse intraneuronal C-terminal end and p-tau protein immuno-reactivities. (A-C) Monoclonal antibody 11.57; (D-F) polyclonal anti-Ser262; (G-I) polyclonal anti-Thr181. Scale bar = 100 μm.
TABLE 2. Tau, Amyloid, and Synaptophysin Measures in the Frontal and Temporal Lobes

<table>
<thead>
<tr>
<th>Measures</th>
<th>Frontal lobe control</th>
<th>VaD</th>
<th>AD</th>
<th>Significance</th>
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</thead>
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<tr>
<td>t-tau RV (mAb7.51)</td>
<td>40 ± 4</td>
<td>37 ± 3</td>
<td>110 ± 27</td>
<td>NS C vs VaD; p = 0.021 C vs AD; p = 0.009 VaD vs AD</td>
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<tr>
<td>p-tau (Ser202/Thr205) RV</td>
<td>0.44 ± 0.04</td>
<td>0.49 ± 0.03</td>
<td>0.65 ± 0.05</td>
<td>NS C vs VaD; p &lt; 0.0001 C vs AD; 0.004 VaD vs AD</td>
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<tr>
<td>p-tau (Ser262) RV</td>
<td>7.99 ± 0.61</td>
<td>8.27 ± 1.28</td>
<td>8.11 ± 0.31</td>
<td>NS C vs VaD; NS C vs AD; NS VaD vs AD</td>
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<tr>
<td>p-tau (Thr181) RV</td>
<td>40.57 ± 2.60</td>
<td>36.09 ± 4.47</td>
<td>37.50 ± 4.91</td>
<td>NS C vs VaD; NS C vs AD; NS VaD vs AD</td>
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<tr>
<td>t/ptau (Ser262)</td>
<td>4.89 ± 0.98</td>
<td>5.30 ± 1.00</td>
<td>54.29 ± 40.28</td>
<td>NS C vs VaD; NS C vs AD; NS VaD vs AD</td>
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<td>t/ptau (Thr181)</td>
<td>1.04 ± 0.29</td>
<td>0.90 ± 0.14</td>
<td>3.22 ± 1.33</td>
<td>NS C vs VaD; 0.095 AD vs C; VaD vs AD, NS</td>
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<td>Aβ40, pmol/g</td>
<td>1,938 ± 1,670</td>
<td>2,701 ± 2,276</td>
<td>2,451 ± 435</td>
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<tr>
<td>Aβ42, pmol/g</td>
<td>4,600 ± 1,442</td>
<td>5,940 ± 1,290</td>
<td>20,855 ± 2,353</td>
<td>NS C vs VaD; p &lt; 0.0001 C vs AD; p &lt; 0.0001 VaD vs AD</td>
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<tr>
<td>Aβ42/40* (ratios)</td>
<td>33 ± 10</td>
<td>66 ± 22</td>
<td>709 ± 234</td>
<td>NS C vs VaD; p = 0.063 C vs AD; p = 0.060, VaD vs AD</td>
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<td>Synaptophysin RV (mAb EPI10)</td>
<td>154 ± 12</td>
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<table>
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<th>VaD</th>
<th>AD</th>
<th>Significance</th>
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<td>t-tau RV (mAb7.51)</td>
<td>172 ± 21</td>
<td>112 ± 13</td>
<td>211 ± 4</td>
<td>p = 0.021 C vs VaD; NS C vs AD; p &lt; 0.001 VaD vs AD</td>
</tr>
<tr>
<td>p-tau (Ser202/Thr205) RV</td>
<td>0.58 ± 0.02</td>
<td>0.61 ± 0.08</td>
<td>0.83 ± 0.13</td>
<td>NS C vs VaD; p &lt; 0.0001 C vs AD; p &lt; 0.006 VaD vs AD</td>
</tr>
<tr>
<td>p-tau (Ser262) RV</td>
<td>4.53 ± 0.39</td>
<td>4.46 ± 0.51</td>
<td>10.59 ± 1.89</td>
<td>NS C vs VaD; p &lt; 0.001 C vs AD; p &lt; 0.0001 VaD vs AD</td>
</tr>
<tr>
<td>p-tau (Thr181) RV</td>
<td>36.87 ± 2.29</td>
<td>37.47 ± 2.34</td>
<td>35.09 ± 2.11</td>
<td>NS C vs VaD; NS C vs AD; NS VaD vs AD</td>
</tr>
<tr>
<td>t/ptau (Ser262)</td>
<td>63.82 ± 21.30</td>
<td>18.41 ± 3.29</td>
<td>30.03 ± 8.82</td>
<td>NS C vs VaD; NS C vs AD; NS VaD vs AD</td>
</tr>
<tr>
<td>t/ptau (Thr181)</td>
<td>84.94 ± 28.45</td>
<td>24.36 ± 5.21</td>
<td>75.48 ± 22.28</td>
<td>NS C vs VaD; NS C vs AD; 0.046 VaD vs AD</td>
</tr>
<tr>
<td>Aβ40, pmol/g</td>
<td>663 ± 498</td>
<td>3,784 ± 1,693</td>
<td>2,175 ± 1,707</td>
<td>NS</td>
</tr>
<tr>
<td>Aβ42, pmol/g</td>
<td>9,060 ± 2,492</td>
<td>13,975 ± 2,563</td>
<td>24,786 ± 1,775</td>
<td>p = 0.079 C vs VaD; p &lt; 0.0001 C vs AD; p &lt; 0.008 VaD vs AD</td>
</tr>
<tr>
<td>Aβ42/40* (ratios)</td>
<td>138 ± 48</td>
<td>165 ± 64</td>
<td>951 ± 357</td>
<td>NS C vs VaD; p = 0.027 C vs AD; p = 0.030 VaD vs AD</td>
</tr>
</tbody>
</table>

Numbers represent mean ± SE for n = 16 to 18 cases. Age ranges: controls, 49 to 99 years; VaD subjects, 52 to 91 years; AD, 58 to 91 years.

T-tau, p-tau, and synaptophysin represented in relative values (RVs) per indirect ELISA (24). Significance: differences in means determined by Mann-Whitney U test, followed by Bonferroni correction to adjust the probability.

*A represent mean ratios calculated from individual values for each case.

Aβ, amyloid-β; AD, Alzheimer disease; C, controls; NS, no significant difference; VaD, vascular dementia.
Another factor causing the reduction in t-tau protein could be its removal via certain cellular mechanisms, including elimination by macrophages/microglia (39). The activated microglia contribute to tau protein accumulation within neurites juxtaposed to the microglia sites rather than at the cell body (40). Studies in mice transgenic for human P301S tau protein suggest that the increase in microglial inflammatory markers (e.g., interleukin-2 and -4) is associated with a decrease in both soluble and phosphorylated tau protein before the onset of neuronal neurodegeneration (41).

Besides the genetic and microglia links, other animal studies also provide further evidence for the relationship between altered blood vessel function and brain/neuronal changes. The impact on the neurovascular unit, including nerve endings with cytoskeletal proteins such as tau and tubulin, depends on the degree of tissue damage. Thus, the loss will be varied whether there is a direct hit (i.e., focal [ischemic] injury) or in the adjacent hypoperfused (penumbral) region or in the normal-appearing possibly remote sites. However, long-term changes in blood pressure (in particular, increased systolic blood pressure) are associated with reduced gray matter volumes, particularly affecting the temporal lobes (42); this argues that blood pressure may influence neuronal integrity, including tau protein expression.

Our findings of a lower and selective decrease in Thr181 phosphorylation corresponding to the higher tau protein isoforms are intriguing. They suggest a putative role of the higher tau isoforms in generating the VaD phenotype. A previous study reported that phosphorylation affects each of the tau isoforms differently and influences distinct tau protein polymerization profiles (43). Although the overall Thr181 measures in the frontal and temporal lobes were similar between the 3 groups, the Thr181 68-kDa band in the VaD cases was 20% to 50% lower compared with those in controls and AD cases. Further work is needed to determine whether there are specific phosphokinases or phosphatases involved in this molecular change.

In contrast to the measurable Thr181 and Ser262 tau phosphorylation sites, we found relatively low levels of p-tau Ser202/Thr205. Although we cannot exclude the contribution of postmortem endogenous dephosphorylation of tau protein that can occur as early as 2 hours postmortem at room temperature (44), the observed results may be attributed to distinct temporal profiles of tau protein phosphorylation. Thus, Thr181 and Ser262 p-tau have been associated with both pretangle (45, 46) and early stages of neurofibrillary pathology (47), whereas the phosphorylation at Ser202/Thr205 site appears to be linked with advanced AD stages and immunolabels predominantly extracellular (ghost) neurofibrillary tangles. The intracellular staining is largely confined to neuritpl plates and tangle-bearing neurons with collapsed dendrites (47). A confocal imaging study showed that mAb AT8 incorporation within both early (e.g., pretangles) and late (coexisting with the truncated tau protein in the extracellular tangles) neurofibrillary pathology may not necessarily be associated with typical β-sheet conformation (48).

Furthermore, a biochemical study comparing sarcosyl and formic acid–extracted PHFs (immunolabeled with mAbs AT8 and 7.51, respectively) found only a minor portion (1:7) of the paired helical filaments to have either N-terminally intact or phosphorylated tau protein (49). This and the lack of β-sheet sheets in end-stage tangles may explain the low yield of p-tau in the AD subjects. A key limitation of our observations relates to the lengthy postmortem delays of the autopsy sample. Although we did not observe any relationship between postmortem interval and any of the protein assay measures, it may be necessary to avoid introducing any biochemical bias in terms of protein loss and altered phosphorylation state (44).
This suggests that t-tau increases in the CSF in tandem with age-related brain atrophy likely due to different causes.

In contrast to the age-related accumulation of soluble Aβ proteins in VaD, our observations support the notion that age-related vascular injury or tissue microinfarcts lead to loss of tau proteins with the consequent impact on phosphorylation and accumulation of the neurofibrillary pathology of AD (56). Thus, cerebrovascular pathologic (57) mechanisms are among the factors that influence the progression of distinct dementia syndromes.

ACKNOWLEDGMENT

We are indebted to all of the patients and their families who have participated in the study.

REFERENCES
