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Review

Astrocytic Tau Pathologies in Argyrophilic Grain Disease and Related Four-repeat Tauopathies

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Neurodegenerative diseases in which tau accumulation plays a cardinal role in the pathogenic process are called tauopathies, and when tau isoforms having four repeats of the microtubule binding sites, four-repeat tau, are selectively accumulated as pathological hallmarks, the term four-repeat tauopathy is used. The major four-repeat tauopathies are progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and argyrophilic grain disease (AGD). Historically, neuronal cytopathologies, e.g., neurofibrillary tangles and ballooned neurons, were emphasized as characteristic lesions in PSP and CBD. Now, however, astrocytic tau pathologies, *i.e.*, tufted astrocytes (TAs) and astrocytic plaques (APs), are considered to be highly disease-specific lesions. Although granular/fuzzy astrocytes (GFAs) frequently develop in the limbic system in AGD cases, the specificity is not conclusive yet. Some AGD cases have a few TAs, and to a lesser frequency, a few APs in the frontal cortex and subcortical nuclei. The number of astrocytic tau pathologies including TAs and GFAs increases with the progression of AGD. In this paper, histopathological features of astrocytic tau pathologies in PSP, CBD, and AGD are first reviewed. Then, recent findings regarding the coexistence of these tauopathies are summarized from a viewpoint of astrocytic tau pathologies. Further biochemical and pathological studies focusing tau-positive astrocytic lesions may be useful to increase understanding of the pathological process in four-repeat tauopathies and to develop novel therapeutic strategies for patients with these diseases.

Key words: astrocytic plaque, four-repeat tau, globular glial inclusion, granular fuzzy astrocyte, tufted astrocyte

Historically, neurodegenerative diseases were pathologically diagnosed only according to the anatomical distribution of tissue degeneration characterized by neuronal loss with gliosis. Later, cytopathological features (e.g., neurofibrillary tangles (NFTs) and Lewy bodies, *etc.*) were regarded to be definitive bases in the diagnosis of degenerative diseases. These inclusions were first defined almost exclusively by morphological and histochemical features. However, along with the accumulation of biochemical findings, it has been revealed that disease-specific proteins, such as tau, α-synuclein, and transactive response DNA-binding protein of 43 kDa (TDP-43), are accumulated in distinctive neuronal and glial inclusions. These proteins are often hyperphosphorylated, which changes their conformations, resulting in protein misfolding. The protein misfolding can lead to protein aggregation, causing the impairment of cellular functions [1].

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Therefore, the pathological diagnosis of most neurodegenerative diseases is now based on morphological, immunohistochemical, and biochemical features associated with disease-specific cytopathologies.

Neurodegenerative diseases in which the pathological process is mainly associated with the abnormal accumulation of tau protein are called tauopathies. Tau is a microtubule-associated protein that stabilizes microtubules [2]. Tau protein exists in six predominant isoforms by alternative mRNA splicing of exon 2, exon 3, and exon 10 in a single gene, *MAPT* [3,4]. Exon 10 encodes the fourth microtubule-binding repeat. Three isoforms have three repeats of the microtubule binding sites, being called three-repeat tau (3R tau). The remaining three isoforms have four repeats of the microtubule binding sites and are called four-repeat tau (4R tau) [5,6]. Tau immunoblotting of sarkosyl-insoluble brain extracts demonstrates 60,64, and 68 kDa bands in AD cases, while 60 and 64 kDa bands are revealed in Pick's disease cases. In PSP and CBD cases, 64 and 68 kDa bands are revealed. A doublet of 60 and 64 kDa bands consists of 3R tau isoforms, while a doublet of 64 and 68 kDa bands consists of 4R tau isoforms. Therefore, these findings suggest that the composition of filamentous tau is different between tauopathies. Indeed, immunostaining using 3R tau- and 4R tau-specific antibodies demonstrates that NFTs in Alzheimer's disease (AD) are composed of both 3R tau and 4R tau, while Pick bodies in Pick's disease are basically composed of 3R tau alone. Several tauopathies in which 4R tau is selectively accumulated are called four-repeat tauopathies. The major four-repeat tauopathies are progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and argyrophilic grain disease (AGD). Their cardinal cytopathological features are NFTs, threads, coiled bodies, and tufted astrocytes (TAs) in PSP; NFTs, threads, ballooned neurons, and astrocytic plaques (APs) in CBD; and argyrophilic grains in AGD. These lesions are labeled with 4R tau- but not 3R tau-specific antibodies.

In the first report of PSP by Steele *et al.* in 1964 [7], the formation of NFTs in the subcortical nuclei was emphasized as a pathological feature. In contrast, they did not note any glial inclusions, probably because only relatively insensitive conventional stains were available. About 30 years later, morphologically distinct argyrophilic astrocytic lesions, so-called tufted astrocytes (TAs), were discovered in PSP brains [8-11]. In the pathological diagnostic criteria proposed in 1994, the presence of TAs was noted as a supportive feature of PSP [12].

Like PSP, ballooned neurons were emphasized as characteristic histological changes in the first description of CBD by Rebeiz *et al.* in 1968 [13], although no glial inclusion was noted. Now, the occurrence of astrocytic plaques (APs) is considered to be highly disease specific and have diagnostic value in CBD [14].

Recently, the classification of major tau-positive astrocytic lesions was proposed [15]. In the consensus paper, TAs and APs are defined as astroglial lesions specific to PSP and CBD, respectively. Further, globular astroglial inclusions and ramified astrocytes are classified as disease-specific lesions of globular glial tauopathy and Pick's disease, respectively. On the other hand, thorn-shaped astrocytes and granular/fuzzy astrocytes (GFAs) are considered to be astrocytic lesions that are not always specifically associated with degenerative diseases, and they were named 'age-related tau astrogliopathy'. GFAs basically have morphological features identical to those of the bush-like astrocytes that were originally reported in AGD cases. However, in a consensus paper, it was noted that the specificity of GFAs was not established yet [15].

In this paper, we first summarize the pathological features of tau-positive astrocytic lesions in the major four-repeat tauopathies, *i.e.*, PSP, CBD, and AGD. Then, we show recent findings regarding the significance of astrocytic tau pathologies in the evaluation of the coexistence of tauopathies and early pathological changes that are usually found incidentally.

Tau Astrogliopathy Specific to PSP

The occurrence of TAs is one of the cytopathological hallmarks of PSP, in addition to 4R tau-positive NFTs, pretangles (diffuse or granular tau accumulations in neuronal cytoplasm with rare fibrillary formation), threads, and coiled bodies [12]. TAs are clearly visualized by the Gallyas method and tau immunohistochemistry (Fig. 1A, 1B, 1C). TAs immunopositive for glial fibrillary acidic protein (GFAP) are rare [16-18], and in general, TAs are not found associated with infarcts or senile plaques [17]. Therefore, it is considered that TAs may result from a degenerative process rather than a reactive change of astrocytes [17]. TAs are morphologically characterized by densely packed tau

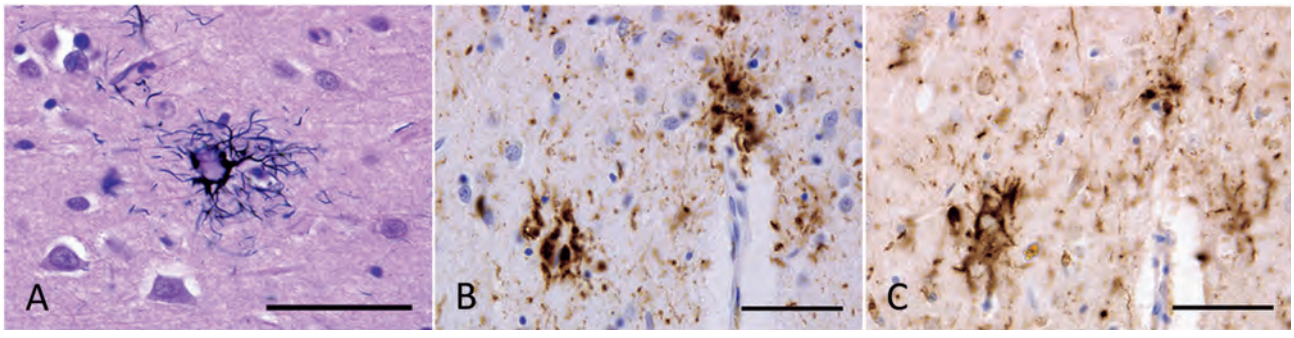

Fig. 1 Tufted astrocytes in the frontal cortex in a case of progressive supranuclear palsy (PSP). (**A**) Gallyas method, (**B**) immunohistochemistry using phosphorylation-dependent anti-tau antibody (AT8), (**C**) tau immunohistochemistry using 4R tau-specific antibody (RD4). (**B**, **C**) The identical region is shown in mirror serial sections after reversal. All scale bars = 50 μm. Figures used with permission from reference [39].

accumulation in astrocytic processes. Tau accumulation is more prominent in the proximal portion than the distal portion of processes and cytoplasm of astrocytes [18]. This intracellular distribution of aggregated tau in TAs is in contrast to that in APs in CBD cases. An astrocytic nucleus is often noted in the central portion of the radially arranged tau-positive processes. TAs are often observed near vessel walls [19], and radiating tau-positive processes are attached to them [18]. The distance between TAs and blood vessels is shorter than that between APs and blood vessels [19].

According to the pathological diagnostic criteria proposed in 1994 [12], the diagnosis of PSP is primarily made according to the distribution and quantity of neuronal tau accumulations, such as NFTs, pretangles, and threads. Predilection sites of these lesions in PSP cases are the caudate nucleus, putamen, globus pallidus, subthalamic nucleus, oculomotor nucleus, substantia nigra, pontine nucleus, inferior olivary nucleus, and dentate nucleus in the cerebellum [20-22]. In these criteria, the presence of TAs is regarded as a supportive feature of PSP. Predilection sites of TAs are not necessarily identical to those of NFTs: TAs frequently occur in the primary motor cortex, parietal cortex, putamen, caudate nucleus, globus pallidus, substantia nigra, red nucleus, and inferior olivary nucleus [20-25]. It was reported that neuronal and glial cell pathologies are not spatially correlated [26]. These findings suggest that TAs do not always occur secondarily after NFTs are formed.

Immunohistochemically, TAs are labeled with major anti-tau antibodies, including AT8, MC-1, or Alz-50 [10, 16]. TAs are hardly labeled with anti-ubiquitin antibodies [21, 27], but often labeled with anti-p62 antibodies [27]. 4R tau is selectively accumulated in TAs as well as other tau pathologies including subcortical NFTs [28].

An ultrastructural study demonstrated that straight tubules forming loose bundles with a diameter of about 15 nm were found in the astrocytic perikaryon [9]. In an ultrastructural study using filament-enriched fractions extracted from the caudate nucleus and motor cortex in which the presence of many TAs was confirmed, tau filaments were straight, their contours were jagged, and the mean width was 22 ± 5.5 nm [16].

Tau immunoblotting of the Sarkosyl-insoluble, urea-soluble fraction in the basal ganglia and brain stem in PSP cases demonstrates the doublet of 64 and 68 kDa bands and 33 kDa tau fragments [29, 30]. Western blotting using filament-enriched fractions extracted from the caudate nucleus and motor cortex in which many TAs were present demonstrated that PHF-1 revealed 68 and 64 kDa bands, and E-10, tau-1, and tau 46 revealed 2-6 bands of 45-68 kDa [16].

Which anatomical sites are affected by PSP pathology in the early stage of the course remains unclear. In an early PSP case reported by Sakai *et al.* [31], TAs were found only in the caudate nucleus, putamen, globus pallidus, and subthalamic nucleus, while NFTs were widely distributed in the basal ganglia and brain stem nuclei. In this case, neuronal loss was noted only in the subthalamic nucleus and substantia nigra. Thereafter, Nogami *et al.* [32] explored early PSP pathology in 324 consecutive autopsy cases. They first screened for 4R tau-positive NFTs, pretangles, and TAs in the midbrain, and selected 29 cases probably having early PSP

pathology but lacking other degenerative diseases. Tau immunohistochemistry in the motor cortex, opercular cortex, basal ganglia, and brain stem nuclei revealed TAs in the putamen in 8 cases (27.6% of 29 cases), in the substantia nigra in 6 cases (20.7%), and in the subthalamic nucleus in 5 cases (17.2%). Few TAs were noted in the other regions. These findings suggest that sites where TAs initially occur may be the putamen, substantia nigra, and subthalamic nucleus.

Tau Astrogliopathy Specific to CBD

The most important pathological hallmark of the diagnosis of CBD is the occurrence of APs (Fig. 2A, 2B, 2C). In addition to APs, CBD cases consistently have NFTs, pretangles, threads, and ballooned neurons in the subcortical nuclei and frontoparietal cortex. The occurrence of APs with typical morphology and distribution strongly supports the pathological diagnosis of CBD [15, 20]. The presence of ballooned neurons was originally emphasized as an essential pathological feature of CBD [13]. However, subsequent studies revealed that ballooned neurons can also develop in the limbic system in other degenerative diseases, especially AGD [33]. The anatomical distribution of APs may not be necessarily identical to that of TAs in PSP. Although both TAs and APs are frequent in the frontoparietal cortex and striatum, TAs rather than APs tend to develop frequently in the globus pallidus, subthalamic nucleus, red nucleus, and substantia nigra [21]. APs are clearly visualized by the Gallyas method and tau immunohistochemistry. APs are labeled with anti-p62 antibodies, but hardly labeled with anti-ubiquitin antibodies [21, 27]. Like TAs in PSP, some APs are GFAP-positive [18]. Although APs are often observed near the vessels, the distance between APs and vessel walls may be longer than that between TAs and vessel walls [19]. Tau immunoblots demonstrated 64 and 68 kDa bands and low molecular weight tau fragments of 37 kDa [29, 30].

A previous study demonstrated that TAs and APs did not coexist in 1 case when strict criteria were applied [8]. On the other hand, Katsuse *et al.* reported an autopsy case that had both TAs and APs, and tau immunoblotting demonstrated 33 and 37 kDa bands as well as 64 and 68 kDa doublets [34]. However, in this case the presence or absence of mutations in *MAPT* was not explored. Tan *et al.* reported 2 autopsy cases that had both APs and TAs. These 2 cases lacked any *MAPT* mutation. However, tau immunoblotting demonstrated only 64, 68, and 37 kDa bands, being consistent with the band pattern in CBD cases [35]. In cases that appear to have both CBD and PSP pathologies, biochemical and genetic analyses should be done because some reported cases with *MAPT* mutations show PSP- or CBD-like pathology [36].

Like PSP, the available data regarding early lesions of CBD cases are very limited because it is difficult to accumulate early CBD cases. Recently, Ling *et al.* reported pathological features in 3 preclinical autopsy-confirmed CBD cases [37]. Compared with six advanced CBD cases, tau pathology in the preclinical CBD cases was mild, and APs were more prominent than neuronal tau accumulation, suggesting that the formation of APs might precede that of NFTs and pretangles. Neurons in the substantia nigra were spared in number. APs were noted in the anterior frontal white matter, posterior frontal cortex, parietal cortex, amygdala, caudate nucleus, putamen, globus pallidus, subthalamic nucleus, and substantia nigra. These patho-

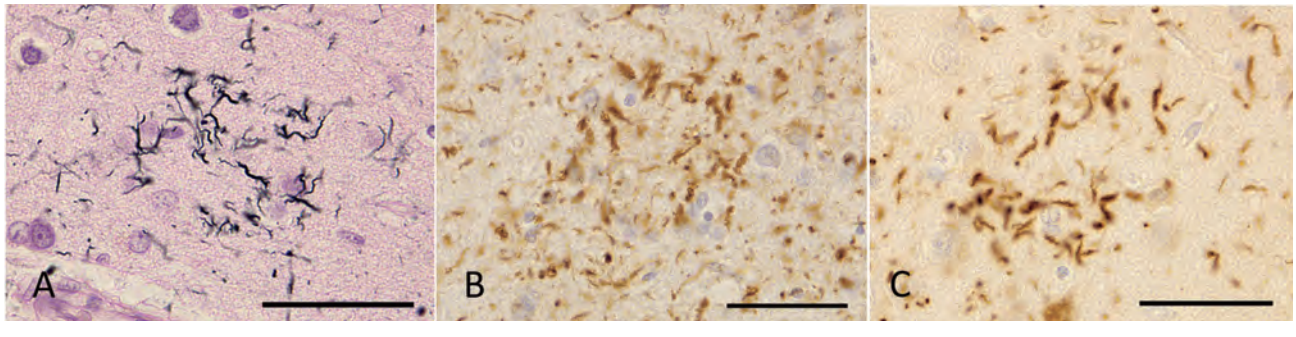

Fig. 2 Astrocytic plaques in the frontal cortex in a case of corticobasal degeneration (CBD). (**A**) Gallyas method, (**B**) AT8 immunohistochemistry, (**C**) RD4 immunohistochemistry. All scale bars = 50 μm. Figures used with permission from reference [39].

logical features are consistent with those observed in our previously reported case having very early CBD pathology (Fig. 3A-3L) [38]. In the present pathological criteria of CBD, neuronal loss in the substantia nigra is emphasized [20]. However, given these findings, it is likely that the neuronal loss in the site may be a characteristic in advanced cases of CBD.

GFAs as an Age-related Tau Astrogliopathy

GFAs are tau-positive astrocytic lesions that are classified as a disease-nonspecific, aging-related tau astrogliopathy (ARTAG), which usually occurs in the gray matter rather than the white matter (Fig. 4) [15, 39, 40]. In GFAs, phosphorylated tau is densely accumulated in the perinuclear region of astrocytes, and fine or granular tau accumulation in the astrocytic processes is radially distributed [15]. The Gallyas method occasionally demonstrates argyrophilia in the soma but not in the processes [15].

The pathophysiological significance of GFAs remains unclear. GFAs are immunohistochemically identical to

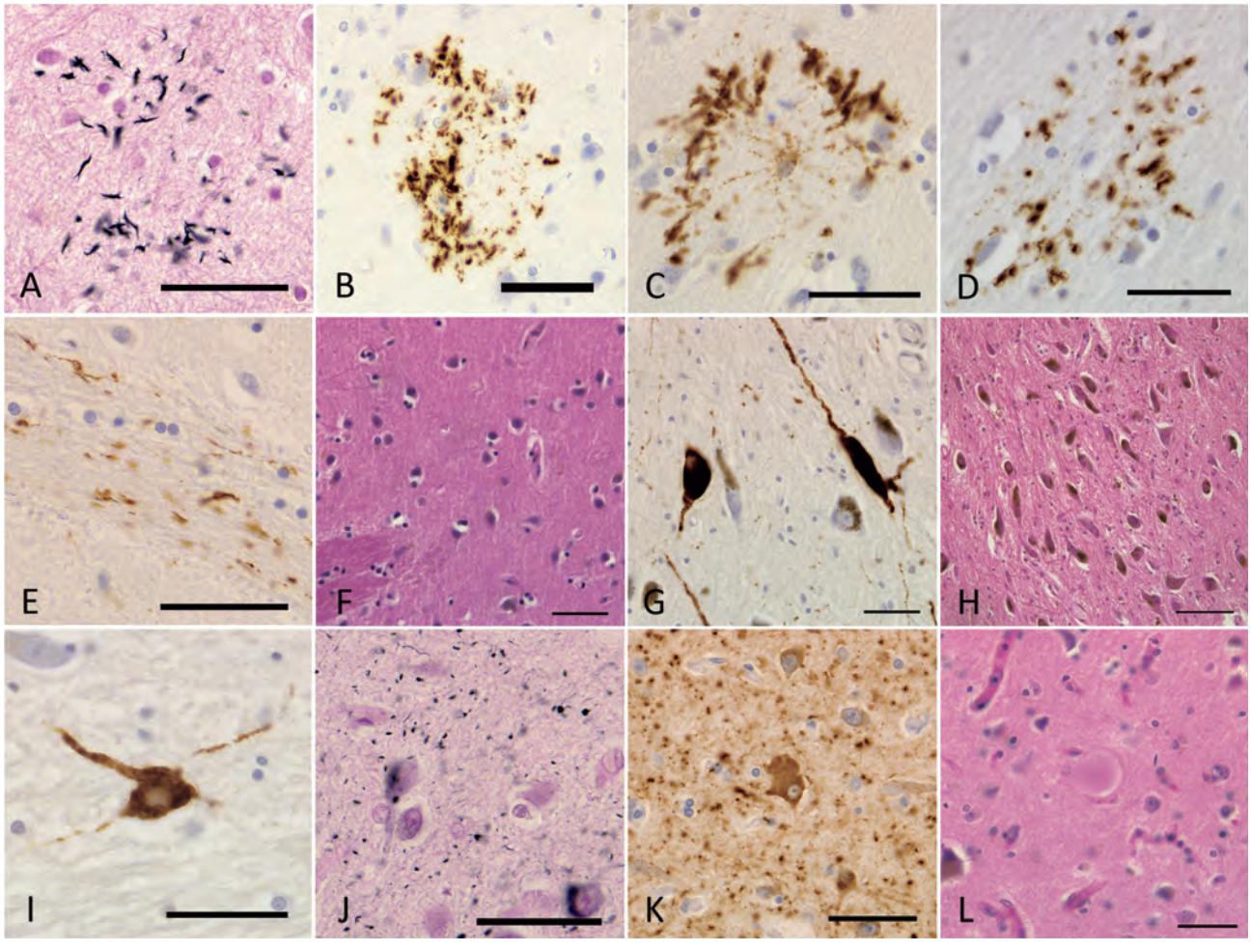

Fig. 3 Early corticobasal degeneration (CBD) pathology with astrocytic plaques (APs). (**A-D**) Astrocytic plaques in the superior frontal gyrus (**A**), motor cortex (**B**), caudate nucleus (**C**), and putamen (**D**). (**E**) A small number of tau-positive threads. The putamen. (**G, I**) A small number of NFTs and threads in the substantia nigra (**G**) and pontine nucleus (**I**). (**F, H**) Unlike classic CBD cases, neuronal loss with glial proliferation was not seen in the putamen (**F**) or substantia nigra (**H**). (**J**) Argyrophilic grains. The amygdala. (**K**) A ballooned neuron and argyrophilic grains. The amygdala. (**L**) A ballooned neuron. The amygdala. (**A, J**) Gallyas-Braak silver stain, (**F, H, L**) hematoxylin-eosin stain, (**B, C, D, E, G, I, K**) AT8 immunohistochemistry. Scale bars = (A, C, D, E, F, G, I, J, K, L) 50 μm, (B) 25 μm, (H) 100 μm. Figures used with permission from reference [38].

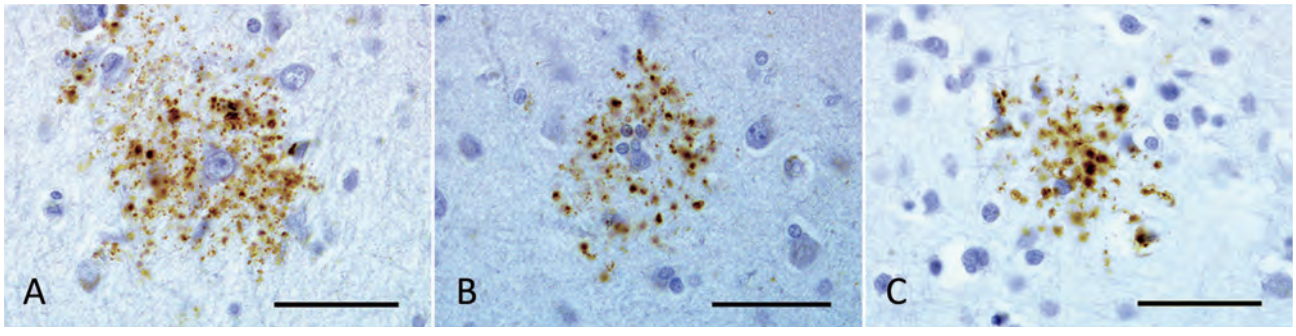

Fig. 4 Granular/fuzzy astrocytes (GFAs) in argyrophilic grain disease (AGD) cases. All scale bars = 50 μm. Figures used with permission from reference [39].

the bush-like astrocytes that were originally found in the limbic system in AGD cases [39-42]. In our recent study, 6 of 20 AGD cases (30.0%) had GFAs but no TAs, and 5 (25.0%) had both GFAs and TAs [39,40]. That is, GFAs were found in 55% of AGD cases [39,40]. However, GFAs are not always specific to AGD [15]. For example, some PSP cases without AGD have not only TAs but also GFAs [39,40]. Kovacs *et al.* reported that elderly cases with mild to moderate NFTs and various quantities of neuritic plaques had GFAs in the frontal, temporal, and cingulate cortices, amygdala, basal ganglia, and brain stem nuclei [43-45]. In the consensus paper on tau astrogliopathy, it was recommended that in cases having GFAs, underlying primary tauopathies should be also documented [15].

The data of the distribution of GFAs are limited. Botez *et al.* reported that GFAs (bush-like astrocytes in their paper) were found in the amygdala and entorhinal cortex, but they did not examine any other regions [41]. In our six AGD cases with GFAs but without TAs that did not meet the pathological criteria of other tauopathies [39], areas most frequently affected by GFAs were the putamen (66.7%), followed by the caudate nucleus (50.0%), frontal cortex (50.0%), globus pallidus (33.3%), oculomotor nucleus (16.7%), and pontine nucleus (16.7%).

Pathological Similarities of GFAs and TAs

Some AGD cases have not only GFAs but also a small number of TAs in the subcortical nuclei [42]. In our recent study, 5 of 20 AGD cases (25.0%) had TAs [39]. The morphological features of TAs in AGD cases were indistinguishable from those of TAs in PSP cases. Further, AGD cases having TAs also had GFAs without exception. Fig. 5 shows the distributions of TAs (red bars) and GFAs (purple bars) in AGD cases. These findings suggest that both TAs and GFAs preferentially developed in the putamen, caudate nucleus, and superior frontal cortex in AGD cases [39,40]. Further, the predilection sites of TAs in PSP cases (green bars) were consistent with those of GFAs and TAs in AGD cases [39,40].

On double staining by AT8 immunohistochemistry and the Gallyas method, a small number of fine Gallyas-positive glial thread-like structures are observed in the distal portion of some GFAs in AGD cases (Fig. 6) [39,40]. In PSP cases without AGD also, GFAs with and without a varying quantity of Gallyas-positive glial threads were found [39,40]. These findings support the possibility that at least some GFAs can evolve into Gallyas-positive TAs.

Increase of Astrocytic Tau Pathologies Associated with Progression of AGD

In our previous study, the number of tau-positive astrocytic lesions (including GFAs and TAs) increased with the progression of AGD (Fig. 7) [39]. Likewise, the quantity of TAs stained with the Gallyas method increased with the progression of AGD (Fig. 8) [39,40]. Interestingly, in this study, abnormal neuronal tau accumulation in the subcortical nuclei also increased with the progression of AGD [39]. Several other studies also demonstrated that some AGD cases had neuronal tau accumulation (*i.e.*, NFTs and pretangles) in the subcortical nuclei [42,46-50]. These findings led us to consider that the progression of AGD may be associated with the formation of astrocytic tau pathologies and tau

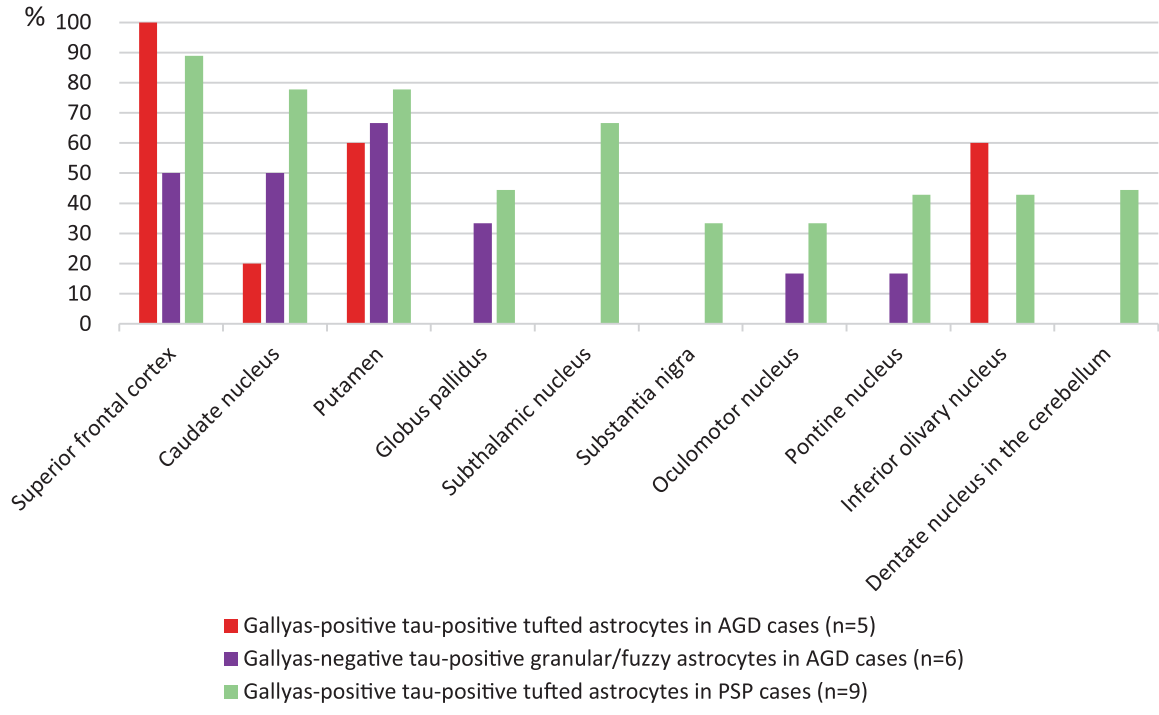

Fig. 5 The predilection sites of Gallyas-positive tau-positive tufted astrocytes (TAs) in argyrophilic grain disease (AGD) cases (n = 5), Gallyas-negative tau-positive granular/fuzzy astrocytes (GFAs) in AGD cases (n = 6), and TAs in PSP cases (n = 9). The proportions of cases having these astrocytic lesions by anatomical region are indicated. The predilection sites of TAs (red) and GFAs (purple) in AGD cases are similar to that of TAs in PSP cases (green). A graph produced using the data from Ikeda *et al*. [39]. A graph used with permission from reference [40].

accumulation in neurons in the subcortical nuclei.

Coexistence of AGD with PSP and CBD: Implications of Astrocytic Tau Pathologies

PSP cases often have coexisting AGD at frequencies ranging from 18.8% [28] to 80% [46]. On the other hand, as noted above, a small number of TAs is frequently found in AGD cases, which comprised 25.0% in our series [39]. It is also known that AGD frequently coexists with CBD pathology at a frequency ranging from 41.2% [51] to 100% [46,52,53], being higher than those in PSP. In contrast, the occurrence of APs in AGD cases may be rare, and the frequency was only 5.0% in our series [39]. Why the frequency of APs is far lower than that of TAs in AGD cases remains unclear. It might be explained by the possible low frequency of CBD in general populations. It is also unclear why the frequency of APs in AGD cases is low (5%), although the frequency of AGD in CBD cases was reported to be very high (41% to 100%). These findings led us to consider that argyrophilic grains may frequently occur parallel to the progression of CBD pathology, and that it may be rare that CBD pathology is secondarily formed after the progression of AGD.

Conclusions

A recently proposed classification of tau-positive astrocytic lesions may provide a novel viewpoint from which to examine the pathophysiology of four-repeat tauopathies. Several findings focusing on astrocytic tau pathologies support the possibility that at least some, although not all, four-repeat tauopathies share a common pathophysiological background. Further biochemical and pathological studies focusing astrocytic lesions may be useful to increase understanding of the pathological process in four-repeat tauopathies and to develop novel therapeutic strategies for patients with these diseases.

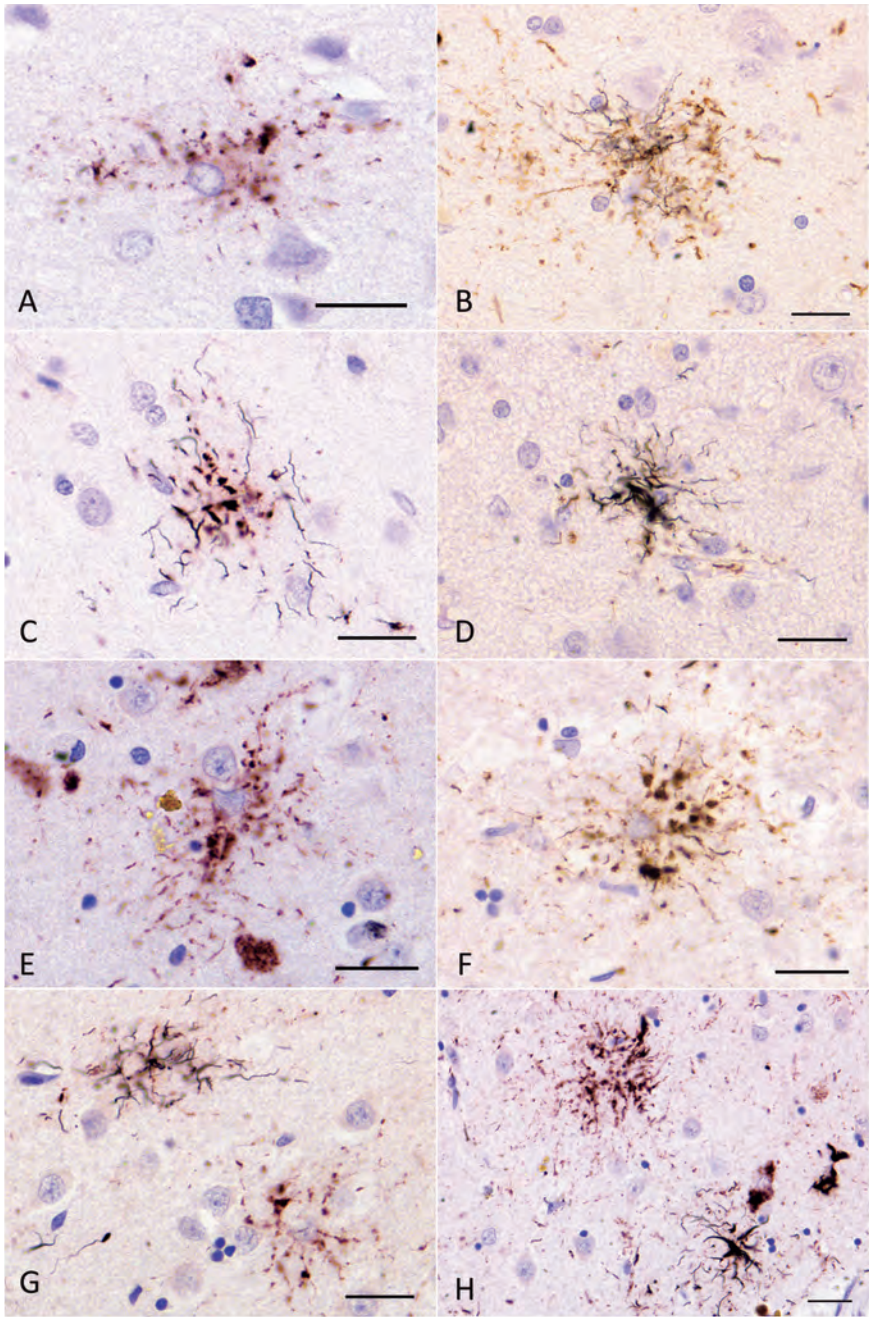

Fig. 6 Tufted astrocytes (TAs) and granular/fuzzy astrocytes (GFAs) in AGD and PSP cases. Double staining of AT8 immunohistochemistry and Gallyas method. (**A**, **B**, **C**, **D**) Astrocytic tau pathology in the superior frontal cortex in an AGD case having both GFAs (**A**) and TAs (**B**, **C**, **D**). The proportion of Gallyas-positive structures varied between lesions. A small number of fine Gallyas-positive glial threads were often observed in the distal portion of astrocytic lesions. (**E**, **F**, **G**, **H**) GFAs and TAs in the putamen in a PSP case. (**E**) GFAs had fine granular tau accumulations that were radially arranged. It is hard to morphologically distinguish GFAs in PSP cases (**E**) from GFAs in AGD cases (**A**). (**F**) When the number of Gallyas-positive thread-like structures was small, they were often found in the distal portion of each astrocytic lesion. (**G**) The lesion on the right (GFA) lacks a Gallyas-positive structure, while the one on the left (TA) has Gallyas-positive thread-like structures. (**H**) A right-hand lesion (TA) has Gallyas-positive thread-like structures, while the left-hand one (GFA) lacks Gallyas-positive structures. The peroxidase labeling was visualized with DAB (**B**, **D**, **F**) or Vector NovaRED (**A**, **C**, **E**, **G**, **H**). All scale bars = 20 μm. Figures used with permission from reference [39].

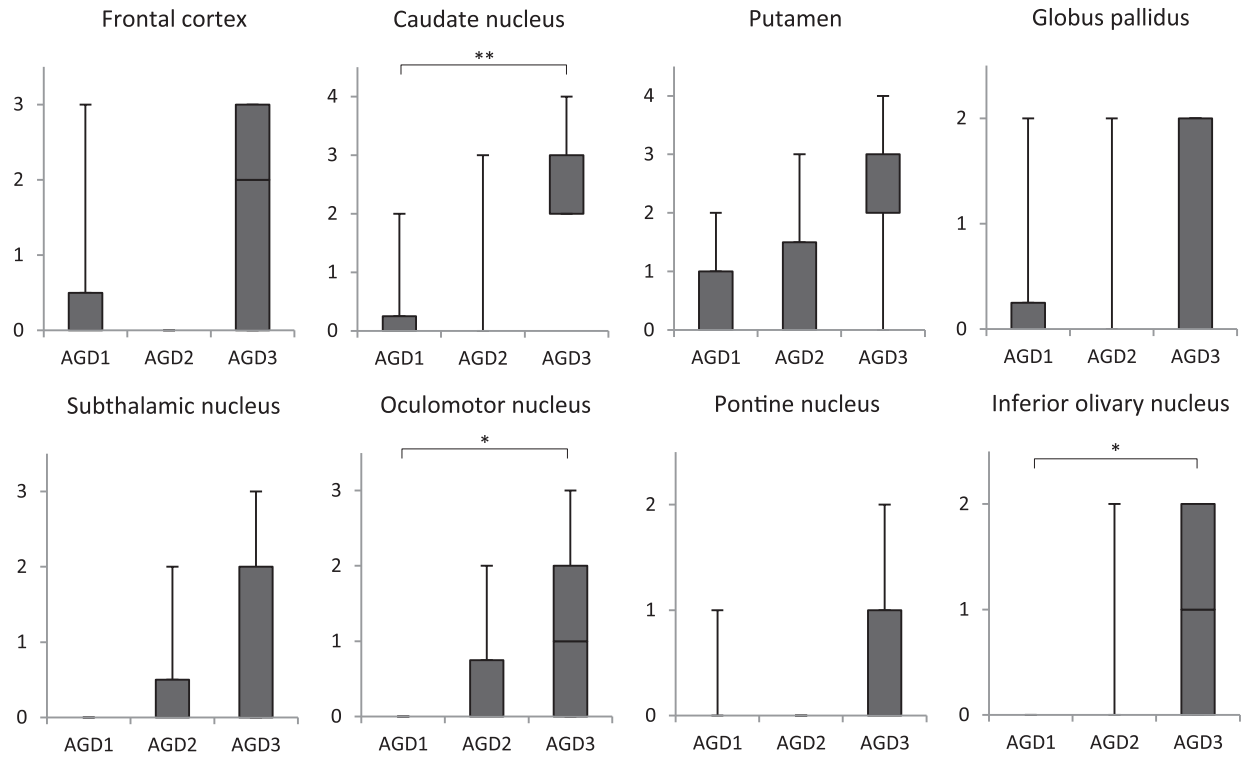

Fig. 7 Quantity of tau (AT8)-positive astrocytic lesions by AGD stage in AGD cases. The number of astrocytic lesions (TAs and GFAs) is shown on the vertical axis: stage 0, no lesion; stage 1, one TA in the anatomical region; stage 2, two to four TAs in the anatomical lesion but less than one TA per × 200 visual field; stage 3, one TA per × 200 visual field; stage 4, 2 to 10 TAs per × 200 visual field; stage 5, 11 to 20 TAs per × 200 visual field; stage 6, over 20 TAs per × 200 visual field. In all regions, the number of tau-positive astrocytic lesions was sequentially increased with the progression of AGD stage. In the caudate nucleus, oculomotor nucleus, and inferior olivary nucleus, the differences between the quantities of astrocytic lesions in different AGD stages reached statistical significance. AGD1: Saito stage I; AGD2: Saito sage II; AGD3: Saito stage III. Kruskal-Wallis and Steel-Dwass tests; $^{*}p < 0.05$; $^{**}p < 0.01$. Graphs used with permission from reference [39].

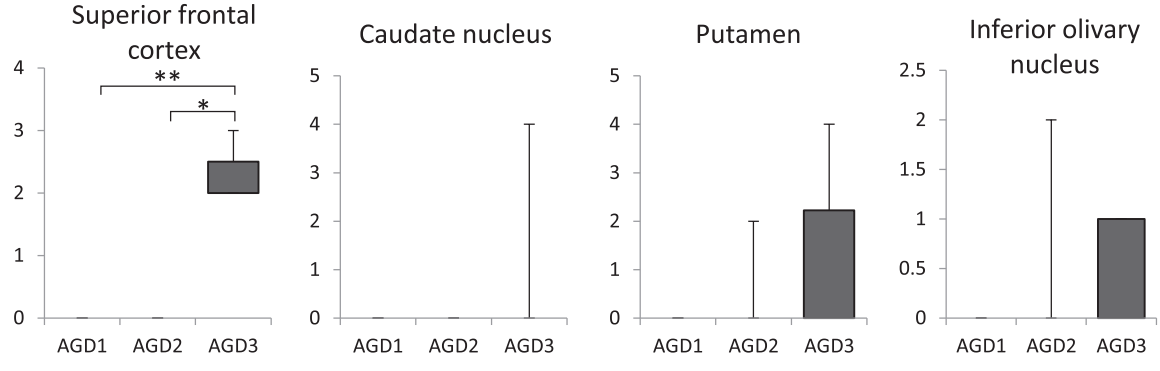

Fig. 8 The relationship between the quantity of Gallyas-positive TAs and the severity of argyrophilic grains (Saito stage) in AGD cases. The number of TAs is shown on the vertical axis: stage 0, no lesion; stage 1, one TA in the anatomical region; stage 2, two to four TAs in the anatomical lesion but less than one TA per × 200 visual field; stage 3, one TA per × 200 visual field; stage 4, 2 to 10 TAs per × 200 visual field; stage 5, 11 to 20 TAs per × 200 visual field; stage 6, over 20 TAs per × 200 visual field. AGD1: Saito stage I; AGD2: Saito sage II; AGD3: Saito stage III. Kruskal-Wallis and Steel-Dwass tests; $^{*}p < 0.05$; $^{**}p < 0.01$. The quantity of TAs tends to be sequentially increased with the progression of AGD, and a significant difference between AGD stages was found in the frontal cortex. Graphs produced using the data in our previous study [39]. Graphs used with permission from reference [40].

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