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Ferritin Is Associated with the Aberrant Tau Filaments Present in Progressive Supranuclear Palsy

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Tau-containing filaments purified from the brain of progressive supranuclear palsy (PSP) patients were isolated and characterized. These filaments co-purify with regular particles that biophysical and biochemical methods identified as ferritin shells. In vivo, brain tau accumulation in PSP co-localized with ferritin. These results suggest that ferritin/iron could modulate the formation of tau aggregates in PSP. ***(Am J Pathol 1998, 152:1531–1539)***

Progressive supranuclear palsy (PSP), also known as Steele-Richardson-Olszewki syndrome,[1] is a neurodegenerative disease clinically characterized by subcortical dementia, akinesia, dystonia, supranuclear gaze palsy, dysarthria, dysphagia, and disturbances of equilibrium. The pathological process, which mainly involves the cortex, basal ganglia, and brain stem nuclei,[2,3] is characterized by atrophy, loss of neurons, and gliosis in these three brain areas and by the formation, in affected neurons, of accumulations of neurofibrillary tangles derived from the microtubule-associated protein tau. These tangles are mainly composed of straight filaments with a diameter of 10 to 16 nm.[2,4–12] Additionally, other filamentous abnormalities have been noted as well as glia.[13,14]

Abnormal tau filaments occur in other neurological disorders, including Alzheimer's disease (AD),[15–25] corticobasal degeneration,[26] Nieman-Pick type C disease,[27] and familial dementia.[28] However, the localization of the brain lesions, the presence of tau-aberrant structures in different cell types, and the morphology of those filament structures are disease specific. In damaged neurons, helical and straight filaments are the characteristic filaments of AD and PSP,[4–12] respectively.

Although native or recombinant tau[29–33] on the absence of other proteins or cofactors is able to assemble into polymers resembling paired helical filaments (PHFs) from AD, it has been described that molecules such as heparin or other sulfated glycans facilitate assembly.[34,35] Moreover, it has also been suggested that the helicity of PHFs is modulated by sulfated glycans.[36] It is not known whether other tau filaments present in other disorders, such as PSP, may also require cofactors to facilitate their assembly and to determine their final morphology.

In this work we have focused in the isolation and characterization of the tau polymers present in PSP. We have tested whether other structures or molecules co-purify with tau filaments to identify tau polymer-associated factors that could be involved in the facilitation of tau aggregation in PSP.

Our results suggest that ferritin may be one of those factors. Ferritin is constituted by a protein shell composed of 24 subunits and an 8-nm inorganic core containing several microcrystals of iron oxide.[37] Identification of ferritin molecules can be carried out by biochemical methods (in the case of the protein shell) and/or physical methods (in the case of the inorganic core). One of the most powerful techniques for studying the structure of ferritin is electron microscopy, by which the morphology of the inorganic core or its composition can be studied by transmission or analytical electron microscopy, respectively.

Materials and Methods

Ferritin and antibodies to ferritin were purchased from Sigma Chemical Co., St. Louis, MO, and from Ramco Laboratories, Houston, TX. These antibodies mainly react with glia cells. Tau antibodies PHF-1 and 333 have been described elsewhere.[38,39] PHF-1 was a kind gift from Dr. Davies (A. Einstein University). Chondroitin antibodies were purchased from Seikagaku Corp., Tokyo, Japan.

Brain Samples

PSP samples were provided by the Banco de Tejidos para Enfermedades Neurológicas, Madrid. The Netherlands Hersen Bank (Dr. Ravid) provided us with samples from two nondemented controls and from two AD patients. The brain

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areas used for these studies were the frontal and cerebral cortex, the basal ganglia, and the brain stem.

Isolation of Filaments

Filaments from the brain of PSP patients were isolated as previously described for PHF-tau, from AD, by Greenberg and Davies.[38] Three sources were used to isolate PSP filaments: temporal and frontal cerebral cortex and brain stem. Many more filaments were isolated from the region constituting the basal ganglia and brain stem (an area preferentially damaged in PSP).

Immunohistochemistry

After fixation by immersion in 4% formaldehyde, tissue blocks were obtained and subsequently embedded in paraffin. For immunocytochemistry, serial 8-μm paraffin sections were obtained from several blocks corresponding to different areas of basal ganglia and brainstem. For colocalization purposes, a double immunohistochemical technique was performed. Tissue sections were first incubated with a monoclonal antibody, PHF-1 (1:100 dilution), for 1 hour at room temperature. Immunostaining was subsequently amplified with an ABC method (Vectastain, Vector Laboratories, Burlingame, CA) and developed using a glucose oxidase/diaminobenzidine/nickel developing solution.[39] Sections were incubated with a polyclonal antibody to ferritin (1:1000 dilution) for 1 hour at room temperature and developed with a highly sensitive alkaline phosphatase procedure (LSAP plus, Dako Laboratories, Glostrup, Denmark) using Fast Red as chromogen.

Electron Microscopy

Sample Preparation

One drop of isolated filaments solution was placed on a carbon-coated grid for 2 minutes and then either stained with 2% uranyl acetate for 1 minute or left unstained. For immunoelectron microscopy, samples were adsorbed to the grids and incubated with rabbit antiserum 333 (1:10 dilution) raised against tau[40] for 1 hour at room temperature. After extensive washing, the grids were incubated with goat antiserum to rabbit IgG conjugated with 5-nm-diameter gold particles. Finally, the samples were negatively stained as described above.

Transmission Electron Microscopy

Grids with or without uranyl acetate contrasting were observed in a TEM JEOL 1200 EXII operated at 120 kV. Electron micrographs were obtained at a magnification of ×50,000 on Kodak SO-163 film developed with D19 developer at full strength for 12 minutes. For measuring purposes, micrographs were digitized using an Eikonix IEEE-488 and processed using the Digital micrograph 2.1 software from Gatan (Pleasanton, CA).

Analytical Electron Microscopy

Unstained isolated filaments were observed in a TEM JEOL 1200 EXII operated at 120 kV and equipped with an electron energy loss spectrometer PEELS Gatan 666-9000 and an Image filter GIF-100. This instrument allows the separation of acquisition of parallel electron energy loss spectra and energy filter images (EFTEM). The energy loss spectrometer obtains plasmon images (images related to the density of the chemical components of the sample) and the elemental maps (images of the distribution of a chemical element). In the case of ferritin, Fe atoms can be identified in EELS due to the two characteristic inner shell ionization edges, the $M_{2,3}$ at 54 eV and the $L_{2,3}$ at 707 eV. Elemental maps of Fe can be obtained from these two peaks. For this purpose, it is necessary to remove the background contribution. The $M_{2,3}$ edge has been used to obtain the Fe map whereas the removal of the background has been performed by a three-windows method that uses a power law model.[41] The three windows, two pre-edge-filtered images at 40 ± 5 and 50 ± 5 eV, respectively, and a post-edge-filtered image at 60 ± 5 eV, were obtained using a slow scan CCD of 512 × 512 pixels.

Preparation of Tau Protein

Recombinant human tau, containing three tubulin-binding motifs and one extra exon near the amino-terminal end or tau fragments 4RC, 3RC, 4R, and 3R containing four or three tubulin-binding motifs with or without the carboxy-terminal region, were expressed and purified as previously indicated.[42,43] The nomenclature used for the residues of these tau isoforms is that of the longest tau isoform.[44]

Iron Sepharose Chromatography

The procedure described by García de Ancos *et al*[45] was followed. Columns were prepared with chelating Sepharose Fast Flow (Pharmacia Fine Chemicals, Uppsala, Sweden) as described previously.[45] Briefly, the gel bed was washed with 4 vols of distilled water, charged with 4 vols of 50 mmol/L $FeCl_3$, and finally washed again with distilled water. The column was equilibrated in binding buffer (0.1 mol/L MES, 1 mol/L NaCl, and 1 mmol/L dithiothreitol (DTT), pH 5.7) before protein loading.

Tau protein was loaded onto the ICAC column after exchanging buffer A (0.1 mol/L 2-(*N*-morpholin) ethanesulfonic acid, 0.5 mmol/L $MgCl_2$, and 2 mmol/L EGTA, pH 6.4) for binding buffer. This was achieved by passing an aliquot of tau protein through a Sephadex G-25 column previously equilibrated with binding buffer. Once tau protein was loaded onto the ICAC column, it was washed with binding buffer and eluted by a stepwise pH gradient composed of the following solutions: 0.1 mol/L MOPS, 1 mol/L NaCl, and 1 mol/L DTT at pH 6.00, 7.00, 7.10, 7.20, 7.40, and 7.60 and 0.1 mol/L Tris /HCl, 1 mol/L NaCl, and 1 mmol/L DTT at pH 7.80, 8.00, and 8.50. Protein fractionation was analyzed by SDS-PAGE sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

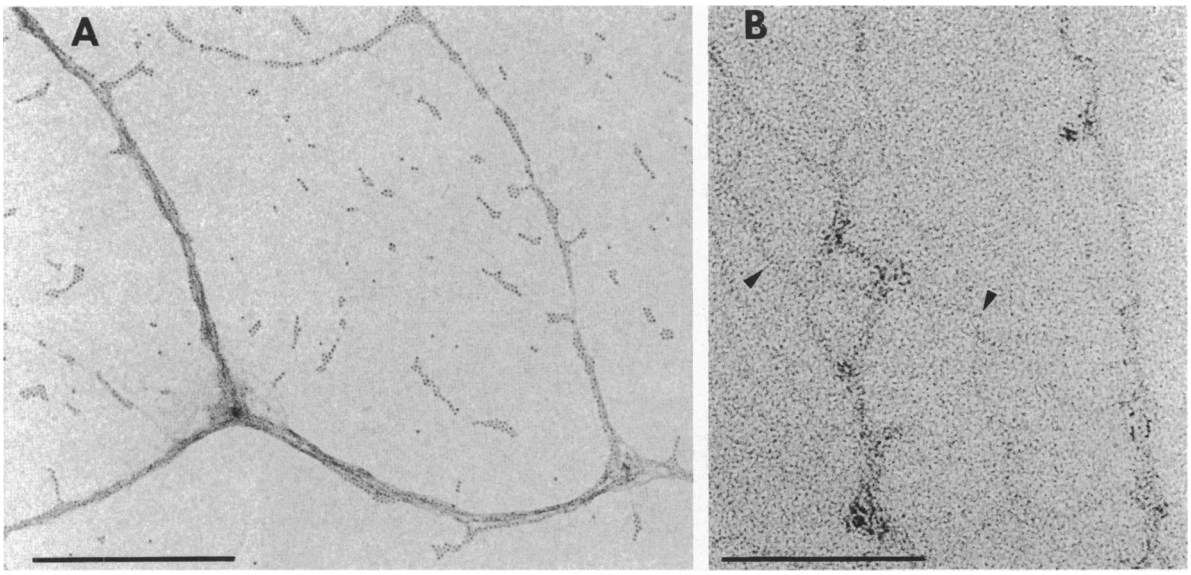

Figure 1. Electron micrographs of negatively stained PSP filaments. **A:** Low-magnification field of PSP filaments covered with electron-dense particles forming a paracrystalline array. The filaments can be several microns long. **B:** High-magnification field of PSP filaments with some electron-dense particles interacting with them. **Arrowheads** indicate the basic filament (2-nm diameter). Bar, 500 nm (**A**) and 100 nm (**B**).

Immunoblot Analysis

Electrophoresis was carried out as previously described[45] on 1-mm-thick 8% polyacrylamide slab gels.

Immunoblot analysis was also done as previously described.[45]

Assembly of Recombinant Tau into Filaments

Tau filaments were assembled as previously indicated by Pérez *et al.*[34] Briefly, filaments were grown by vapor diffusion in hanging drops in the standard way used for protein crystallization.[30] Fourteen micrograms of recombinant tau proteins was lyophilized and resuspended in 10 to 15 μl of buffer A (see above) plus 50 mmol/L NaCl in either the absence or the presence of 0.05 μmol/L ferritin and 10 μmol/L ascorbate (see Ref. 46). The reservoir contained 0.2 mol/L NaCl in buffer A. Filaments were obtained after incubation for 4 days at 4°C.

Aggregation of tau protein in the presence of ferritin was tested by incubation of tau protein (or fragments of it) at 1 mg/ml with increasing amounts of ferritin for 4 days at 4°C in a final volume of 20 μl. Samples were centrifuged for 15 minutes at maximal speed in an Airfuge (Beckman, Palo Alto, CA), and the protein present in supernatant and pellet was analyzed by SDS-polyacrylamide gel electrophoresis. The amount of protein was quantified by densitometry of the fractionated protein after Coomassie Brilliant Blue staining of the gel and comparison with the results obtained using known amounts of bovine serum albumin.

Results

Tau Filaments from PSP

Previous reports[4–12] have indicated that the neurofibrillary tangles of PSP are composed of filaments with a diameter of 10 to 16 nm, when fixed brain specimens were examined by electron microscopy.

Here we use the method of Greenberg and Davies[38] to isolate PHF-tau from AD to isolate analogous polymers from PSP.

We found that the isolated filaments of PSP (Figure 1A) are much longer than the average filament found in PHF-tau preparations from AD (up to several microns). Furthermore, the diameter (2 nm; Figure 1B, see arrows), was thinner than that described in fixed brain specimens of PSP[4–12] but was similar to that of tau filaments described by Ruben *et al.*[46] The 2-nm filaments often occurred in parallel bundles of up to 20 nm.

Immunoelectron microscopy with the tau antibody demonstrated that it is a component of the isolated PSP filaments (Figure 2, A and B). Moreover, when the filament preparation was isolated by sedimentation, its protein fractionated by gel electrophoresis, and blotted to nitrocellulose, a clear reaction with the tau antibody was observed with proteins corresponding in electrophoretic mobility to phosphorylated tau (data not shown). Additionally, two proteins with an electrophoretic mobility of ~20 k_d, not immunologically related to tau, are present (data not shown).

Co-Purification of Regular Particles with Tau Polymers from PSP

Electron microscopy analysis of tau filaments from PSP indicated regular particles in close apposition to tau-containing filaments. Electron microscopy of contrasted specimens show that the particles have an intrinsic high electron density, suggesting the presence of a heavy element. Two possibilities could explain the presence of electron-dense particles in our preparations of PSP fila-

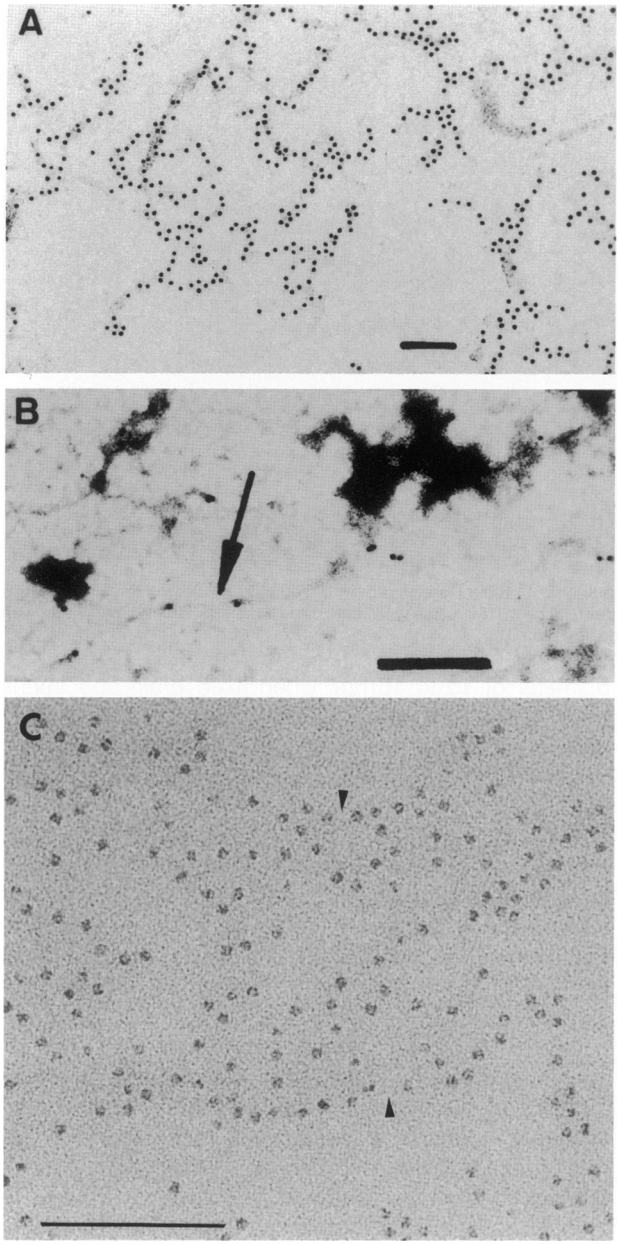

Figure 2. Electron microscopy of PSP filaments. **A** and **B:** Interaction of the PSP filaments with antibodies raised against tau protein can be seen. A fraction containing PSP filaments in the aggregated form (**A**) or in the form of thin filaments (**B, arrow**) was incubated with anti-tau antibody (ab333), and the reaction was tested by using a secondary antibody coupled to gold particles. (**C:**) Field of unstained PSP filaments in which particles with electron-dense core can clearly be observed. **Arrowheads** point to PSP filaments that are also detected. Bar, 200 nm (**A** and **B**) and 100 nm (**C**).

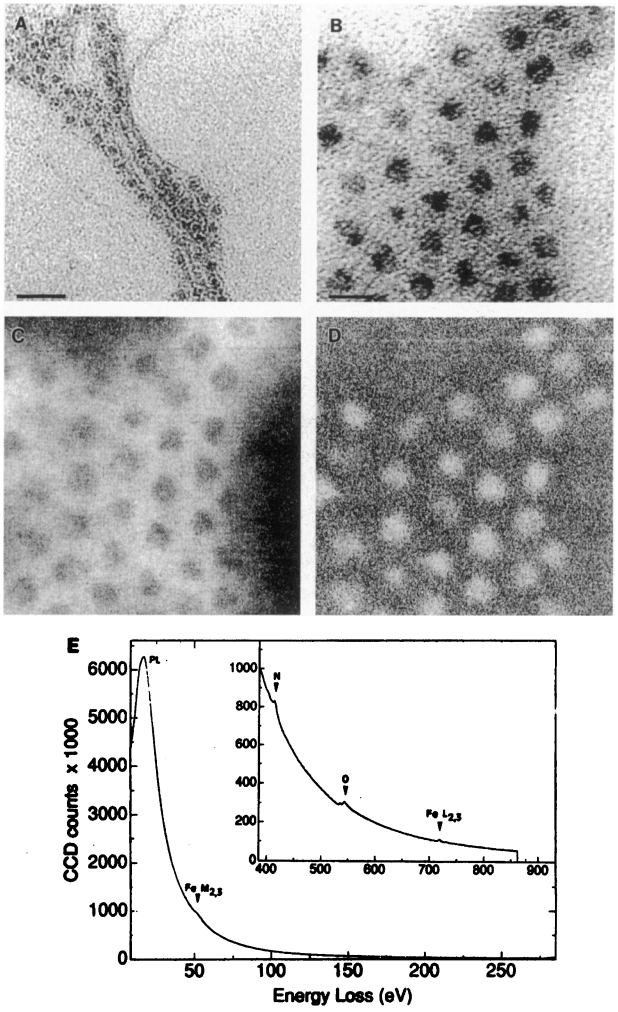

Figure 3. Elemental Fe map of PSP filament. **A:** Electron micrograph of negatively stained PSP filaments. **B:** Higher magnification of an unstained area, showing the ferritin-like particles. **C:** Plasmon image of the same area, showing the presence of proteinaceous material around the electron-dense cores. **D:** Fe map of the same area obtained according to the procedures described in Materials and Methods. The Fe signal is located exclusively on the proposed ferritin cores. **E:** Energy loss spectrum from the sample with PSP filaments and the putative ferritin particles. The spectrum shows the plasmon (PL) and the $M_{2,3}$ Fe-containing region. The **inset** shows a magnification of the higher-energy region (400 to 900 eV), containing the N_K, O_K, and $L_{2,3}$ Fe regions, indicating that the sample is a protein containing Fe. Bar, 40 nm (**A**) and 20 nm (**B–D**).

ments. One is an association between the structures. The other is their nonspecific co-purification. To examine these possibilities, the same protocol was used for the isolation of filaments, but in this case a brain sample from a patient without neurological disorders, but with a similar age to that of the PSP patients, was tested. We found no electron-dense particles in the control sample. As this result supports a specific interaction, we were interested in the molecular analysis of that interaction.

Ferritin Molecules Interact with PSP-Tau Filaments

Having an electron-dense core (Figure 3A) strongly suggested that the particles could be ferritin molecules. The dimension of the particle (13.4-nm diameter) is similar to that reported for ferritin.[37] Consistent with ferritin (Figure 3B) the particles revealed no contrast in the periphery of the particles whereas the core had a strong contrast. The diameter of the core (8 nm) again coincided with that of the ferritin core. Figure 3C shows the plasmon image of the same area as in Figure 3B, which reveals the presence of proteinaceous material around the electron-dense core.

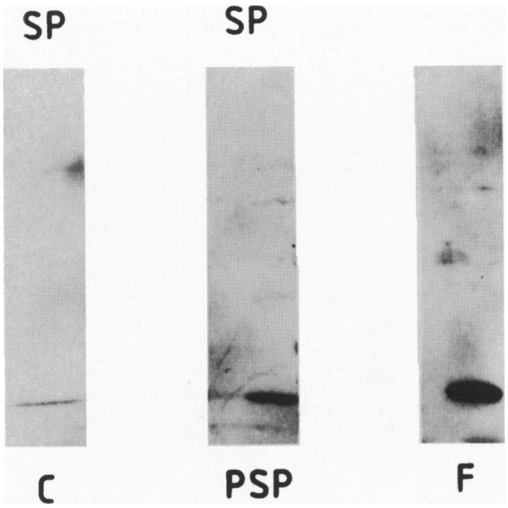

Figure 4. Presence of ferritin in the particulate fraction of PSP brain. Identical amounts of protein from particulate (P) and soluble (S) fractions from a PSP (PSP) or a control (C) brain were fractionated by gel electrophoresis and blotted with an anti-ferritin antibody. In parallel, the reaction of the antibody with purified ferritin (F) was tested.

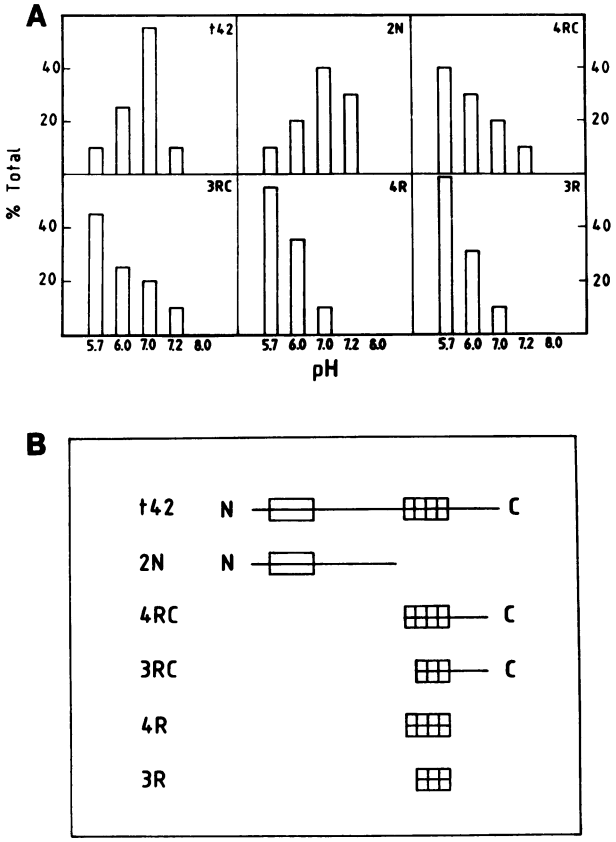

Figure 5. Interaction of iron with tau fragments. **A:** The whole recombinant tau or fragments of it (see **B**) were chromatographed on an iron-Sepharose gel, and the attached protein was eluted stepwise by increasing the pH of the eluting buffer. The key for the identification of the different tau peptides is in **B**. On the ordinate is shown the percentage of eluted protein at different pH values with respect to the total protein chromatographed. **B:** Scheme of tau fragments. The whole tau protein (t42) containing exons 2 and 3 (large box) and tau fragments containing the amino-terminal region (2N), the three or four tubulin-binding motifs, identified as small boxes, together with the carboxy-terminal region (4RC, 3RC), or only the (three or four) tubulin-binding motifs (4R, 3R) are indicated. These different peptides were used for the experiment shown in **A**.

To test whether these particles contain iron atoms, electron energy loss spectrometry experiments were carried out as described in Materials and Methods. Figure 3E shows the spectra obtained from approximately 20 particles, which revealed the presence of the characteristic $M_{2,3}$ and $L_{2,3}$ peaks from the iron atoms. The K peaks from N (nitrogen) and O (oxygen) can also be observed, which suggested that the structure under study is a protein with a strong iron content. Finally, the iron map from the same area as in Figure 3, B and C (Figure 3D) reveals the presence of iron in the cores of these particles.

A biochemical characterization of the PSP-tau filament fraction was also carried out (Figure 4). When the fraction was separated by electrophoresis in SDS, a protein running with the mobility corresponding to 20 kd (the identical molecular weight of the polypeptides constituting ferritin particles) was found. The 20-kd protein reacted with a ferritin antibody. The reaction was clearly found in the particulate fraction of a brain stem extract from PSP patients whereas only a weak reaction was observed in a similar fraction from a control brain.

Tau-Ferritin/Iron Interaction

These results suggest that ferritin may be specifically bound to tau polymers. Indeed, in other studies, it was observed that recombinant tau protein associates with ferritin shells, suggesting that tau could bind to other iron-containing proteins.[47]

To study this further, tau and ferritin iron were mixed and the formation co-assemblies noted. Furthermore, based on a previous report[48] indicating that iron, in the presence of ascorbate, is able to induce formation of tau polymers, we also added ascorbate. Electron microscopy analysis showed increased tau filament formation in the presence of ferritin/iron and ascorbate. Filaments are straight and wider (3.5-nm diameter) than those isolated from PSP brain (data not shown).

As an additional experiment, we tested whether tau protein was able to bind iron. Figure 5A shows that recombinant tau and its fragments can bind to an iron-Sepharose column. Previous reports have indicated that such binding could be due in part to the phosphorylation of tau protein.[45] However, in the experiment shown in Figure 5, recombinant (unmodified) tau was tested, which indicates that tau protein can bind to iron in the absence of phosphate groups (although, as previously indicated,[45] presumably in the phosphorylated form the affinity for iron is increased). Figure 5A shows that the whole tau molecule binds to iron-Sepharose and that it elutes at the same pH to that of a tau fragment containing the amino-terminal half of the molecule, whereas in contrast, elution of the fragments containing the tubulin-binding motifs or those motifs plus the carboxyl-terminal region require only a lower pH to be eluted from the column (Figure 5B).

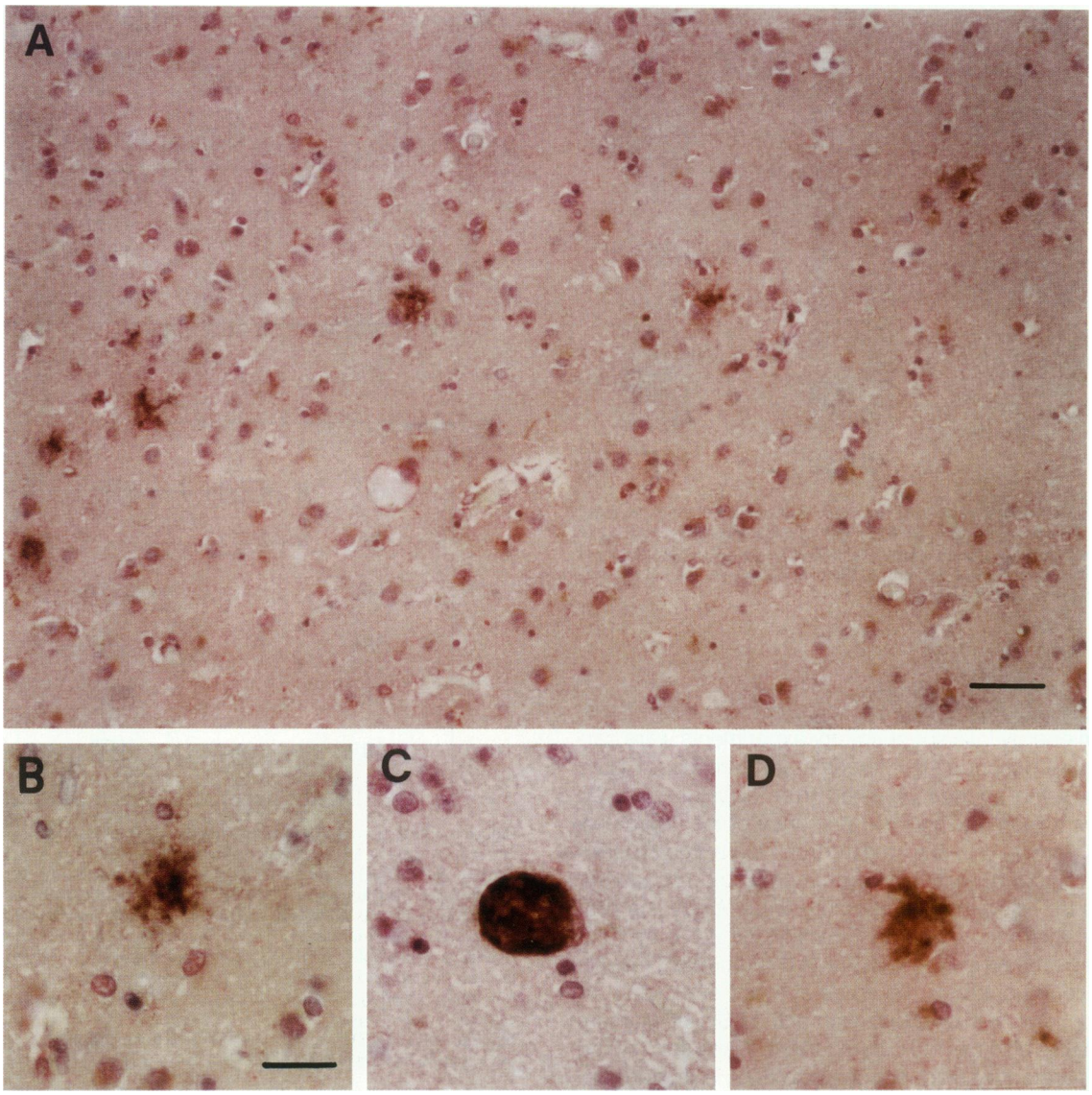

Figure 6. Ferritin and tau cell staining. **A:** A section of the caudate nucleus from a case of PSP was subjected to double-immunofluorescence analysis (red for ferritin, black for PHF-tau.) The photograph shows several double-stained glial cells. **Insets** show the appearance of some of these cells (**B** and **D**) together with that of a neuron (**C**) at higher magnification. Bars, 50 μm (**A**) and 15 μm (**B**).

Co-Localization of Tau and Ferritin in Brain Cells

It has been suggested that ferritin and tau could associate to form filaments and thus could co-localize in brain areas, such as basal ganglia, that are mainly affected in PSP patients. To test for that, the reaction with anti-ferritin antibodies and anti-tau antibody in slices of fixed brain specimens was done in a field from the caudate nucleus of a PSP patient. Some of the cells in which we found the co-localization had a glial morphology (Figure 6, more evident in the inset). In Figure 6, a neuron containing tau filaments and a much lower amount of ferritin is also shown.

Discussion

The isolation of filaments from the brain of PSP patients reveals filaments with a diameter from 2 to 20 nm. A possible interpretation for this variability could be that the narrow filaments are able to interact with each other to form wider filaments, which are analogous to the filaments observed in brain specimens.

The width of the basic PSP filament (2 nm) is very similar to that described by Ruben *et al*[46] for tau filaments. Tau protein could have variable conformations.[49,50] If we consider tau as a cylinder (something very reasonable given the narrowness of the filament) of 1-nm radius, and given the average molecular weight of tau (40 kd) and the average density of a protein (0.8 kd/nm^3), the dimensions of a tau monomer would be those of a cylinder of 16-nm length and 2-nm diameter. Therefore, the PSP filaments would be composed of tau monomers interacting head to tail, that is, the carboxyl terminal of a monomer interacting with the amino-terminal of the following one.

To identify molecules that favor assembly of tau filaments into the straight filaments we examined those pro-

teins that co-purify with PSP filaments. A major 20-kd protein, characterized like ferritin, was observed.

A previous report[51] indicates the association of ferritin with tau filaments from AD, an association that could be destroyed in the presence of ionic detergents.[52]

The association of the PSP filaments with ferritin prompted the study of whether there is a specific interaction between the PSP filaments and ferritin or whether they merely co-purify. The results clearly indicate that there is an interaction.

First, ferritin was not isolated by the same procedures from control brains. *In vitro* ferritin particles were directly bound to the tau filament. Finally, ferritin and tau co-localize in brain cells and levels of iron and ferritin are increased in PSP.[53,54]

Tau-iron binding experiments suggest that the iron-binding site is in the amino terminal. In this region, residues 40 to 48 (HQDQEGDTD) are similar to the iron-binding site in ferritin.[37,55] However, other possible binding sites, such as the region containing acidic residues at the carboxyl-terminal part of the molecules, cannot be excluded.

Iron abnormalities have been postulated in neurological diseases. Iron entry by mammalian cells is through a cell receptor (transferrin receptor) whereas homeostasis is additionally maintained by the coordinated expression of ferritin present inside the cell.[56] In the diseases involving iron overload, oxidation stress occurs together with other secondary effects, such as an increase in collagen formation (fibrosis) (for a review see Ref. 37). In neurological diseases, such as AD, Parkinson's disease, and PSP, changes in iron content are reflected in an increase of ferritin compared with that of control samples.[53] In PSP, elevation of iron in the substantia nigra correlates well with that of ferritin.[53] If both tau and ferritin/iron are involved in the formation of tau polymer in PSP, it should be a co-localization of both proteins in a single cell, and in this work we found that this co-localization may occur in brain cells. A co-localization has been previously suggested in glia cells from AD patients.[57]

Based on the previous results, we can propose a working model to explain the mechanism for the formation of tau accumulations in PSP. It involves the association of tau with ferritin. Iron is sequestered in a nontoxic but accessible form in ferritin.[55] Thus, if ferritin is bound to tau, accessible iron will also be closed to tau molecules as there is an equilibrium between iron that is in the free form and iron bound to ferritin, and the first form could be available to bind to tau. Ferritin can be composed of two different subunits, H and L.[56] The proportion of these subunits is different in different cell types, such as glia and neurons, being H-subunit predominant in neurons.[55,58] Also, it has been suggested that the interchange between free and ferritin-bound iron is more dynamic for the H subunit, where iron can be more rapidly utilized than that bound to the L subunit.[58,59]

On the other hand, we cannot exclude another possibility to explain the association of ferritin with tau filaments. This possibility suggests an antitoxic effect for ferritin. In this way, ferritin will associate with the iron bound to tau filaments to remove the toxic metal. As a lower proportion of ferritin is found in neurons compared with glia cells, neurons will be more sensitive to iron. Additionally, this possibility suggests that the association of ferritin with tau filaments will take place mainly in glia cells.

Thus, a mechanism involving an oxidation process mediated by iron to promote tau polymerization in PSP can be taken into account. A similar mechanism has been proposed mainly by Perry's group for other neurological disorders, such as AD.[60–63] It has been indicated that iron can mediate a variety of redox reactions involving H_2O_2 to produce oxidative species[64] and that the presence of iron will facilitate oxidative protein modifications by reducing sugars, modifications that have been observed in AD.[39,65] Moreover, in a recent publication, it has been also indicated that iron accumulation that co-localizes with neurofibrillary tangles could be an important contributor toward the oxidative damage found in Alzheimer's disease.[66] Interestingly, it was also indicated in that work that the binding of iron to neurofibrillary tangles could be blocked by a compound that binds to histidine residues, and that amino acid is in the putative iron-binding motif of tau, as indicated above.

Thus, in PSP, as in different neurological diseases, iron could have an important role to promote tau aggregation.

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