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Argyrophilic grain disease and Alzheimer's disease are distinguished by their different distribution of tau protein isoforms

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Abstract Prominent neuronal and glial tau filamentous inclusions are hallmarks of neurodegenerative tauopathies, among them Alzheimer's disease (AD), progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease (PiD), and argyrophilic grain disease (AgD). AgD is a late onset dementia in which pathologically aggregated tau proteins are found in limbic structures in the shape of distinct argyrophilic grains and coiled bodies. Until now tau protein deposits in AgD have not been assessed biochemically. We therefore decided to investigate the electrophoretic profile of pathological tau protein as well as the tau protein isoform composition of filamentous inclusions in AgD cases. A distinct pathological tau doublet at 64 and 69 kDa and a minor 74-kDa band was obtained in two AgD cases with only very mild concomitant AD pathology (Braak stage I), while in two AgD cases with moderate AD pathology (Braak stage II and III, respectively), an additional minor band at 60 kDa was detected. Thus, the pathological tau profile (PTP) in pure AgD cases differs from both the PTPs in AD (tau triplet at 60, 64 and 69 kDa, minor band at 74 kDa) and PiD (major tau doublet at 60 and 64 kDa, minor band at 69 kDa) but not from those in PSP and CBD. Using a two-dimensional gel electrophoresis approach anti-exon 10 antiserum strongly stained the AgD doublet and the minor 74-kDa band, while anti-exon 2 and 3 antisera only faintly stained the 69- and the minor 74-kDa component, thus suggesting that pathological tau aggregates in AgD are mainly made of four-repeat (4R) tau isoforms. Furthermore, in contrast

to earlier immunohistochemical studies, we now show biochemically that Ser262 indeed is phosphorylated in the PTP of AgD. Finally, expression of normal tau protein was not found to be altered in AgD. Altogether, our results demonstrate that AgD is characterized by a major tau doublet that is distinct from AD and PiD. AgD, however, shares the pathological tau doublet (64 and 69 kDa) as well as the predominance of 4R tau isoforms with CBD and PSP.

Keywords Alzheimer's disease · Argyrophilic grain disease · Microtubule-associated protein tau · Neurodegenerative disorders · Protein phosphorylation

Introduction

Alzheimer's disease (AD) is neuropathologically characterized by the presence of abundant neurofibrillary lesions (NFL) formed by the hyperphosphorylated microtubule-associated protein tau, and deposits of fibrillar amyloid β peptides. Prominent filamentous tau inclusions in the absence of β amyloid deposits are also hallmarks of a heterogeneous group of dementias and movement disorders, collectively known as neurodegenerative tauopathies [7, 20]. They include among others, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease (PiD), frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), and argyrophilic grain disease (AgD).

Hallmark lesions of AgD, a late-onset dementia first described by Braak and Braak [4, 5], are abundant argyrophilic grains (ArGs) in neuronal processes and coiled bodies in oligodendrocytes. Both ArGs and coiled bodies consist of the microtubule-associated protein tau in a hyper- and abnormally phosphorylated state. We have recently shown that the tau protein of ArGs and coiled bodies shares a high number of phosphorylated sites with the NFL of AD [33]. Moreover, a striking feature in AgD cases consists of a widespread hyperphosphorylation of the tau protein in the somatodendritic domain of limbic

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projection neurons [6, 32], similar to the pretangle neurons in AD [1].

One of the reasons why AgD is still questioned as a disease entity distinct from AD, is the observation that most AgD cases show at least some associated changes of the AD type, e.g., NFL [6, 35]. Therefore, AgD has often been considered as a variant of AD. Furthermore, co-occurring ArGs have been reported in a variety of neurodegenerative disorders, among them PiD, PSP, CBD, Parkinson's disease and the tangle-predominant form of senile dementia (for review see [18, 35]). Thus, it has been suggested that AgD may be classified as a variant of the lobar atrophy complex (Pick's complex) or PSP [23].

Biochemical analysis of tau protein abnormalities recently emerged as a powerful tool complementing neuropathology for a rational classification of tauopathies. Thus, in AD, all six brain tau isoforms are abnormally phosphorylated, and are detected by immunoblotting as a major tau triplet (tau 60, 64 and 69). In CBD and PSP, mainly tau isoforms with four microtubule-binding domains (4R) aggregate into filaments, and they appear as a major tau doublet (tau 64 and 69). This contrasts with another major tau doublet in PiD (tau 60 and 64), in which filamentous tau deposits are predominantly made up of isoforms with three microtubule-binding domains (3R) (reviewed in [7, 20]).

To further characterize AgD within the heterogeneous group of tauopathies we now assessed biochemically the electrophoretic profile of pathological tau protein as well as the tau protein isoform composition of filamentous inclusions in AgD cases.

Material and methods

Brains from four demented subjects with ArGs were investigated in the study (Table 1). Routine neuropathological examination, histology (including Gallyas staining) and immunohistochemistry were performed as previously described [33].

SDS-PAGE and Western blot

For electrophoresis and Western blot analysis, brain tissues were processed as previously described [14]. Briefly, brain tissues were homogenized in lysis buffer (TRIS-HCl 100 mM pH 6.8, 5% SDS, 20% glycerol, 20 mM DTT) using a Teflon potter and boiled 10 min at 100°C. Protein, 100 µg, was loaded and run on a 8–16% gradient SDS-PAGE gel. Following electrophoresis, proteins were transferred onto nitrocellulose using the Semi-Dry system Nov-

ablot (Amersham-Pharmacia Biotech, Orsay, France) according to manufacturer's instructions. The nitrocellulose membrane was then blocked in TRIS-HCl 50 mM pH 8.4, 150 mM NaCl, 0.1% Tween-20 (TNT) buffer added with 5% of dry skimmed milk. Following three washes with TNT, the membrane was incubated with the primary antibody for 2 h at room temperature. The membrane was washed three times in TNT and incubated with a mouse or rabbit secondary antibody coupled with horseradish peroxidase (Sigma-Aldrich, St. Quentin Fallavier, France). The immunoreactive complexes were revealed using the ECL Western blot detection system and Hyperfilm (Amersham-Pharmacia Biotech) in accordance with manufacturer's instructions. Total brain tissue homogenates from sporadic cases of PiD, CBD, PSP and AD, were used for comparison. These homogenates were already used in previous studies [13, 27].

Two-dimensional gel electrophoresis

Two-dimensional (2D) gel electrophoresis of total brain tissue protein was performed as previously described [25]. 100 µg of protein in SDS-lysis buffer) was added to 1 vol of 2D electrophoresis loading buffer (9.5 M Urea, 2% Triton X-100, 4% pharmalytes, 20 mM DTT) and loaded on the top of polyacrylamide gels in capillary tubes, and 100,000 V/h was applied. Capillary gels were extruded and the second dimension followed by Western-blotting was performed as for SDS-PAGE and Western blot.

Purification of aggregated tau protein and isoform characterization in AgD

Brain tissues were homogenized in TRIS-HCl 10 mM pH 7.4, 800 mM NaCl (buffer A), using a Teflon potter and the homogenate was spun at 100,000 g for 30 min at 4°C in a TLA-100.4 fixed angle rotor in a Beckman microcentrifuge ultracentrifugation system. The supernatant (S1) was collected and the pellet was homogenized in buffer A supplemented with 2% Triton X-100 and spun with the same parameters. Supernatant S2 was collected and the pellet was then homogenized in buffer B (TRIS-HCl 10 mM pH 7.4, 250 mM NaCl) containing 0.5% SDS and centrifuged following the same parameters. Supernatant S3 was collected and the pellet was washed twice in buffer C (TRIS-HCl 10 mM pH 7.4) containing 1% SDS. Supernatants S4 and S5 were collected and the final pellet (P6) was treated with 100% formic acid. Protein concentration was determined using the BCA protein quantification kit (Pierce) and 50 µg protein was loaded on a gel. The formic acid homogenate was evaporated under nitrogen and the same volume of SDS-lysis buffer was added and boiled for 10 min; 5 µg protein was loaded. In parallel, the evaporated material was rinsed twice in the alkaline phosphatase buffer in 80% acetone to neutralize the residual formic acid. The acetone solution was discarded and 50 µl alkaline phosphatase buffer was added. Following sonication, 400 U/ml alkaline phosphatase from calf intestine (EC 3.1.3.1, Roche Molecular Biochemicals, Meylan, France) was added, followed by incubation at 37°C overnight. The reaction was stopped by adding 1 vol of SDS-lysis buffer and boiling. Of the sample 30 µl was loaded on a gel as well as the six bacterial recombinant

Table 1 Clinicopathological features of AgD cases (*pmd* post-mortem delay, *b.wt.* brain weight, *B. stage* Braak stage, *Aβ* amyloid deposits (almost exclusively in the shape of diffuse plaques),

PD Parkinson's disease, *MCA* middle cerebral artery, *CAA* cerebral amyloid angiopathy, *c.s.* cribriform state of the basal ganglia, + scarce, ++ moderate)

Case	Age (years)	Sex	Dementia	pmd (h)	b.wt. (g)	B. stage	Aβ	Other findings
AgD1	98	F	Yes	2.5	1,150	I	+	c.s.
AgD2	91	F	Yes	7	1,235	I	+	no
AgD3	76	M	Yes + PD	18	1,240	II	++	Lewy pathology (brain stem), old encephalomalacia MCA
AgD4	86	M	Yes	30	1,370	III	+	CAA

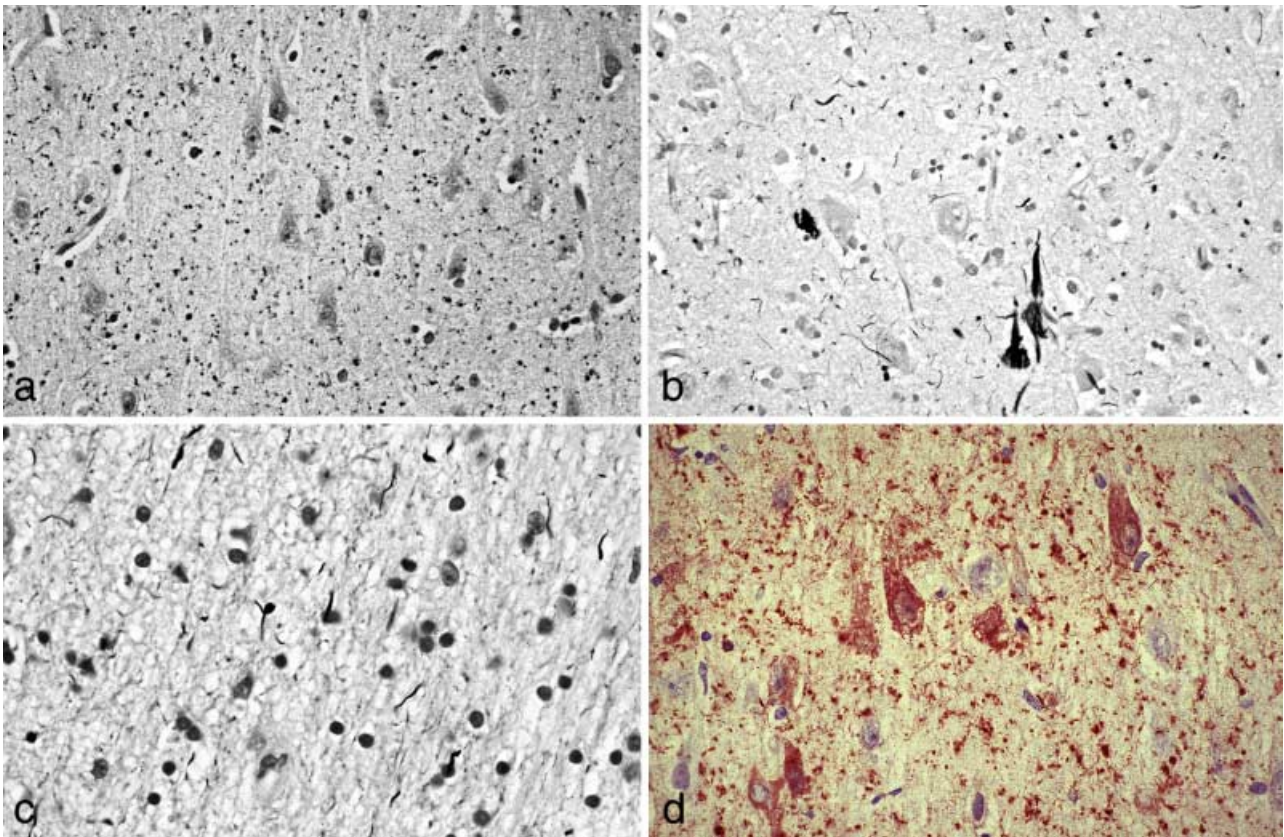


Fig. 1a–d Histological and immunohistochemical features of the AgD cases. **a** Abundant ArGs in sector CA1 of the hippocampus in AgD case 1 (Table 1). Note the total absence of NFTs and NTHs in this area. **b** Entorhinal cortex of AgD case 2. In upper cortical layers there is an admixture of ArGs, NFTs and NTHs. **c** AgD case 3. Entorhinal cortex. Coiled bodies and thread-like structures in the subcortical white matter. **d** AT8 immunostain of sector CA1 of AgD case 1 as shown in **a**. In addition to strongly stained ArGs there are many “pretangle” pyramidal neurons (AgD argyrophilic grain disease, ArGs argyrophilic grains, NFTs neurofibrillary tangles, NTHs neuropil threads). **a–c** Gallyas stain; **a, b** $\times 180$; **c, d** $\times 220$

rected against the last 15 amino acids of tau proteins was used to visualize both normal and pathological tau protein [28].

Results

Histology and immunohistochemistry

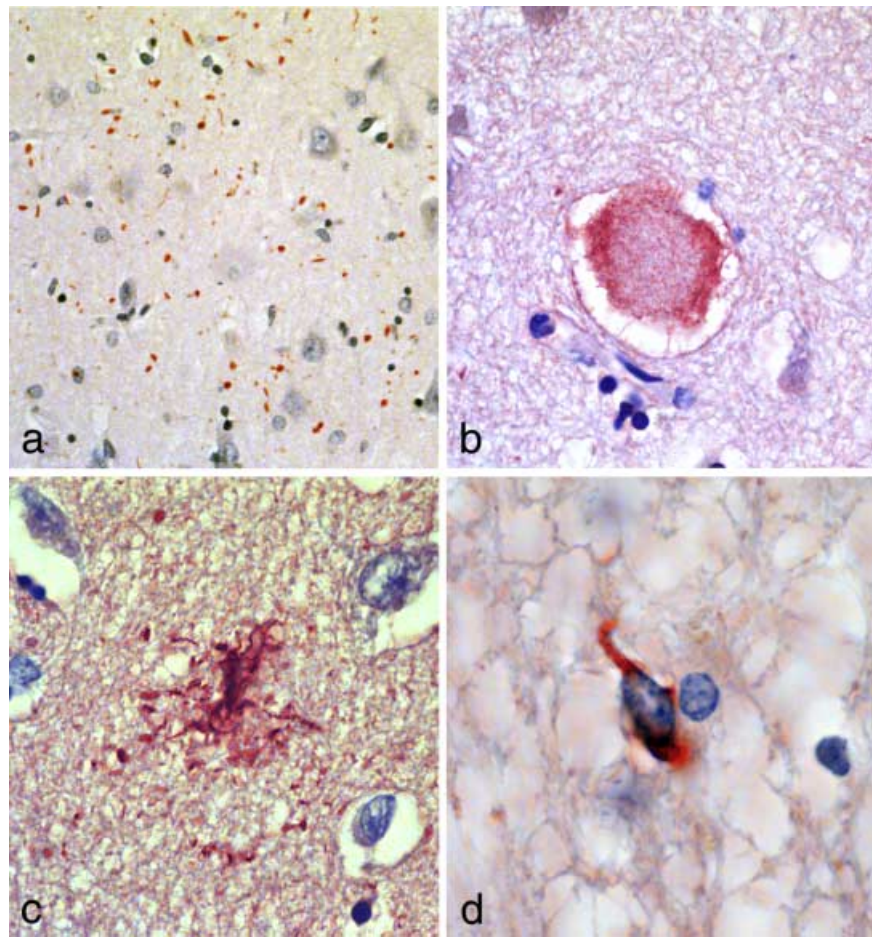
In all cases, Gallyas stain revealed high densities of ArGs throughout sector CA1 of the hippocampus (Fig. 1a), the entorhinal and transentorhinal cortices (Fig. 1b), and the basolateral nuclei of the amygdaloid complex. Only a few grains were found in the granular and molecular layer of the dentate gyrus, in sectors CA2–4, in the subiculum and the temporal neocortex. There were many oligodendroglial coiled bodies scattered predominantly in the subcortical white matter (Fig. 1c). NFL of the AD type were found interspersed between ArGs corresponding to Braak stages I (two cases), II, and III, respectively (Fig. 1b, Table 1) [5]. As previously shown, AT8 strongly immunolabeled ArGs, coiled bodies and many pyramidal neurons in areas rich in ArGs (Fig. 1d) [33]. In limbic areas, AT8-immunoreactive non-argyrophilic astrocytes [2] and α B-crystallin-expressing ballooned cells [31] were found in all cases. ArGs (Fig. 2a), ballooned cells (Fig. 2b), non-argyrophilic astrocytes (Fig. 2c) and coiled bodies (Fig. 2d) were also strongly stained with antibody Tau-E10. A β immunohistochemistry revealed scarce to moderate amounts of senile plaques, which were almost exclusively of the diffuse type, thus corroborating earlier reports [30, 34] (see Table 1 for additional neuropathological findings).

human tau isoforms (kindly provided by M. Goedert, MRC, Cambridge, UK).

Antibodies

For immunohistochemistry monoclonal phosphorylation-dependent anti-tau antibodies AT8 (directed against phospho-epitopes Ser202 and Thr205, dilution 1:1,000) [17] and 12E8 (against phosphorylated Ser262 and Ser356, dilution 1:100) [29] were used. Antiserum Tau-E10 detects a highly conserved microtubule-binding motif encoded by exon 10 in the C terminus of tau proteins [25]. For immunohistochemistry Tau-E10 was purified by affinity chromatography on the longest tau isoform as already described [28], and used by a dilution of 1:5. For Western blotting phosphorylation-dependent anti-tau antibodies 12E8, anti-tau^{Ser262P} (against phosphorylated Ser262; Calbiochem, San Diego, Calif.), AD2 (against phosphorylated Ser396 and Ser404) [9], and AP422 (against the phosphorylated Ser422) [11] were used, as well as antiserum Tau-E10. Tau-1 recognizes the unphosphorylated sequence ranging from amino acids 189–207 (Boehringer Mannheim, Germany). Exon-specific tau antisera Tau-E2 and Tau-E3 are directed against the corresponding sequences of exons 2 and 3 [27]. A phosphorylation-independent tau antiserum (TauCter) di-

Fig. 2a–d Immunohistochemical features of AgD cases. **a** AgD case 1, entorhinal cortex. ArGs are strongly labeled with antibody Tau-E10. **b** Tau-10E-stained ballooned cell in the amygdala of AgD case 2. **c** AgD case 1. Tau-E10-stained non-argyrophilic astrocyte in the amygdala. **d** Tau-E10-stained coiled body in the sub-cortical white matter of AgD case 2. **a** $\times 200$; **b**, **c** $\times 400$; **d** $\times 500$



Western blot analysis of pathological tau in AgD

Biochemically, we analyzed the pathological tau profile (PTP) in three brain regions (hippocampus, amygdala, temporal neocortex) of AgD cases 1 and 3, and in two brain regions (hippocampus and temporal neocortex) of AgD cases 2 and 4, and compared the results with the PTPs obtained in AD, PiD, PSP and CBD. The characteristic tau triplet at 60, 64 and 69 kDa with a minor band at 74 kDa was observed in AD (Figs. 3, 4, 6). In contrast, a doublet at 60 and 64 kDa and a minor 69-kDa component was detected in PiD, whereas in both PSP and CBD a doublet was detected at 64 and 69 kDa as previously described [13, 27]. In tissue samples from all AgD brains, antibody AD2 stained two major pathological tau bands at 64 and 69 kDa, which were very similar to those observed in PSP and CBD but distinct from PTPs in PiD and AD (Fig. 3). In addition, AD2 very faintly stained a minor 74 kDa component (Figs. 3, 4, 5). In AgD cases 3 and 4, the presence of a minor band at 60 kDa is most likely due to the concomitant AD pathology [neurofibrillary tangles (NFTs) corresponding to Braak stages II and III, respectively]. In accordance with the histopathological findings, PTPs were mainly found in the hippocampus formation of AgD cases. In AgD cases 1 and 3, pathological tau was also found in the amygdala and, in slight amounts, in the

temporal neocortex of AgD case 1 (Figs. 3, 6). No PTPs were obtained in temporal neocortices of AgD cases 2–4, which is likely explained by the low densities of ArGs in this region.

Two-dimensional analysis of pathological tau in AgD

We recently analyzed tau isoform composition of the tau triplet and of the minor 74-kDa band in AD [25]. The 60-kDa component is made up of the shortest 3R tau isoform lacking sequences encoded by exons 2 and 3 (2–, 3–, 10–), while the 64-kDa component contains both 4R (2–, 3–) and 3R (2+, 3–) tau isoforms. Also, 4R (2+, 3–) and 3R (2+, 3+) tau isoforms constitute the 69-kDa band, while the minor 74-kDa component is exclusively made of the longest 4R (2+, 3+) tau isoform. To further determine the isoform composition of the pathological tau doublet in AgD, tau proteins were resolved by 2D electrophoresis and labeled with antiserum Tau-E10 (AgD case 3). AD2 strongly stained the pathological tau components at 64 and 69 kDa, and to a lesser extent the 74- and 60-kDa components (Fig. 4). Again, we assume that the minor 60-kDa band is most likely due to an admixture of mild AD-type pathology (NFTs corresponding to Braak stage II). When compared to pathological tau of AD, the

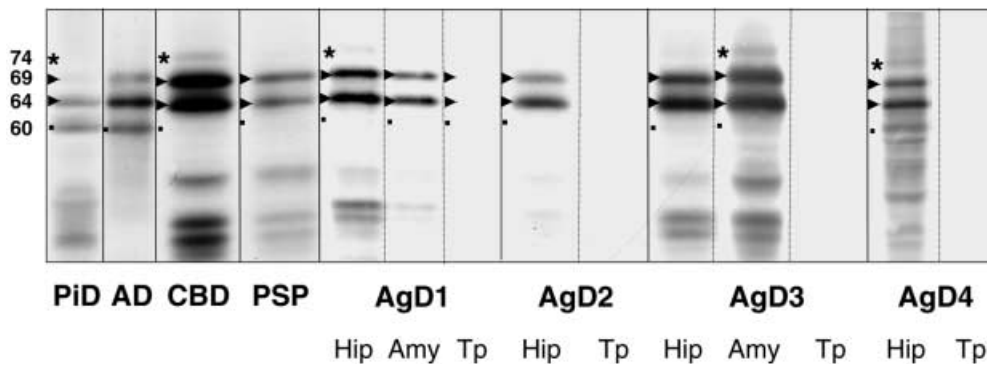


Fig. 3 Western blot analysis of AD2-labeled pathological tau proteins in AgD. The same amount of total brain tissue protein (100 μ g) from PiD, CBD, PSP, and AD cases diluted five times in SDS-lysis buffer and four AgD cases (*AgD1-4*) were loaded on SDS-PAGE. Three brain regions were studied in AgD cases 1 and 3: the hippocampus (*Hip*), the amygdala (*Amy*), and the temporal cortex (*Tp*). *Hip* and *Tp* were analyzed in the two other AgD cases. Following labeling with antibody AD2, a doublet of pathological tau protein at 60 and 64 kDa and a minor 69-kDa component were detected in PiD. The characteristic triplet at 60, 64 and 69 kDa was obtained in AD. The major doublet at 64 and 69 kDa was observed in both CBD and PSP. Note more intense signal in CBD and the presence of a 74-kDa pathological tau component. In AgD cases, a major doublet at 64 and 69 kDa is shown in the *Hip* and *Amy* of AgD1 and 3, and in the *Hip* of AgD2 and 4. A minor band at 74 kDa is also detected in AgD cases 1, 3 and 4. Most likely due to concomitant AD-type pathology an additional minor pathological tau component is labeled in AgD cases 3 and 4. The pathological tau doublet was slightly visible in the *Tp* of AgD1 (see also Fig. 6) but not detected in the *Tp* of AgD2–4 (*PiD* Pick's disease, *CBD* corticobasal degeneration, *PSP* progressive supranuclear palsy, *AD* Alzheimer's disease)

isoelectric points (pI) in AgD were found to be higher than in AD. A maximum acidic pI value of 6.2 was observed in AgD samples, while pI values of pathological tau in AD were below 5.5 [12, 25]. Anti-Tau-E10 strongly stained the pathological tau components at 64, 69 and 74 kDa. However, in contrast to antibody AD2, anti-Tau-E10 consistently failed to label the 60-kDa component (Fig. 4). Antisera Tau-E2 and Tau-E3 showed very faint staining of the 69- and 74-kDa components but not of the pathological 60- and 64-kDa bands (data not shown), even on purified fractions of pathological tau aggregates (see below). These results suggest that the pathological tau doublet in AgD is mainly composed of 4R tau isoforms.

Purification of pathological tau in AgD

Because of its high sensitivity, AD2 detects very low amounts of pathological tau proteins in total brain tissue homogenates [11]. Pathological tau in AgD, however, is found in only low amounts and a purification assay was applied prior to the use of other phosphorylation-dependent anti-tau antibodies. The purification protocol was based on that used for purifying PHF-tau in AD [16]; however, following the latter protocol pathological tau in AgD became completely solubilized (not shown). Using

our approach, normal tau proteins were detected only in fractions S1 and S2 (Fig. 5a). Using SDS, normal tau was solubilized and was therefore not co-purified with aggregates of pathological tau. Thus, after two washes in SDS the aggregates were concentrated in the pellet and further solubilized in formic acid (Fig. 5a). Antibody Tau-1 was used to make sure that normal tau, which becomes dephosphorylated in post-mortem brain tissue, is not co-purified with aggregates of pathological tau. No Tau-1 staining was observed in the P6 fraction (Fig. 5b). In contrast, AP422 strongly labeled the pathological tau components at 64 and 69 kDa and less intensively the minor 60-kDa component (Fig. 5b). To corroborate our isoform characterization of pathological tau in AgD, the aggregates of pathological tau purified in the P6 fraction were subjected to dephosphorylation, and the dephosphorylated tau protein isoforms were aligned with the six human recombinant tau (TauR) isoforms. Four tau isoforms were detected that aligned with TauR as follows: 3R, 4R, 3R (+2), and 4R (+2) (Fig. 5b). The 3R (2+, 3+) isoform was faintly detected, and the 3R tau isoforms were only visible following long time of exposure. These findings are in line with the view that the 4R tau isoforms constitute the major isoforms of aggregated tau in AgD. Normal tau protein expression was investigated in the first two fractions of the extraction assay (Fig. 5c). The amount and distribution pattern of normal tau proteins was found to be the same in both fractions in a control and an AgD brain (case 3), suggesting that expression of normal tau proteins is not altered in AgD.

Western blot analysis of phosphorylated residue Ser262 of pathological tau in AgD

We have previously shown immunohistochemically that ArGs and coiled bodies are not stained with antibody 12E8 directed against phosphorylated residues Ser262 and Ser356 of tau [33]. In the present study, again, no immunostaining of AgD changes was obtained when using the 12E8 antibody (data not shown). In contrast, Western blot analysis revealed a distinct staining of the two major 64- and 69-kDa pathological tau bands in AgD cases using both, antibody 12E8 and anti-tau^{Ser262P} (Fig. 6). In some cases (AgD cases 3 and 4), however, staining was only obtained after a long incubation time (2 h or more). In the temporal cortex of AgD case 1, in which a slight

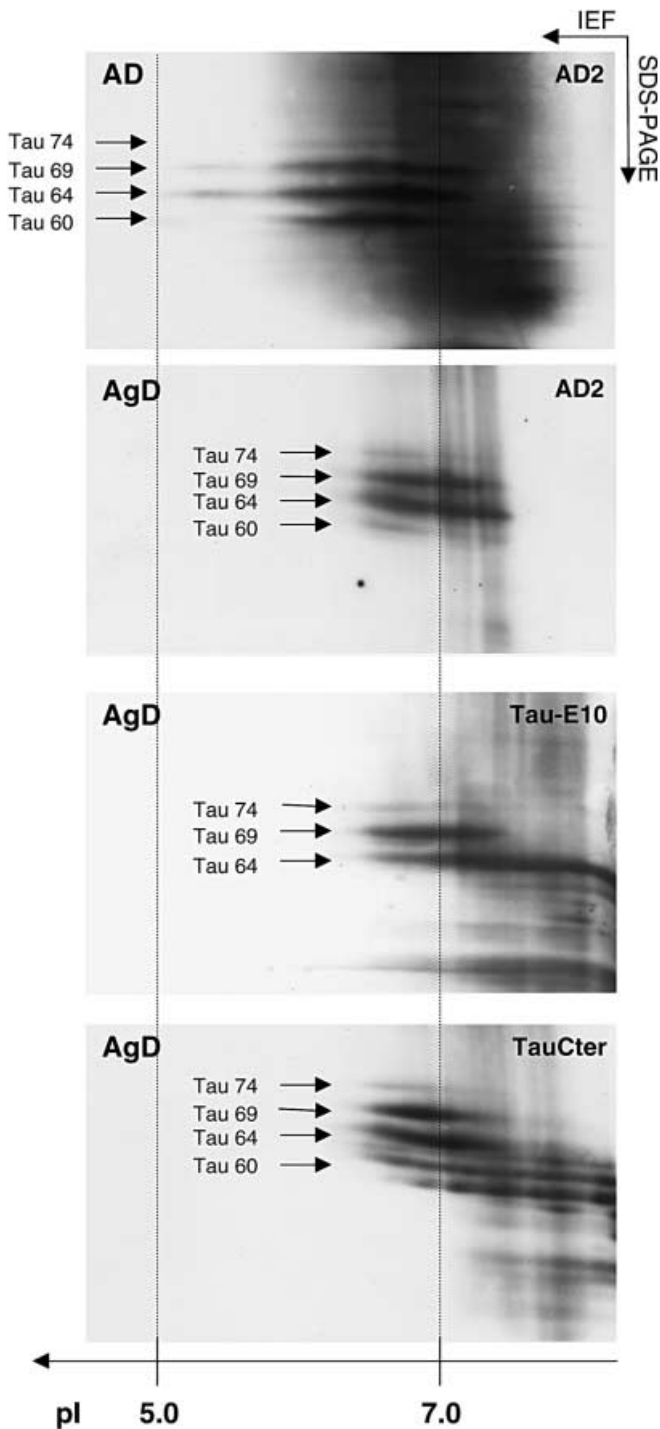


Fig. 4 2D analysis of pathological tau and tau isoform content in AgD. Total brain proteins were resolved by 2D gel electrophoresis in an AD case and in the hippocampus of AgD case 3. Pathological tau proteins were detected with antibody AD2 (*first and second panel*). Tau proteins containing the sequence corresponding to exon 10 were labeled with antiserum Tau-E10 (*third panel*). Antiserum TauCter was used to visualize both normal and pathological tau proteins (*fourth panel*). Pathological AD2 (Tau 60, 64, 69 and 74) are indicated by *arrows*. The isoelectric points (pI) are indicated on the *x-axis*. Note that pathological tau proteins in AgD are less acidic (pI 6.2) than pathological tau in AD (pI 5.5). Pathological tau components at 64 and 69 kDa and the minor 74-kDa component are detected with antiserum Tau-E10 (*third panel*), while pathological tau at 60 kDa is not (*second panel*) (2D two dimensional)

amount of ArGs was found (see above), antibody AD2 only faintly stained the pathological tau doublet. Most likely due to the small amount of ArGs and concomitant phosphorylated Ser262 epitopes, antibodies 12E8 and anti-tau^{Ser262P} failed to stain the pathological tau doublet, even after long time of exposure (Fig. 6).

Discussion

In the present study we demonstrate a different electrophoretic profile of hyperphosphorylated tau proteins in AgD and AD. Biochemical analysis of brain tissue samples from two AgD cases (case 1 and 2) revealed a distinct tau doublet at 64 and 69 kDa and a minor band at 74 kDa, while the characteristic major tau triplet at 60, 64 and 69 kDa and a minor band at 74 kDa was found in the AD case. In two further AgD cases (cases 3 and 4), antibody AD2 again labeled the major tau doublet and the minor band at 74 kDa but also an additional 60-kDa band which, however, was less labeled than the tau doublet. These biochemical data match quite well the histopathological findings in our AgD cases. In AgD cases with the major tau doublet at 64 and 69 kDa and the minor 74-kDa component, tau filamentous inclusions were almost exclusively found in the shape of ArGs and coiled bodies, while NFL of the AD type were only sparse (Braak stage I). However, AgD cases with the additional minor tau band at 60 kDa were characterized by the presence of moderate amounts of AD-type NFL (Braak stage II and III, respectively) in addition to ArGs and coiled bodies. Therefore, a characteristic major tau doublet at 64 and 69 kDa and a minor 74-kDa component are likely to define pure AgD cases (e.g., without or very mild additional AD-type pathology), and an additional minor band at 60 kDa probably reflects concomitant NFL of the AD type.

We also compared the PTP in AgD with the PTPs obtained in PiD, PSP and CBD. PiD is characterized by a tau doublet at 60 and 64 kDa and a minor 69-kDa component [8, 13, 21], and clearly differs from PTP in AgD. In CBD and PSP the biochemical profile of insoluble tau consists of two major bands at 64 and 69 kDa, and a variable, minor band of 74 kDa [8, 15, 19, 36], and is therefore quite similar to the PTP obtained in AgD.

Several studies have shown that filamentous tau inclusions in AD are made of all six brain tau isoforms (reviewed in [7, 20]). In PiD, the major tau doublet appear to lack the microtubule-binding repeat encoded by exon 10, and is believed to be composed mainly, if not exclusively, of 3R tau isoforms [22, 26]. This contrasts with CBD and PSP in which the pathological tau doublet are mainly made of hyperphosphorylated tau isoforms with four microtubule repeats [27].

In 2D gel electrophoresis normal tau proteins are found in the basic range, whereas PTPs are focused in the acidic range of the pH gradient [12, 25]. When compared to pathological tau of AD, the pI in AgD were found to be higher than in AD. A maximum acidic pI value of 6.2 was observed in AgD samples, while pathological tau in AD reached pI values below 5.5. Comparable to the 1D gel

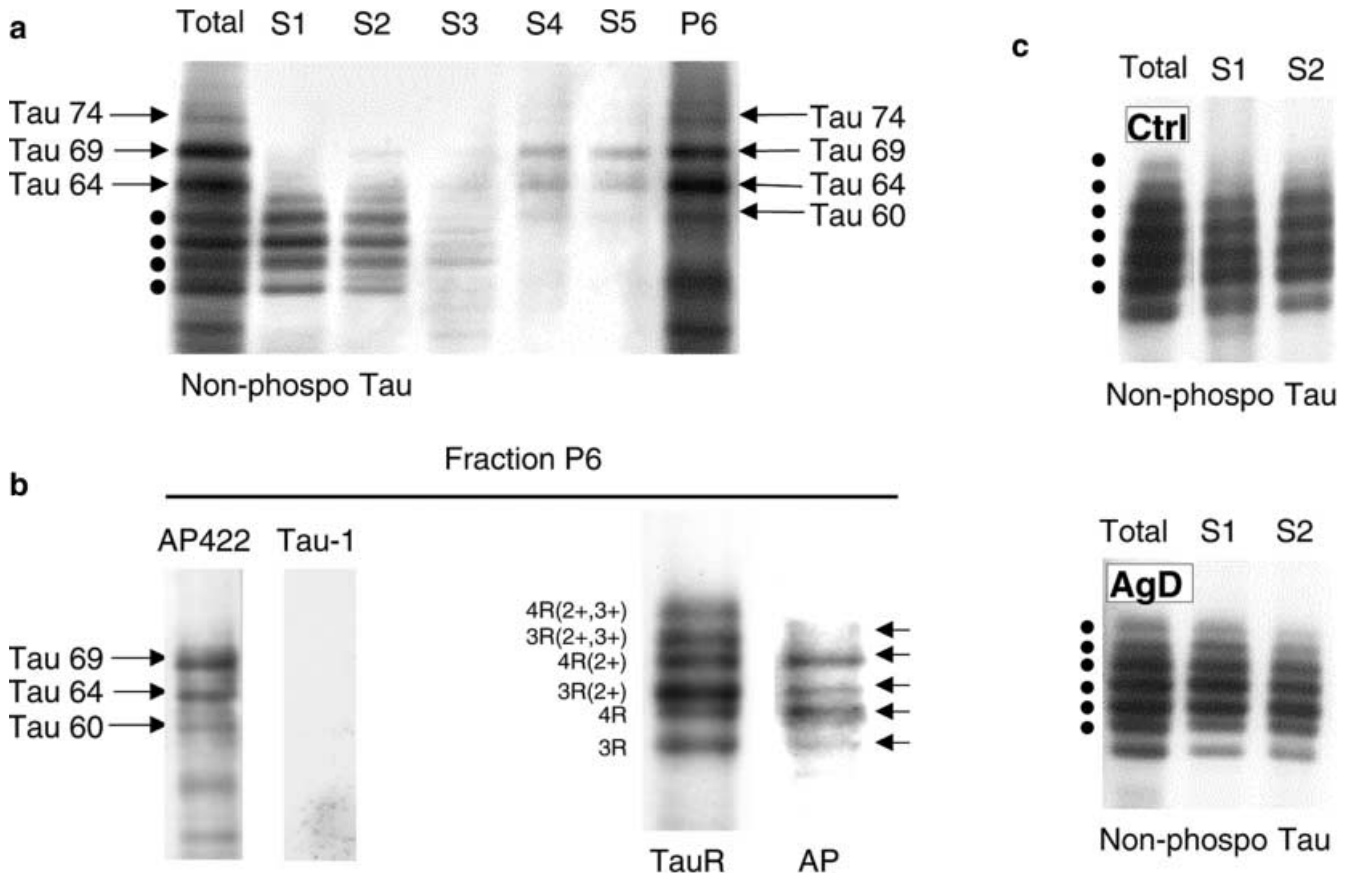


Fig. 5a-c Purification of tau protein aggregates, phosphorylation state, isoform content and normal tau protein expression in AgD. A stepwise approach was used to purify aggregates of tau in AgD brain tissue (see Material and methods). **a** Staining of tau proteins in supernatants (S1–S5 and the final pellet P6, homogenized in formic acid). Note that both normal tau (indicated by dots) and pathological tau components at 64, 69 and the minor 74 kDa are detected in the hippocampus of AgD case 3. Normal tau are labeled in the TRIS-salt and Triton X-100 supernatant. Pathological tau are slightly labeled during the two washes in SDS buffer and concentrate in the final pellet. Note that the pathological components of 64 and 69 kDa are strongly detected, whereas the 60 and 74 kDa pathological tau components are stained to a lesser extent in the vertical smear. **b** Pathological tau proteins were labeled with antibody AP422 in the P6 pellet, whereas no staining was obtained with Tau-1 antibody. Alkaline phosphatase treatment of aggregated tau from the P6 fraction (AP) are aligned with recombinant tau (TauR) and stained with TauCter antiserum. Note the predominance of 4R tau isoforms. **c** Normal tau protein expression and solubility was evaluated in the TRIS-salt and in the Triton X-100 supernatants in temporal cortices of a control case (Ctrl) and AgD3 case (AgD). Tau proteins were stained with antiserum TauCter (indicated with dots). Note that the same pattern and distribution is observed in Ctrl and AgD

electrophoresis, antibody AD2 strongly stained the main pathological tau components at 64 and 69 kDa and to a lesser extent the 60- and 74-kDa components (AgD case 3). Antibody Tau-E10, which exclusively detects 4R tau isoforms [25], strongly labeled the 64- and 69-kDa components and faintly the minor 74-kDa band, while the 60-kDa component made of the shortest brain tau isoform

(2-, 3-, 10-) was not stained. Antisera Tau-E2 and Tau-E3 detected only very slightly the 69- and 74-kDa components but not the pathological 60- and 64-kDa tau bands. These findings are corroborated by dephosphorylation of the purified fraction of PTP, showing that the typical doublet of pathological tau in AgD is mainly composed of 4R isoforms. Moreover, as suggested from 2D gel electrophoresis, pathological tau aggregates in AgD are less phosphorylated when compared to those of AD.

In AgD, ArGs are most abundant in the hippocampal region (prosubiculum, sector CA1), in the pre- β layer of the entorhinal cortex, in the basolateral nuclei of the amygdaloid complex and in the hypothalamic lateral tuberal nucleus, while ArGs are usually found in smaller amounts in the temporal neocortex [6, 35]. These histological findings are confirmed by our biochemical data. In all AgD cases, PTPs were most prominent in the hippocampus formation. In AgD cases 1 and 3, in which frozen tissue was also available from the amygdala, antibody AD2 strongly labeled the pathological tau doublet in this area. However, due to the small amount of ArGs, AD2 only faintly stained the tau doublet at 64 and 69 kDa in the temporal neocortex. In AgD cases 2–4, in which only scattered ArGs were present in the temporal neocortices, antibody AD2 failed to reveal the pathological tau doublet.

Due to its high sensitivity, antibody AD2 may detect trace amounts of pathological tau protein in total brain tissue homogenates [14]. However, as pathological tau is

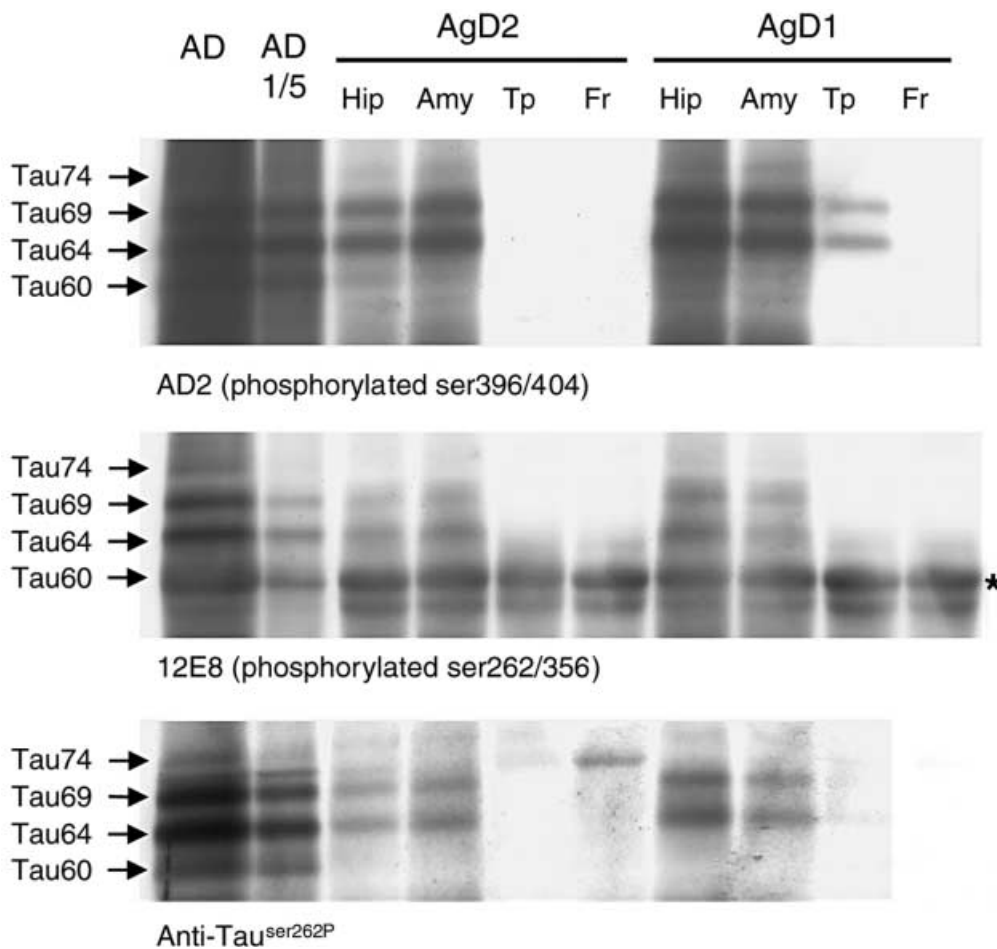


Fig. 6 Western blot analysis of phosphorylated residue Ser262 of pathological tau in AgD. The same amount of total brain tissue protein (100 μ g) from AgD cases 1 and 2 as well as an AD case (undiluted and fivefold diluted in SDS-lysis buffer) were loaded on SDS-PAGE. Four brain regions were analyzed in the AgD cases: the hippocampus (*Hip*), the amygdala (*Amy*), the temporal cortex (*Tp*) and the frontal cortex (*Fr*). Total amounts of the presence or absence of pathological tau is evaluated with AD2 staining (AD2 panel). Note that the signal for pathological tau in the affected brain regions of the two AgD cases is similar or lower than that observed in the diluted AD sample. 12E8 and anti-tau^{Ser262P} were used to analyze phosphorylation of pathological tau at phosphorylated Ser262 and Ser356, and phosphorylated Ser262 alone, respectively. The pathological tau doublet at 64 and 69 kDa was stained in both AgD cases, albeit to a lesser extent than in the diluted AD sample. Note that pathological tau are not labeled with either 12E8 or anti-tau^{Ser262P} in the temporal cortex of AgD case 1. The bands indicated by an *asterisk* correspond to an artifactual staining of 12E8 on total brain tissue homogenates. Pathological tau bands at 60, 64, 69 and 74 kDa are indicated by *arrows*

found in much lower amounts in AgD than in AD, we applied a purification assay prior to the use of other phosphorylation-dependent anti-tau antibodies. With this purification technique we were able to demonstrate that the tau doublet obtained in AgD indeed is due to aggregates of pathological tau. When using phosphorylation-dependent anti-tau antibodies AP422 and AD2 on Western blots, the characteristic tau doublet at 64 and 69 kDa was

strongly labeled, while no staining was obtained with antibody Tau-1. Phosphorylation of Ser422 has recently been shown to be a common feature of pathological tau aggregates in several neurodegenerative disorders [10]. Conversely, Ser422 was found to be in a non-phosphorylated state in biopsies or autopsy-derived control samples of aged patients [10]. Thus, the strong AP422 labeling in our AgD cases further supports the view that AgD changes are made of pathological tau aggregates, and that they do not constitute a normal age-related change. A selective reduction or total loss of all six brain tau isoforms have been described recently in both sporadic and familial forms of frontotemporal lobar degeneration [37]. We therefore measured normal tau protein expression in the first two fractions of the purification assay. On Western blots the amount and distribution of tau proteins were found to be the same in AgD and control cases in both fractions, thus suggesting that expression of normal tau protein is not altered in AgD.

In a recent immunohistochemical study we have shown that tau proteins in ArGs and coiled bodies share a high number of phosphorylation sites with the NFL of AD [33]. A notable exception was antibody 12E8 directed against phosphorylated Ser262 and/or Ser356 of tau [29]. While NFL in AD were strongly stained, this antibody did not stain ArGs and coiled bodies of AgD [33]. In the present study, again, no staining of ArGs and coiled bodies

was obtained with the 12E8 antibody. However, by the use of more sensitive biochemical analysis, we now demonstrate that epitope Ser262 indeed is phosphorylated in pathological tau aggregates of AgD cases. The pathological tau doublet in AgD was found to be stained with both antibody 12E8 and anti-tau^{Ser262P}. These results are confirmed by a recent immunohistochemical study in which staining of AgD changes is reported with anti-tau^{Ser262P} (Ferrer et al., manuscript submitted). In addition to AgD, Ser262 has been shown to be phosphorylated in AD, PSP and CBD [27], while discrepant results have been obtained concerning phosphorylation of Ser262 in PiD ([13, 21, 24], and Ferrer et al., manuscript submitted).

In summary we have demonstrated that the biochemical tau profile in AgD (Tau 64, 69, minor 74; predominantly 4R) is distinct from that in AD (Tau 60, 64, 69, minor 74; 3R + 4R) and PiD (Tau 60, 64, minor 69; mainly 3R). AgD shares the pathological tau doublet as well as the predominance of 4R tau isoforms with PSP and CBD. Thus, following the biochemical classification of tauopathies [11, 20], which distinguishes PTP type I (as in AD), type II (as in PSP and CBD), type III (as in PiD), type IV (as in myotonic dystrophy type 1 [28]), and finally type 0 (as reported in some sporadic and inherited cases of frontotemporal dementia [37]), our present results suggest that AgD belongs to the family of 4R tauopathies of PTP type II. Further morphological and genetic studies (e.g., tau haplotype analysis) should now be aimed at further classifying AgD within the spectrum of 4R tauopathies, and at defining the clinical characteristics of AgD since the definite diagnosis still is based on post-mortem neuropathological analysis.

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Note added in proof: Togo et al. [38] very recently reported that filamentous tau deposits in AgD are predominantly made of 4R tau isoforms, thus corroborating our own results.

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