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# Argyrophilic Grain Disease Is a Sporadic 4-Repeat Tauopathy

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**Abstract.** Argyrophilic grain disease (AGD) was first reported as an adult-onset dementia, but recent studies have emphasized personality change, emotional imbalance, and memory problems as clinical features of AGD. AGD is characterized by spindle- or comma-shaped argyrophilic grains in the neuropil of entorhinal cortex, hippocampus, and amygdala. Immunohistochemistry with monoclonal antibodies specific to tau isoforms with four (4R) or three (3R) repeats in the microtubule-binding domain showed immunostaining of grains with 4R, but not 3R, tau antibodies, suggesting that AGD was a 4R tauopathy. The tau isoform composition of AGD was confirmed with densitometric analysis of Western blots of sarkosyl-insoluble tau from the medial temporal lobe of AGD brains with a range of concurrent neurofibrillary pathology and compared with Alzheimer controls. The 4R/3R ratio was 1 or less for Alzheimer disease; the 4R/3R ratio was more than 1 for AGD, decreasing with increasing neurofibrillary pathology and demonstrating that insoluble tau in AGD was enriched in 4R tau. The frequency of the extended tau haplotype was not different in AGD compared to other sporadic 4R tauopathies, progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD). Furthermore, AGD occurred in PSP and CBD more frequently than in dementia controls, including Alzheimer disease. These results suggest that AGD, PSP and CBD are 4R tauopathies that share common pathologic, biochemical, and genetic characteristics.

**Key Words:** Alzheimer disease; Argyrophilic grain disease; Corticobasal degeneration; Progressive supranuclear palsy; Tau.

## INTRODUCTION

Argyrophilic neurofibrillary lesions constitute a defining neuropathologic characteristic of a number of neurodegenerative disorders, including Alzheimer disease (AD), Pick disease, progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD). Ultrastructurally, these lesions contain paired helical filaments and straight filaments composed of microtubule-associated protein tau in a hyperphosphorylated state (1). In adult human brain, alternative mRNA splicing of a single gene transcript yields 6 tau isoforms (2–4). Alternative splicing of exon 10 gives rise to tau isoforms with 3 (exon 10−) or 4 (exon 10+) repeats in the microtubule-binding domain, so-called 3 repeat (3R) tau and 4 repeat (4R) tau. The 3R and 4R tau isoforms have different properties in terms of microtubule binding, while alternative splicing of exons 2 and 3 at the amino-terminus (producing inserts of 29 or 58 amino acids) has unclear functional significance (4). Recent studies have revealed that tau protein accumulates within neurons and glia in neurodegenerative diseases in a disease-specific manner. Both 3R and 4R tau isoforms are present in AD (5), though not necessarily in equal amounts (6). While 3R tau predominates in Pick disease (7), evidence suggests that 4R tau predominates in PSP and CBD (8, 9). In addition to PSP and CBD, some forms of frontotemporal dementia and Parkinsonism linked to chromosome 17, particularly those associated with 5′-spice site or coding region mutations in exon 10 of tau, are associated with preferential accumulation of 4R tau within pathologic neuronal and glial lesions that are the histologic features of these disorders (10–13). It is reasonable to consider that the tauopathies characterized by the accumulation of 4R tau in neurons and glia as a distinct group of disorders, ''the 4R tauopathies.''

Argyrophilic grain disease (AGD), first reported by Braak and Braak (14, 15) as an adult-onset dementia, is characterized by spindle- or comma-shaped argyrophilic grains in the neuropil of entorhinal cortex, hippocampus, and amygdala. Argyrophilic grains are clearly demonstrated with the Gallyas silver stain and are also immunoreactive with antibodies to phospho-tau (17). Ultrastructurally, argyrophilic grains are aggregates of straight filaments (15–17) or smooth tubules (18, 19) and are located mainly in dendrites of neurons (17, 18, 20). AGD is under-recognized but not rare, with grains present in 5%–9% of autopsies, increasing in frequency with age (17, 19, 21, 22). The clinical features of AGD are not fully understood. Early studies suggested that dementia was characteristic of AGD (14, 15), but there have been no large clinicopathologic studies of AGD in prospectively evaluated individuals. More recently personality change and emotional imbalance preceding memory failure have been emphasized as clinical features of AGD (21, 23).

In addition to grains in neuronal cell processes, AGD is usually accompanied by argyrophilic inclusions in oligodendroglia, so-called coiled bodies (15), in the white

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matter underlying affected cortices and ballooned neurons in the limbic lobe (22, 24). The frequency of AGD increases with age, consequently it is common to find AD-type pathology or other neurodegenerative disorders that increase in frequency with age, including Lewy body disease, CBD, and PSP (19, 21, 22), concurrently with AGD. In some series, a high frequency of AGD has been noted in PSP (19). These findings suggest that AGD may share common genetic or pathogenetic features with PSP and CBD.

While it is known that argyrophilic grains contain hyperphosphorylated tau, the isoform composition of grains has not been clarified, presumably because grains have not been isolated to homogeneity and biochemical analysis is confounded by concomitant Alzheimer neurofibrillary pathology. In this study, we performed Western blotting of sarkosyl-insoluble tau from a series of AGD cases with a range of Alzheimer neurofibrillary pathology to determine the isoform composition of grains in concert with immunohistochemistry with monoclonal antibodies specific to 3R and 4R tau. We also examined variants in the tau gene in AGD, because polymorphisms (25) and an extended tau haplotype, referred to as the H1 haplotype (26), are significantly more frequent in PSP and CBD compared with normal controls (26–29). Our results suggest that argyrophilic grains are composed of tau enriched in 4R tau and that AGD is a sporadic 4R tauopathy. We show that AGD shares genetic, biochemical, and pathologic features with PSP and CBD and that, moreover, AGD is found in PSP and CBD more frequently than in controls.

## MATERIALS AND METHODS

### Case Material

The major neuropathologic features of the cases studied are summarized in Table 1. The diagnosis of AGD was confirmed by the presence of argyrophilic grains in both the hippocampus and amygdala by tau immunostaining and with Gallyas silver stain. In all cases, coiled bodies were detected in white matter underlying the affected cortex, and ballooned neurons were detected in limbic regions as previously reported (22). A subset of cases was chosen for biochemistry based on presence of minimal concurrent AD-type pathology and the availability of frozen tissues. The control AD cases met NIA-Reagan diagnostic criteria (31) and all had advanced disease based upon Braak staging (Stage VI) (32). All AGD and AD cases were devoid of concomitant pathology, in particular PSP and CBD.

### 3R and 4R Tau Monoclonal Antibodies

Two monoclonal antibodies, anti-human 3R tau [RD3 (clone 3D1/A9)] and anti-human 4R tau [RD4 (clone 1E1/A6)], were developed by one of the authors (RDS) by immunizing mice with synthetic peptides containing sequences that bridged the splice site for exon 10 (RD3: VQIINKKLDLSNVQSKC) or to a unique sequence in exon 10 (RD4: KHQPGGGKVQIVYKPV). For RD4 the conjugation to hapten was through the intrinsic cysteine residue, but for RD3 a C-terminal cysteine was added to the peptide. Specificity of RD3 and RD4 was determined with immunohistochemistry in 3R tauopathy (Pick disease), 4R tauopathy (PSP), and AD cases (Fig. 1A). Biochemical confirmation of specificity employed Western blots of Pick disease, PSP, and AD (Fig. 1B), as well as recombinant tau proteins (Fig. 2). In Pick disease, RD3 but not RD4 immunostained Pick bodies (Fig. 1d), while sparse Alzheimer tangles in some cases of Pick disease were immunostained by RD4 (Fig. 3c). In PSP, RD4 immunolabeled neurofibrillary tangles (NFTs) as well as tufted astrocytes (Fig. 1a), while these lesions were not stained with RD3 (Fig. 1c). In AD, NFTs, neuropil threads, and neurites in senile plaques had variable double staining with RD3 and RD4, except that extracellular NFT were preferentially immunolabeled by RD3 (Fig. 3a, d).

Recombinant tau isoforms were expressed individually in *Escherichia coli* and purified by CM sepharose chromatography. The protein content of purified recombinant tau was determined by the bicinchoninic acid method (BCA kit, Pierce,

TABLE 1A.
Cases with AGD and AD used for Analysis of Tau Isoforms

| Case | Age | Sex | Diagnosis | Braak stage | 4R/3R |
|---|---|---|---|---|---|
| 1 | 86 | M | AGD, PA, ASCVD | 1 | 2.58 |
| 2 | 75 | M | AGD, TLBD | 1 | 1.97 |
| 3 | 79 | M | AGD, PA | 2 | 1.15 |
| 4 | 97 | F | AGD, PA | 3 | 1.19 |
| 5 | 87 | M | AGD, PA | 3 | 1.14 |
| 6 | 75 | M | AGD, PA | 3 | 0.68 |
| 7 | 81 | M | AGD, PA | 3 | 0.56 |
| 8 | 82 | F | AD | 6 | 0.73 |
| 9 | 90 | F | AD | 6 | 0.56 |
| 10* | 73 | M | AD | 6 | 1.02 |
| 11* | 82 | M | AD, telangiectasia | 6 | 0.98 |
| 12* | 72 | F | AD | 6 | 0.91 |
| 13* | 70 | F | AD, meningioma | 6 | 0.70 |
| 14* | 72 | M | AD, subdural hematoma | 6 | 0.58 |
| 15* | 72 | M | AD, ASCVD, hematoma (white matter) | 6 | 0.40 |

TABLE 1B.
Spearman Rank Order Correlations with 4R/3R ratio in AGD cases

| Braak Stage | Age | Sex |
|---|---|---|
| $r = -0.797$ | $r = 0.144$ | $r = -0.204$ |
| $p < 0.05$ | n.s. | n.s. |

Cases 10–15 (asterisks) were previously reported from our laboratory using similar methods (6). Pathological aging (PA) (51) are cases with abundant cortical amyloid plaques, usually diffuse plaques, but a low (III or less) Braak stage; 4R/3R: ratio of tau isoforms with 3 or 4 repeats in the microtubule-binding domain. Abbreviations: AGD, argyrophilic grain disease; AD, Alzheimer disease; TLBD, Lewy body disease, transitional type; ASCVD, arteriosclerotic vascular disease; M, male, F, female; n.s., not significant.

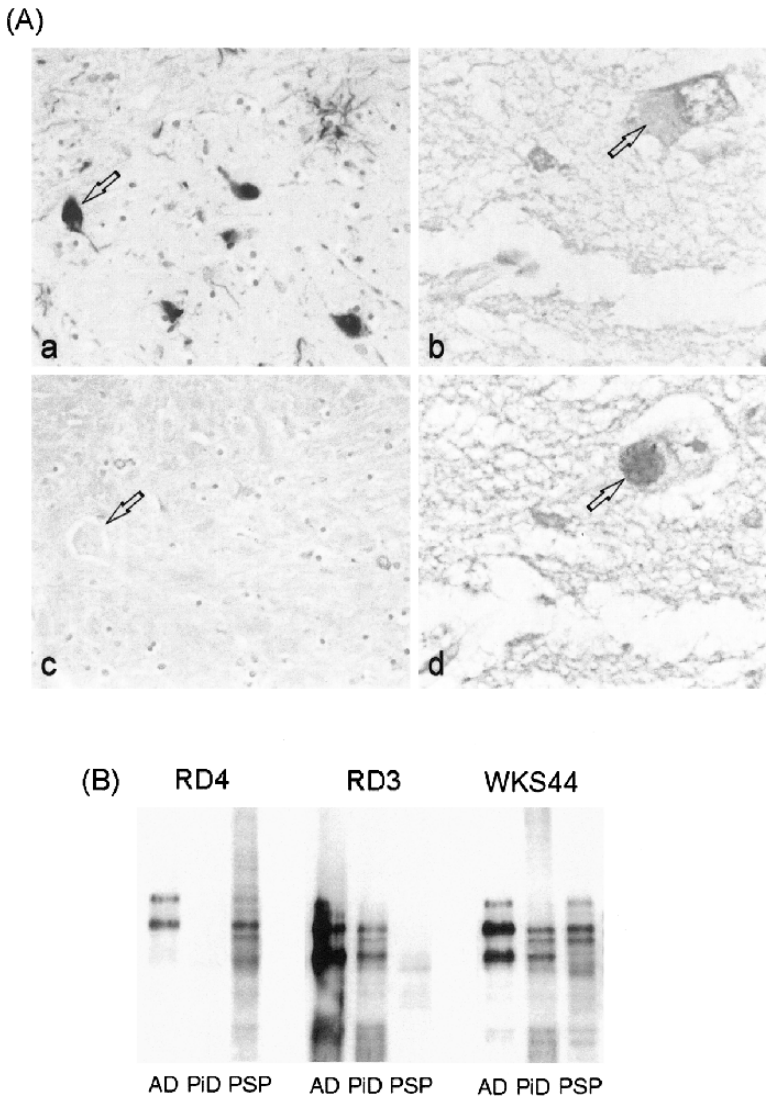


**Fig. 1.** A: Immunohistochemistry of adjacent sections of PSP (a, c) and Pick disease (b, d) with RD4 (a, b) and RD3 (c, d). In the globus pallidus of PSP, RD4 labels NFT (arrow in a), neuropil threads, and tufted astrocytes that are not visible withRD3 (arrow in c). In contrast, Pick bodies (arrows) are immunolabeled with RD3 (d), but not by RD4 (b). Magnifications: a, c ×400; b, d ×1,000. B: Western blots of sarkosyl-insoluble tau in representative cases of AD, Pick disease (PiD), and PSP. Note that RD4 reacts with 2 bands of 68 and 66 kDa in AD and in PSP, but with nothing in Pick disease (PiD). Similarly, RD3 reacts with 2 bands of 60 and 64 kDa in AD and PiD, but nothing in PSP. Low molecular weight species are degradation products. WKS44 shows distinct profiles in AD (60, 64, and 68 kDa triplet), PiD (prominent 60 and 64 kDa bands with a minor band at 62 kDa), and PSP (prominent 64 and 68 kDa bands with a minor band at 62 kDa).

Rockford, IL). For each isoform, 5 ng or 50 ng of protein was loaded on SDS-PAGE and probed with primary antibodies to tau, including a polyclonal antibody (WKS44) with an epitope located in amino acid residues 162–178 that recognizes all 6 isoforms, as well as the 4R and 3R antibodies. For RD3, optimal immunoblotting required loading 5 ng of recombinant tau protein. The 3R tau isoforms (3R0N, 3R1N, and 3R2N) were detected by RD3 (1:100), but no 4R tau isoforms were detected (Fig. 2). In contrast, the 4R tau isoforms (4R0N, 4R1N and 4R2N) were detected by RD4 (1:10) (Fig. 2). Comparing the dilutions needed to obtain optimal immunoblotting, RD3 was at least 10 times stronger than RD4. In contrast, RD3 was weaker in immunohistochemistry, possibly due to differences in epitope binding in denatured tau proteins in the Western blots compared to tau within filamentous lesions, which are physically different in 3R and 4R tauopathies, in tissue sections (33).

## Immunohistochemical Methods

For immunohistochemistry, paraffin embedded tissue sections were cut at a thickness of 5 μm. A variety of dilutions and conditions were tested to determine the optimal detection methods for RD3 and RD4. Sections were processed with and

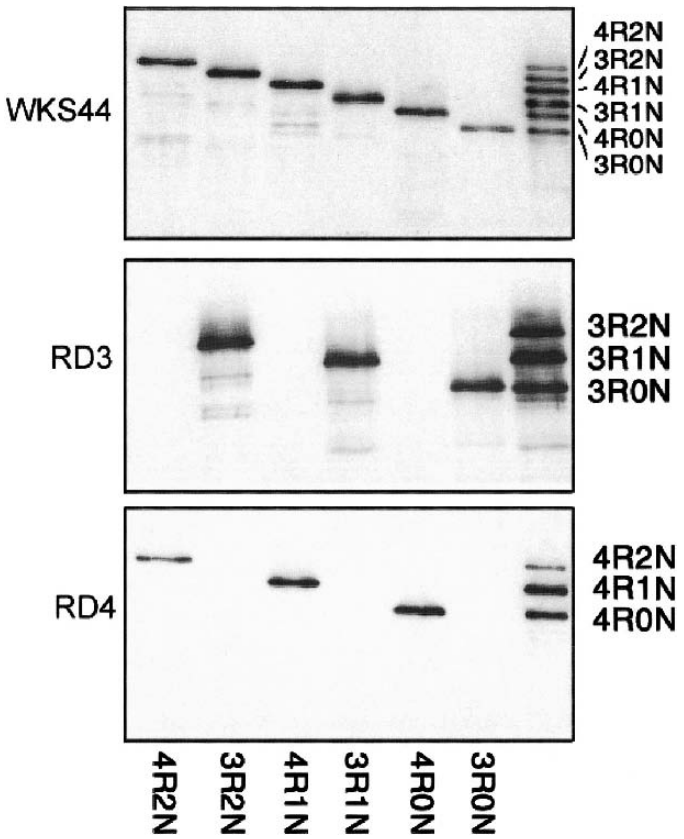


**Fig. 2.** Western blots of recombinant tau protein isoforms with antibodies, WKS44, RD3, and RD4. WKS44 detects all tau isoforms, while RD3 recognizes 3R tau isoforms (3R0N, 3R1N, 3R2N), but not 4R tau isoforms (4R0N, 4R1N, 4R2N). In contrast, RD4 recognizes 4R tau isoforms, but not 3R tau isoforms.

without microwave, formic acid, and proteinase K pre-treatments; with varying antibody dilutions (1:5, 1:10, 1:25; 1:50); and with varying incubation times (16 hours [h], 24 h, 48 h).

For the final double immunostaining studies, the deparaffinized and rehydrated sections were pretreated in 95% formic acid for 30 min and microwaved in distilled water at high power setting for 10 min for 3 times, and then incubated in 0.01 M phosphate-buffered saline (PBS, pH7.4) containing 0.3% hydrogen peroxide ($H_2O_2$) for 30 min. The sections were treated with 5% normal horse serum for 30 min and incubated with RD4 (1:10 in PBS with 5% normal horse serum) for 48 h. After incubation with the primary antibody, sections were treated overnight with biotinylated anti-mouse IgG secondary antibody (1:200, Vector, Burlingame, CA) followed by incubation in the avidin-biotinylated horseradish peroxidase (HRP) complex (1: 200, Vector) for 3 h. Peroxidase labeling was detected by incubation with a solution containing 0.3 mg/ml 3,3′-diaminobenzidine (DAB) and 0.03% $H_2O_2$. After the DAB reaction of the first immunohistochemical cycle, sections were treated with 0.1M glycine, pH 2.5, for 30 min, and 0.3% $H_20_2$ solution in PBS for 20 min. The second immunohistochemical cycle with RD3 (1: 5 in PBS with 5% normal horse serum) was carried out similarly to the first, except that the HRP complex was detected by Vector SG (1:100, Vector) for blue precipitation.

## Western Blot Analyses

Brains from 7 cases of AGD and 2 cases of AD were used for Western blotting and compared with 6 AD cases previously reported from our laboratory using similar methods (6). For the present study, one half of the brain had been frozen at the time of autopsy and the other half had been fixed in formalin for diagnostic evaluation. Frozen tissue blocks were dissected from anterior medial temporal lobe at the level of the uncus, including the amygdala, and were used for Western blot analyses. Formalin-fixed blocks from hippocampus and amygdala from the opposite hemisphere were used for immunohistochemistry.

Western blots and densitometric analyses were performed as described (6). In short, sarkosyl-insoluble fraction was extracted from brain homogenates and dephosphorylated by hydrofluoric acid (34). The dephosphorylated and non-dephosphorylated samples, as well as a mixture of 6 isoforms of recombinant tau prepared as previously reported (35), were loaded on 10% sodium dodecyl sulfate (SDS)-polyacrylamide-Tris/HCl gels and transblotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were incubated with anti-human tau polyclonal antibody, WKS44, followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (Chemicon, Temecula, CA). The HRP complex was detected by the Enhanced Chemiluminescence Plus System (ECL+, Amersham Life Science, Piscataway, NJ). Immunoreactive images of the blots were scanned and the densities of the immunoreactive bands were measured with MCID (Imaging Research Inc, Ontario, Canada). The ratio of 4R to 3R tau was calculated from the appropriate immunoreactive bands.

## Frequency of AGD in PSP and CBD

To examine the frequency of AGD in PSP and CBD, the hippocampal and amygdaloid sections were immunostained with a phospho-tau monoclonal antibody (CP13; 1:100, which recognizes phospho-serine 202 of tau or PHF-1; 1:100, which recognizes phosphoserines 396 and 404 of tau; both antibodies from Peter Davies, Albert Einstein College of Medicine) from 117 cases of PSP (58 females and 59 males, age at death [mean ± SD] 73.9 ± 8.3 yr) and from 17 cases with CBD (8 females and 9 males, age at death 71.7 ± 6.6 yr). All PSP and CBD cases had previously undergone a complete neuropathologic assessment, including immunostaining with tau monoclonal antibodies and silver stains to assess tau pathology. PSP cases were considered typical and had tufted astrocytes (36–38), while all CBD cases had typical astrocytic plaques and numerous gray and white matter neuropil threads (36, 39). Gallyas silver stains were used to analyze adjacent sections of hippocampus and amygdala to confirm the diagnosis of AGD (22).

## Tau Haplotype Determination

Fresh frozen brains in 20 cases of AGD (12 females and 8 males, age at death 80.0 ± 9.8 yr), 96 cases of PSP (47 females and 49 males, age at death 74.6 ± 8.0 yr), 19 cases of CBD (9 females and 10 males, age at death 74.3 ± 7.1 yr), and 651

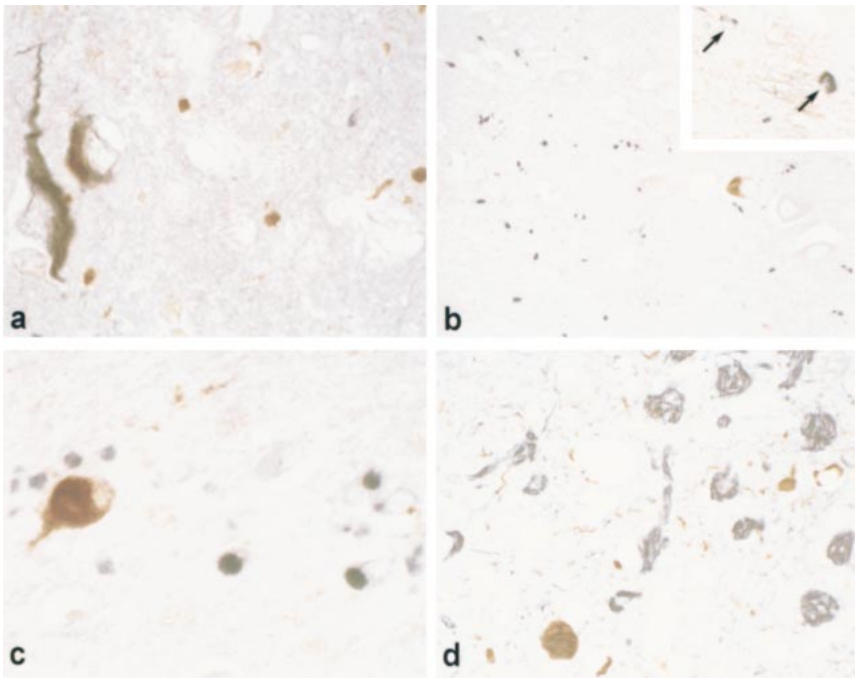


**Fig. 3.** Double immunostained sections of AGD (a, b), Pick disease (c), and AD (d) with RD3 (blue-gray) and RD4 (brown). RD4 labels scattered grains in AGD (a, DAB, brown) while 2 NFT at the left are double stained with RD3 and RD4. Reversing the chromogens produces the same result. RD4 detects grains in CA1 region of the hippocampus in AGD (b, DAB-SG, blue-gray) as well as coiled bodies (b, inset, arrows) in the subjacent white matter. A solitary NFT is positive with RD3 (DAB, brown). In the dentate fascia of Pick disease, RD3 labels many round Pick bodies (c, DAB-SG, blue gray), while a rare NFT shows immunoreactivity for RD4 (DAB, brown). In the entorhinal cortex of advanced AD, where most of the NFT are extracellular lesions, most of the NFT are labeled with RD3 (d, DAB-SG, blue-gray), while a few intracellular NFT are labeled with RD4 (DAB, brown). Note that neuropil threads are heterogenous with respect to RD4 and RD3 immunoreactivity. Magnifications: a, c, d, ×400; b, ×200.

non-tauopathy controls (344 females and 307 males, age at death 78.9 ± 11.1 yr) were used for tau genotyping. The controls were derived from an autopsy database of cases with complete neuropathologic evaluations. All brains in our brain bank that have been submitted with frozen tissue are subjected to routine tau haplotyping and apolipoprotein E genotyping. Some of the cases in this series have been included in previous publications (6, 22). All of the PSP and CBD cases met pathologic criteria and included some cases with concurrent AGD. On the other hand, none of the AGD cases had PSP or CBD, and the non-tauopathy controls did not include AGD, PSP, or CBD.

Genomic DNA was extracted from frozen brain using the QIAamp DNA mini kit (Qiagen, Bothell, WA). The DNA was amplified using a Hybaid Touchdown thermal cycler (Hybaid, Cambridge, UK). Conditions were an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60 to 50°C touchdown annealing for 30 s at 0.5°C/cycle, and 72°C for 45 s, with a final extension at 72°C for 10 min. The primer sequences used for this amplification were as follows: Forward, 5′- GGAAGACGTTCTCACTGATCTG−3′ and reverse, 5′-AGGAGTCTGGCTTCAGTCTCTC−3′. The presence of the intronic 238bp deletion (26) was determined by visualizing PCR product on an agarose gel, giving main fragment sizes of 484 and 246 base pairs. The polymorphic dinucleotide sequence was determined by PCR using conditions as described by Conrad et al (25) followed by analysis on the ABI 377 using Genotyper software (PE Applied Biosystems, Foster city, CA). The primer sequences were 5′-GCCTCGCAAATTGCTGGGAT-3′ (forward) and 5′ AGGTGACTGGGTAGAGACAGAGC-3′ (reverse).

### Statistical Methods

Correlations of Braak stage, age, and sex to the 4R/3R ratio were tested using Spearman rank order correlation. The frequencies of tau haplotypes in various tauopathies, as well as the frequency of AGD in various disorders, were tested with chi-square test or the Fisher exact test. Data were analyzed by using Sigma Stat for Microsoft Windows, version 2.03 (SPSS Science, Chicago, IL), and significance levels were set as $p < 0.05$.

## RESULTS

### Immunohistochemical Analysis of Tau

Argyrophilic grains were immunostained with RD4, but not RD3 (Figs. 3a, b, 4a–d). Reversal of the chromogen detection system gave the same results (Fig. 3a, b). As expected, argyrophilic grains were found with

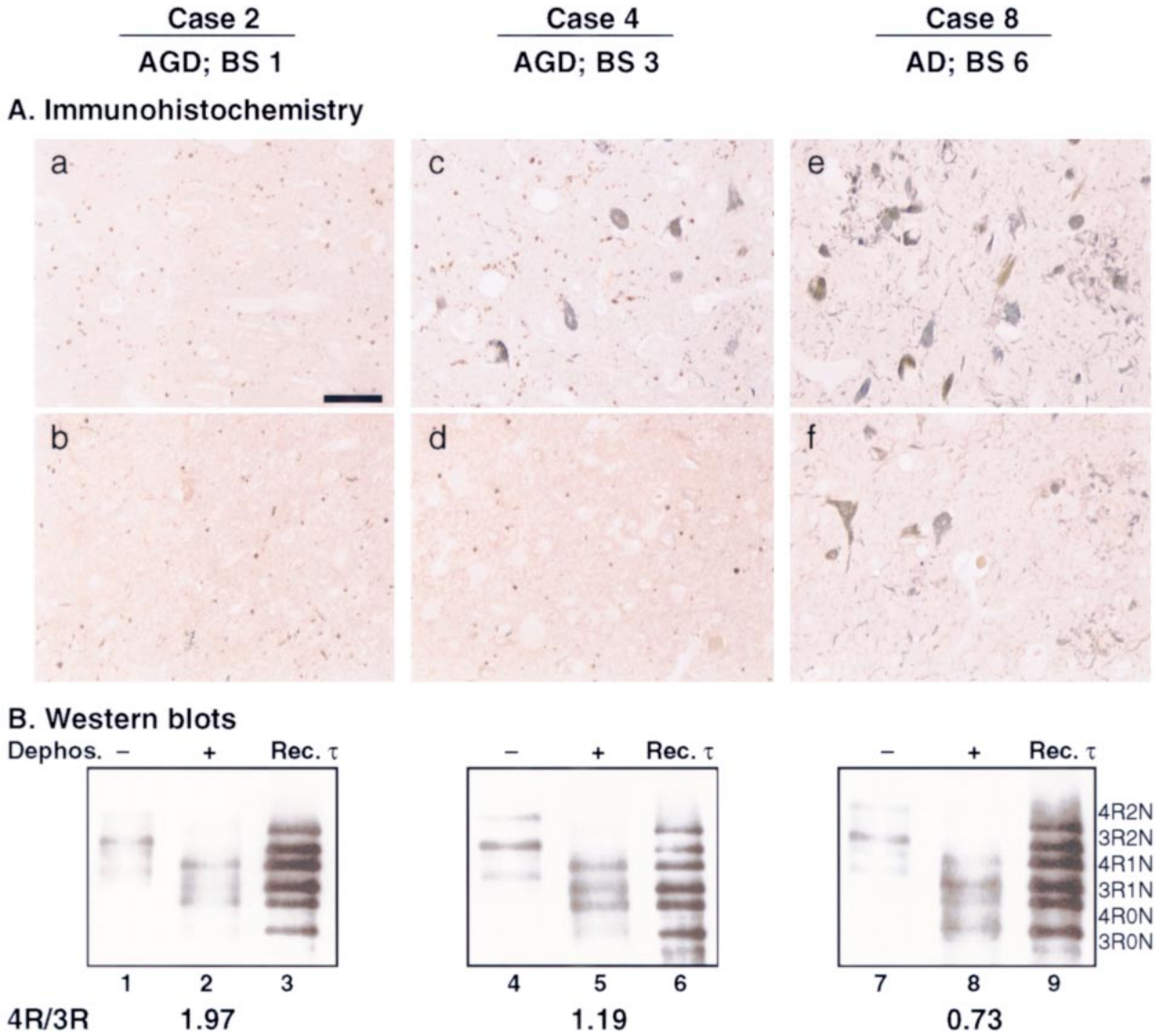


**Fig. 4.** A. Immunohistochemistry. Double-immunostaining of CA1 sector of hippocampus (a, c, e) and amygdala (b, d, f) of 2 AGD cases with BS 1 (case 2) and BS 3 (case 4) and a control AD case (BS 6, case 8) using RD3 (DAB-SG, blue-gray) and RD4 (DAB, brown). A number of AGs are positive with RD4 (a–d). On the other hand, Alzheimer NFT, neuropil threads, and dystrophic neurites in senile plaques are stained with either RD3 or RD4 or double stained with both (c–f). Magnification: a–f, ×200; Scale bar = 25 μm. B: Western blots Sarkosyl-insoluble tau of the same cases as in part (A) from anterior medial temporal lobe at the level of the uncus and including the amygdala are immunoblotted with WKS44. Non-dephosphorylated (−, lanes 1, 4, 7), hydrofluoric acid-dephosphorylated (+, lanes 2, 5, 8) samples and a mixture of all 6 recombinant tau isoforms (lanes 3, 6, 9) were separated with electrophoresis. The blots were scanned and the densities of the immunoreactive bands measured to determine the 4R/3R ratio. Dephosphorylated tau from AGD with BS1 (case 2) is composed of 2 major bands that align with four 4R tau with or without exon 2 and 1 minor band that aligns with 3R tau with exon 2 (lane 2). The dephosphorylated tau from AGD with BS 3 (case 4) is composed of major bands that align with 3R and 4R tau with and without exon 2 (lane 5). Dephosphorylated tau from AD (case 8) is composed of 2 major bands that align with 3R tau with or without exon 2 and 2 minor bands that align with 3R tau with or without exon 2. The numbers below the blots are the 4R:3R ratios. Abbreviations: AGD, argyrophilic grain disease; AD, Alzheimer disease; BS, Braak stage; Dephos., dephosphorylation; Rec τ, 6 recombinant tau isoforms; 3/4R tau, tau isoforms with 3/4 microtubule-binding repeats.

highest density in transentorhinal and entorhinal regions, CA1 sector of hippocampus, and cortical nucleus of amygdala, and the distribution and the density of these RD4-positive grains were comparable to that detected with a sensitive phospho-tau monoclonal antibody, CP13, and with Gallyas silver stains. In contrast, pre-tangles were better stained with CP13 than with RD4 (not shown). Oligodendroglial coiled bodies were also immunopositive for the 4R tau antibody, but not the 3R tau antibody (Fig. 3b, inset). On the other hand, Alzheimer

neurofibrillary pathology, including NFTs, neuropil threads, and dystrophic neurites in senile plaques, were stained with either RD3 or RD4 or double stained with both antibodies (Figs. 3a, d, 4c, e, f). The immunoreactivity of these structures was not different from those in pure AD (Fig. 4e, f).

### Western Blots Analysis of Tau

Western blots of sarkosyl-insoluble tau protein extracted from AGD and AD brains were probed with polyclonal antibody that recognizes all isoforms of tau (WKS44) and quantified with densitometric analysis. Quantitative data on the 4R/3R ratio are summarized in Table 1. After hydrofluoric acid dephosphorylation, sarkosyl-insoluble tau in AGD cases with a low Braak stage appeared as 2 major bands that aligned with recombinant 4R tau with or without exon 2 (Fig. 4B, lane 2). In AD cases, dephosphorylated tau was composed of 2 major bands that aligned with recombinant 3R tau and 2 minor bands that aligned with 4R tau, with or without exon 2 (Table 1A, cases 8, 9; Fig. 4B, lane 8). Quantitative analysis of AD showed that the 4R/3R ratios cases were usually less than 1 (Table 1A, cases 8, 9) and similar to previous data using similar methods (Table 1A, cases 10–15) (6). In AGD cases with more advanced Braak stages (e.g. Braak stages II or III), the 4R/3R ratios were intermediate between pure AGD and pure AD (Table 1A, cases 3–7; Fig. 4B, lane 5). The 4R/3R ratio decreased significantly with increasing neurofibrillary pathology as indexed by Braak stage ($r = -0.797$, $p < 0.05$; Table 1B), but did not show a correlation with age or sex.

### Tau Haplotype in AGD, PSP, CBD, and Non-Tauopathy Controls

The frequencies of the H1 haplotype in PSP and CBD, and H1/H1 genotype in PSP, were significantly higher than non-tauopathy controls ($\chi^2 = 25.400$, $p < 0.001$ [1*df*]; $\chi^2 = 3.943$, $p < 0.05$ [1*df*]; $\chi^2 = 24.513$, $p < 0.001$ [1*df*]). The frequency of H1/H1 genotype in CBD also showed a trend of increased frequency compared to controls, although it did not reach statistical significance ($\chi^2 = 3.287$, $p = 0.070$ [1*df*]). The frequency of the H1 haplotype in AGD was not different from those in PSP and CBD and showed a trend for higher frequency than in controls, although the difference between AGD and non-tauopathy controls was not statistically significant (Table 2B). The frequency of the H1/H1 genotype in AGD was also not different from those in PSP and CBD and showed a nonsignificant trend for higher frequency than in controls (Table 2C).

### Frequency of AGD in PSP, CBD, and Non-Tauopathy Controls

AGD was found in 22 of 117 PSP cases (18.8%; 12 females and 10 males, age at death 76.0 ± 6.7 yr), and in 7 of 17 CBD cases (41.2%; 4 females and 3 males, age at death 72.3 ± 5.1 yr; Table 3). PSP and CBD cases with concurrent AGD were similar to PSP and CBD cases without grains with respect to age and sex distribution. PSP cases, but not CBD cases, with concurrent AGD had a higher Braak stage than PSP cases without AGD (3.0 ± 0.2 vs 2.0 ± 0.1, $p < 0.001$; Mann-Whitney rank sum test). These frequencies were significantly higher than in a series of AD cases in our laboratory and controls in several previously published consecutive autopsy series (Table 3) (17, 19, 21). The possibility of underestimating grains in AD, especially advanced AD with its extensive neuropil tau pathology, has been previously noted (22). The availability of specific methods to detect grains, such as immunohistochemistry with antibodies to 4R tau, may eventually resolve this issue.

TABLE 2A.
Haplotype and Genotype Frequencies in AGD, PSP, CBD, and Non-Tauopathy Controls

| | AGD (n = 20) | PSP (n = 96) | CBD (n = 19) | Non-Tauopathy controls (n = 651) |
|---|---|---|---|---|
| Haplotype | | | | |
| H1 | 0.875 (35) | 0.932 (179) | 0.921 (35) | 0.771 (1004) |
| H2 | 0.125 (5) | 0.068 (13) | 0.079 (3) | 0.229 (298) |
| Genotype | | | | |
| H1/H1 | 0.750 (15) | 0.875 (84) | 0.842 (16) | 0.610 (397) |
| H1/H2 | 0.250 (5) | 0.115 (11) | 0.158 (3) | 0.323 (210) |
| H2/H2 | 0.0 (0) | 0.010 (1) | 0.0 (0) | 0.068 (44) |

TABLE 2B.
H1 Haplotype Frequencies Compared to AGD (Chi-Square Test)

| PSP | CBD | Non-Tauopathy controls |
|---|---|---|
| $\chi^2 = 0.823$ n.s. | $\chi^2 = 0.088$ n.s. | $\chi^2 = 1.838$ n.s. |

TABLE 2C.
H1/H1 Genotype Frequencies Compared to AGD (Chi-Square Test)

| PSP | CBD | Non-Tauopathy controls |
|---|---|---|
| $\chi^2 = 1.189$ n.s. | $\chi^2 = 0.099$ n.s. | $\chi^2 = 1.072$ n.s. |

The number of chromosomes and the number of subjects, respectively, are given in parentheses; AGD: argyrophilic grain disease; PSP: progressive supranuclear palsy; CBD: corticobasal degeneration; n.s.: not significant.

TABLE 3.
Frequency of AGD in PSP, CBD and Control Cases (Chi-Square Test)

| | AGD cases/ total cases | Frequency (percent) |
|---|---|---|
| PSP | 22/117 | 18.8* |
| CBD | 7/17 | 41.2* |
| Alzheimer controls[a] | 10/354 | 2.8 |
| Controls[b] | 28/301 | 9.3 |
| Controls[c] | 17/300 | 5.7 |
| Controls[d] | 125/2661 | 4.7 |

Alzheimer controls[a] investigated in our laboratory; Controls[b]: consecutive autopsies, over age 65 years, Tolnay et al, 1997 (17); Controls[c]: consecutive autopsies, mean age 61.3 ± 15.8 years, Martinez-Lage et al, 1997 (19); Controls[d]: consecutive autopsies, age 27 to 96 years, Braak et al, 1998 (21). *Significantly different ($p < 0.05$) from all controls. AGD: argyrophilic grain disease; PSP: progressive supranuclear palsy; CBD: corticobasal degeneration.

## DISCUSSION

Argyrophilic grains were first described as a novel neuropathologic finding in patients with dementia (14), and subsequent studies established AGD as a medial temporal lobe tauopathy (40). The clinical characteristics of AGD remain to be defined, but given the brunt of the pathology in the medial temporal lobe, it is not surprising that at least some cases of AGD have had a relatively restricted amnestic syndrome consistent with so-called mild cognitive impairment (41).

Because argyrophilic grains are often accompanied by varying degrees of Alzheimer neurofibrillary degeneration, it has been assumed that argyrophilic grains may be a manifestation of AD; however, the present study suggests that AGD is more akin to other 4R tauopathies, such as PSP and CBD, than to AD. The tau isoform composition of neurofibrillary pathology in AD is a variable mixture of 3R and 4R tau, depending upon the nature of the lesion and disease stage, whereas neurofibrillary lesions in 4R tauopathies are enriched in 4R tau. In the present study, grains were shown to be immunoreactive with a monoclonal antibody specific for 4R tau, and densitometric analysis of Western blots of sarkosyl-insoluble tau showed enrichment of 4R tau in AGD, consistent with the interpretation that AGD is also a 4R tauopathy.

The other 2 major sporadic 4R tauopathies (PSP and CBD) share clinical, pathologic, biochemical and genetic characteristics, although there are notable differences that warrant their separation as clinicopathologic entities (42–45). In the present study, we have shown that AGD also shares pathologic, biochemical, and genetic features with PSP and CBD. Pathologically, both PSP and CBD have tau inclusions in neurons and glia, and AGD also has tau-immunoreactive neuronal and glial lesions. A range of tau-immunoreactive neuronal and glial lesions characterizes the 4R tauopathies.

In PSP, typical neuronal lesions are globose-shaped NFTs within vulnerable neurons of the basal ganglia, subthalamic nucleus, and brainstem nuclei. In addition, it is increasingly recognized that non-filamentous precursors to dense fibrillary lesions, so-called pre-tangles, are also common in PSP, especially in the motor cortex and striatum. In CBD, globose NFTs (also referred to as ''corticobasal bodies'') are uncommon except in certain brainstem nuclei. More often, neuronal lesions in CBD are pleomorphic lesions that contain disorganized wisps of cytoplasmic fibrils that occasionally resemble small NFTs or Pick bodies. Neuronal lesions in CBD are more widespread than in either PSP or AGD and include cortical and subcortical neurons. The neuronal lesions in AGD are the most restricted in terms of cytoplasmic domain and regional anatomic distribution. Grains are fibrillar tau deposits within discrete dendritic domains of select populations of neurons in the limbic lobe and are not found elsewhere to any significant degree (17, 18, 20).

Ballooned neurons are another type of neuronal alteration found in 4R tauopathies. They are swollen pyramidal neurons that show neurofilament and variable tau immunoreactivity. Ballooned neurons are also intensely immunoreactive for the small heat shock protein α-B crystallin (46). In CBD, ballooned neurons are typically numerous and most abundant in cortices showing focal atrophy. Ballooned neurons are uncommon in PSP and when detected are usually restricted to the limbic lobe. In most but not all cases, this is due to concurrent AGD (47). In AGD, ballooned neurons are a constant feature in limbic areas and found in virtually all cases in amygdala (24), cingulate cortex, claustrum, or entorhinal cortex (22).

Analogous to neuronal lesions, there is a range of tau-immunoreactive astrocytic lesions in the 4R tauopathies. While astrocytic lesions are not a prominent feature of AGD, they have been described (48). In contrast, specific astrocytic lesions are characteristic of PSP and CBD and aid in their neuropathologic diagnosis (49). In particular, tau-positive astrocytes in PSP have a tufted appearance and are abundant in the neocortex and striatum. The hallmark of CBD is the astrocytic plaque (39), which is characterized by fibrillar tau aggregates within distal domains of astrocytic cell processes. Astrocytic plaques in CBD are most often found in the neocortex, especially in regions that show focal cortical atrophy. Previous studies have shown that tufted astrocytes and astrocytic plaques are positive for 4R tau using an antibody specific to 4R tau (8). The present results extend these observations to AGD.

Oligodendroglial lesions in the 4R tauopathies show the least diversity in terms of morphology, but differ significantly in terms of their anatomic distribution; in AGD,

they are largely restricted to the white matter underlying the limbic cortex. In CBD, widespread distribution of tau-positive threads and coiled bodies is increasingly recognized as perhaps the most significant defining pathologic lesion of the disorder. In PSP, coiled bodies are most numerous in the diencephalon, especially in white matter tracts in the vicinity of the subthalamic nucleus.

Biochemical studies of brain tissue from PSP and CBD also show similar alterations in tau protein. Abnormal tau proteins in PSP and CBD are relatively insoluble and hyperphosphorylated and are enriched in 4R tau (6, 8, 9), which is also true for AGD as shown in the present study.

Genetically, over-representation of an extended tau H1 haplotype and H1/H1 genotype is recognized in both PSP and CBD (26, 28, 29). In this study, we showed that AGD showed a trend for increased H1 haplotype similar to PSP and CBD; however, the small sample size limited statistical significance between AGD and non-tauopathy controls. Nevertheless, there was no significant difference between H1 haplotype frequency and frequency of the H1/H1 genotype in AGD compared to PSP and CBD. That a sample size explains the lack of significance is reasonable since the frequency of H1 is relatively high even in control populations and statistical significance was also not detected for CBD until a sufficient sample size was reached, in one case requiring a multinational collaborative study to assemble a large enough collection of pathologically confirmed cases of CBD to prove this association (29).

The notion that AGD shares common genetic background and pathogenesis with PSP and CBD is also supported by the high frequency of their coexistence. In the present study, AGD was found in conjunction with PSP and CBD frequently, with 19% of PSP and 41% of CBD cases having AGD, compared to 5%–9% of consecutive autopsies (17, 19, 21, 22). This agrees with the previous publications that have reported a high frequency of AGD in PSP (21), but differs from the interpretation of Braak et al (21) and of Tolnay et al (50) that AGD and PSP are distinct disease processes.

In this study, we have shown immunohistochemically and biochemically that argyrophilic grains are composed of tau enriched in 4R tau. In addition, AGD may share a common genetic risk factor with PSP and CBD. These results, together with the high frequency of AGD in PSP and CBD, suggest that these 4R tauopathies define a disease spectrum, with a medial temporal lobe tauopathy (AGD) intermediate between a diffuse cortical and subcortical tauopathy (CBD) and a more restricted basal ganglia and brainstem tauopathy (PSP).

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