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ORIGINAL PAPER

Wide distribution of alpha-synuclein oligomers in multiple system atrophy brain detected by proximity ligation

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Abstract

Multiple system atrophy (MSA) is a fatal adult-onset neurodegenerative disease that is characterized by varying degrees of cerebellar dysfunction and Parkinsonism. The neuropathological hallmark of MSA is alpha-synuclein (AS)-positive glial cytoplasmic inclusions (GCIs). Although severe neuronal loss (NL) is also observed in MSA, neuronal inclusions (NIs) are rare compared to GCIs, such that the pathological mechanism of NL in MSA is unclear. GCIs and NIs are late-stage pathology features relative to AS oligomers and may not represent early pathological changes in MSA. To reveal the early pathology of MSA, it is necessary to examine the early aggregation of AS, i.e., AS oligomers. Here, we adopted a proximity ligation assay (PLA) to examine the distribution of AS oligomers in brain tissue samples from patients with MSA and other diseases. Surprisingly, MSA brains showed a widespread distribution and abundant accumulation of oligomeric AS in neurons as well as oligodendrocytes of the neocortex. In several regions, oligomeric AS signal intensity was higher in cases with MSA than in cases with Parkinson's disease. In contrast to previous studies, AS–PLA revealed abundant AS oligomer accumulation in Purkinje cells in MSA brains, identifying oligomeric AS accumulation as a possible cause of Purkinje cell loss. This wide distribution of AS oligomers in MSA brain neurons has not been described previously and indicates a pathological mechanism of NL in MSA.

Keywords Alpha-synuclein · Multiple system atrophy · Oligomer · Pathogenesis

Introduction

Multiple system atrophy (MSA) is a fatal adult-onset neurodegenerative disease that is characterized by varying degrees of cerebellar dysfunction, Parkinsonism, and autonomic failure [13]. The pathological hallmark of MSA is glial cytoplasmic inclusions (GCIs) [29] that consist of phosphorylated, misfolded, and fibrillized alpha-synuclein (AS) [15, 38]. AS is a 140-amino acid protein that is mainly expressed in neurons and is localized to presynaptic terminals, where it functions to support synaptic vesicle transport [5, 25]. The common presence of fibrillized AS in Lewy bodies and Lewy neurites in Parkinson's disease (PD) and

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dementia with Lewy bodies has led to the classification of these diseases as α-synucleinopathies.

In addition to GCIs, MSA is also characterized by neuronal cytoplasmic inclusions (NCIs) and neuronal nuclear inclusions (NNIs) [7, 41]; however, those neuronal inclusions (NIs) are generally rare compared with GCIs. Accordingly, the pathological diagnosis of MSA emphasizes the presence of widespread GCIs [16]. Moreover, MSA is considered a primary oligodendrogliopathy [39] wherein neuronal loss (NL) is the result of glial cell dysfunction. Yet, previous studies have reported severe NL in regions with mild GCIs [17, 28] and in regions with neurons containing only mild AS aggregates [3]. Therefore, fibrillized AS does not fully explain the observed pathology in MSA.

In other neurodegenerative disorders, the accumulation of protein oligomers plays a common and important pathogenic role; the importance of this mechanism has led to the broad classification of these diseases as oligomeropathies [14]. For example, in Alzheimer's disease (AD), Aβ oligomers are more highly neurotoxic than fibrils [21], and in some cases of familial AD, mutations in the amyloid precursor protein gene cause dementia as a result of enhanced Aβ oligomer formation [26, 36]. Tau oligomeric species are also neurotoxic and are thought to play a pathogenic role in tauopathies [22].

In synucleinopathies, oligomeric species of AS are also thought to be responsible for disease pathogenesis [1, 9, 32]. Yet, previous studies using immunohistochemistry have been unable to examine AS oligomers sufficiently in brain sections of patients with synucleinopathies. More recently, Roberts et al. described the detection of AS oligomers as early lesions in PD brains using a proximity ligation assay (PLA) approach [33], which had originally been developed to detect interactions between endogenous proteins [34]. In PLA, oligonucleotides are attached to antibodies against two target proteins and serve as templates for the circularization of connector oligonucleotides by a ligase when the target proteins are in close proximity (< 40 nm). The circularized DNA strands remain hybridized to the proximity probes; then, after the addition of DNA polymerase, the oligonucleotide arm of one of the PLA probes acts as a primer for a rolling-circle amplification (RCA) reaction using the ligated circle as a template, generating a concatemeric product that extends from the oligonucleotide arm of the PLA probe. The oligonucleotide of the second probe has three mismatched, exonuclease-resistant 2′ *O*-methyl RNA nucleotides at the 3′ end that prevent its use as a primer for RCA. RCA produces a randomly coiled, single-stranded product of up to 1000 complements of the DNA circle. Finally, oligonucleotides labeled with horseradish peroxidase hybridize to the RCA product and a visible signal is detected as a distinct brown dot. Roberts et al. amended the original method by attaching a plus or minus oligonucleotide to the same epitope-blocking anti-AS antibody to detect AS oligomers in PD [33].

The aim of this study was to examine the distribution of AS oligomers in MSA brains. Recently, AS pathology was reported outside of canonical regions of MSA [7]. Moreover, the cognitive impairment in MSA is now considered more common than previously thought [4, 20]. Therefore, in this study, we expanded the regions of analysis to neocortex and hippocampus, as well as striatum and brain stem. Here, we visualized AS oligomers using the modified PLA approach and examined the distribution of AS oligomers in brains from patients with MSA.

Materials and methods

Brain samples and neuropathology

Post-mortem brain samples from neuropathologically confirmed cases of MSA, or PD and disease control patient brain samples were provided by National Hospital Organization Hyogo-Chuo Hospital (Hyogo, Japan) and Kobe University Hospital (Hyogo, Japan). Clinical information was retrospectively collected from the medical chart. This study included five MSA cases (age at death 73.8 ± 8.3 years, disease duration 9.0 ± 6.2 years), five PD cases (age at death 76.4 ± 6.3 years, disease duration: 11.6 ± 4.0 years) and five disease control cases (age at death 63.6 ± 9.6 years, disease duration 26.0 ± 15.5 years). As additional controls, post-mortem brain samples of four subjects without known neurological diseases (age at death 63.3 ± 10.2 years) were used. Written informed consent was obtained from the patients' next of kin. This study was approved by the ethical committee of Kobe University.

Brain samples were examined in accordance with protocols in Research Resource Network Japan [37]. Briefly, the time of autopsy, portions of brain samples were stored at − 80 °C for further biochemical analyses. After fixation in 10% neutral buffered formalin, cerebral samples were dissected along coronal planes, brainstem samples were dissected along axial planes, and cerebellum samples were dissected along sagittal planes. Representative anatomical regions were embedded in paraffin. Serial 6-μm-thick sections were investigated. Sections were routinely stained with hematoxylin and eosin (H&E) and the Klüver–Barrera methods. The following regions were included in the analysis: putamen, substantia nigra pars compacta (SNpc), pontine nuclei, inferior olivary nucleus, cerebellum, hippocampus, and neocortex (frontal, temporal, parietal, occipital, and cingulate). Two sections of each region were analyzed: one with AS-PLA and one with p-AS IHC, with the exception of one PD patient who lacked a cerebellum section.

Clinical and neuropathological assessment

Patients included in this study were evaluated by board-certified neurologists at the National Hospital Organization Hyogo-Chuo Hospital (Hyogo, Japan) and Kobe University Hospital (Hyogo, Japan) between 2006 and 2017. The disease control cases included three patients with amyotrophic lateral sclerosis (ALS), one patient with limb-girdle muscular dystrophy (LGMD), and one patient with myotonic dystrophy (MyD). None of the control cases exhibited Lewy bodies or GCIs on p-AS IHC. The normal control cases included one patient with ileus, one patient with peritonitis, and two patients with acute myocardial infarction. None exhibited known neurological diseases. The demographic and clinical characteristics of the cases are shown in Table 1.

NL was assessed in H&E-stained sections semi-quantitatively and graded as absent (0), mild (1+), moderate (2+), or severe (3+) by a neuropathologist. GCIs and NIs were analyzed using phosphorylated AS (p-AS) IHC slides. The diagnosis of MSA or PD was based on the presence of GCIs or Lewy bodies on p-AS IHC, respectively. The neuropathological grading of MSA patients [17] and the Braak staging of PD patients [2] are summarized in Table 1.

Table 1 Demographic and clinical characteristics of cases

ID	Clinical diagnosis	Age	Sex	Duration (months)	Pathological grade
M1	MSA-C	74	M	89	OPCA III + SND II
M2	MSA-P	75	F	76	OPCA I + SND III
M3	MSA-C	60	F	65	OPCA II + SND II
M4	MSA-P	78	M	67	OPCA II + SND III
M5	MSA-C	82	F	240	OPCA III + SND III
P1	PD	76	M	204	Braak stage 3
P2	PD	85	F	93	Braak stage 6
P3	PD	68	F	142	Braak stage 5
P4	PD	79	M	119	Braak stage 3
P5	PD	74	M	238	Braak stage 6
C1	ALS	63	M	147	–
C2	MyD	60	F	360	–
C3	ALS	55	F	444	–
C4	ALS	80	F	90	–
C5	LGMD	60	M	516	–
C6	Ileus	68	M	–	–
C7	Peritonitis	48	M	–	–
C8	MI	69	M	–	–
C9	MI	68	M	–	–

M male, *F* female, *MSA* multiple system atrophy, *PD* Parkinson's disease, *ALS* amyotrophic lateral sclerosis, *MyD* myotonic dystrophy, *LGMD* limb girdle muscular dystrophy, *OPCA* olivopontocerebellar atrophy, *SND* striatonigral degeneration, *MI* myocardial infarction

Immunohistochemistry (IHC)

Paraffin-embedded brain sections were dewaxed in xylene, then rehydrated in a graded series of alcohol. Antigen retrieval was performed in pH 6 citrate buffer by microwave heating for 15 min. After blocking in 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) at room temperature for 30 min, primary antibody for phosphorylated AS (1:2000; mouse monoclonal, psyn#64, Wako, Osaka, Japan) [15] was added and the samples were incubated at 4 °C overnight. Sections were washed in Tris-buffered saline (TBS) and were then incubated in hydrogen peroxide at room temperature for 30 min to inactivate endogenous peroxidases. Next, sections were washed and incubated with biotin-conjugated secondary antibody (goat anti-mouse IgG) followed by incubation with avidin-biotin complex (VECTASTAIN *Elite* ABC Kit, Vector Laboratories, Burlingame, CA, USA). Phosphorylated AS staining was visualized with 3,3′-diaminobenzidine-tetrahydrochloride-dihydrate and counter-stained with hematoxylin. Samples were dehydrated in a graded series of alcohol and xylene before mounting with Permount mounting medium (Falma, Tokyo, Japan).

AS-PLA procedures

We used PLA Duolink kits [33] supplied by Sigma-Aldrich (St. Louis, MO, USA) for the detection of AS oligomers. Experiments were conducted in accordance with manufacturer's instructions. An AS antibody (mouse monoclonal, syn211, Ab80627, Abcam, Cambridge, UK) was used to make both probes [11]. Briefly, 20 µg of antibody was added to 2 µL of conjugation buffer and the antibody solution was transferred to a vial containing lyophilized oligonucleotides (plus or minus). Then, the solution was incubated at room temperature overnight. The conjugates were incubated with 2 µL of stop solution for 30 min at room temperature and suspended in 24 µL of storage solution. Paraffin-embedded tissues were dewaxed in xylene, rehydrated in a graded series of alcohol, and incubated with hydrogen peroxide for 1 h at room temperature to inactivate endogenous peroxidases. Antigen retrieval was performed by microwave heating for 15 min in pH 6 citrate buffer. All samples were blocked in 3% BSA in PBS at 37 °C for 1 h, followed by incubation with PLA-conjugated antibodies diluted in PLA probe diluent (1:100) at 37 °C for 1 h, and then at 4 °C overnight. Sections were washed in TBS + 0.05% Tween 20 and incubated with ligation solution and ligase at 37 °C for 1 h. After washing, samples were incubated with amplification reagents and polymerase at 37 °C for 2.5 h, washed, and finally incubated with detection solution at room temperature for 1 h, followed by incubation with substrate solution at room temperature for 20 min. Sections were counter-stained

with hematoxylin and dehydrated in a graded series of alcohol and xylene before mounting with bright-field mounting medium.

For proteinase K treatment, sections were incubated with 50 µg/ml proteinase K in 10 mM Tris–HCl pH 7.8, 100 mM NaCl, and 0.1% Nonidet P-40 at 37 °C for 1 min; they were then washed in tap water for 5 min, followed by AS-PLA procedures or p-AS IHC procedures.

For double-labeling immunohistochemistry with the combination of AS-PLA and anti-NeuN (1:500; rabbit-polyclonal, ab104225, Abcam), anti-Olig2 (1:500; rabbit-monoclonal, ab109186, Abcam), or anti-phosphorylated AS (1:500; rabbit-monoclonal, ab51253, Abcam), sections were incubated with PLA-conjugated antibodies diluted in PLA probe diluent (1:100) and primary antibody for NeuN, Olig2 or p-AS at 37 °C for 1 h, and then at 4 °C overnight. After the PLA procedures of ligation, amplification, and incubation with detection solution and substrate solution, sections were washed and incubated with biotin-conjugated secondary antibody (goat anti-rabbit IgG), followed by incubation with VECTASTAIN ABC-AP kit (Vector Laboratories). The IHC staining was visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium. Samples were dehydrated in a graded series of alcohol and xylene before mounting with bright-field mounting medium.

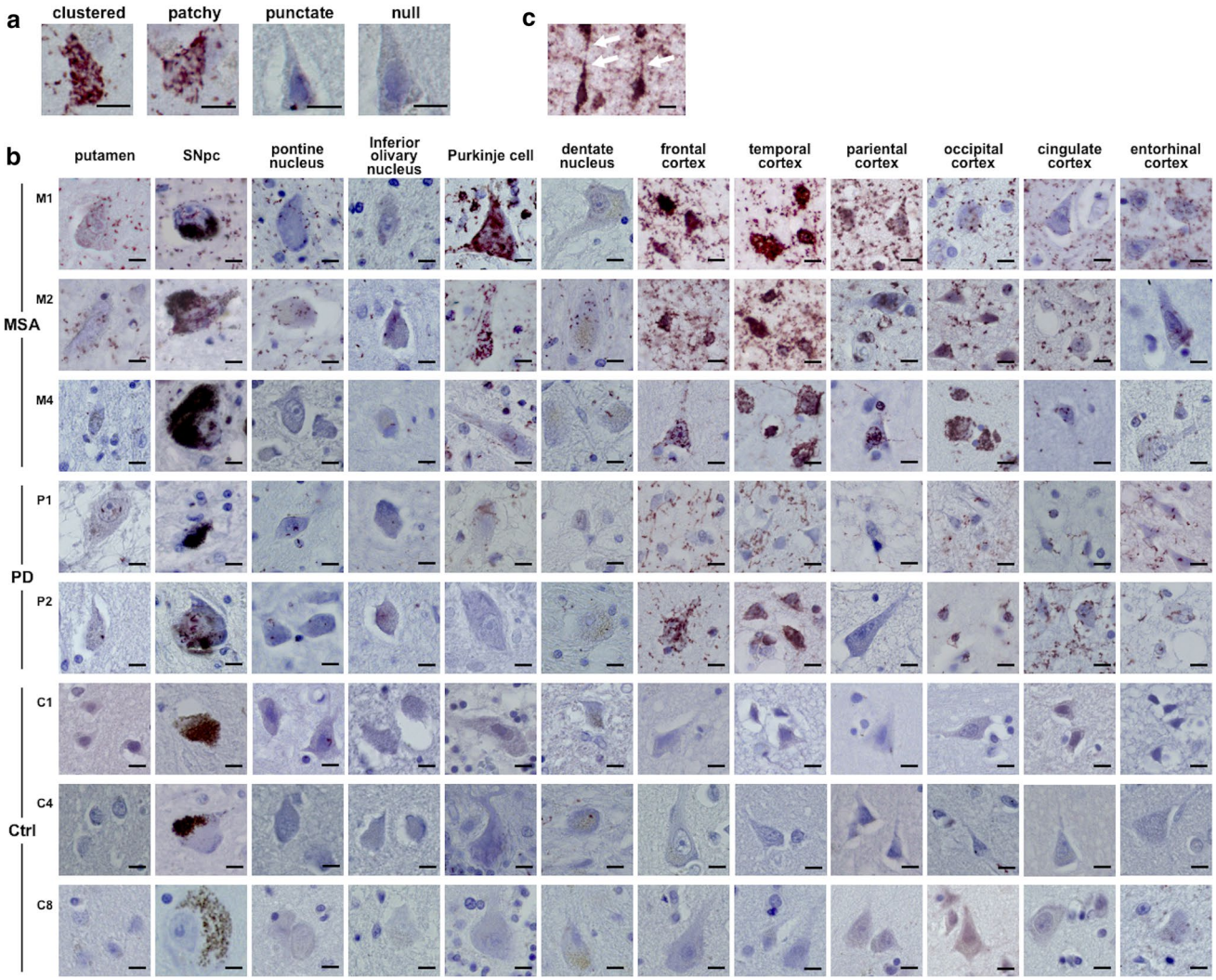

Fig. 1 α-Synuclein proximity ligation assay (AS–PLA) staining. **a** The 4 main patterns of neuronal AS–PLA staining: neuronal-clustered staining, neuronal-patchy staining, neuronal-punctate staining, and no staining, respectively. Scale bar 10 µm. **b** Representative images of AS–PLA staining in each region. Multiple system atrophy (MSA) neurons showed clustered staining and AS oligomer accumulation. Scale bar 10 µm. **c** Some MSA neurons showed linear AS–PLA staining along apical dendrites (white arrow). Scale bar 10 µm. *Ctrl* control group, *MSA* multiple system atrophy group, *PD* Parkinson's disease group, *SNpc* substantia nigra pars compacta

Table 2 AS–PLA staining pattern of neurons

	Putamen	SNpc	Pontine N	ION	Purkinje	Dentate N	Frontal	Temporal	Parietal	Occipital	Cingulate	Entorhinal	CA1	CA2	CA3	CA4
M1	punctate	punctate	punctate	punctate	clustered	punctate	clustered	clustered	patchy	punctate	punctate	punctate	punctate	punctate	punctate	punctate
M2	punctate	patchy	punctate	punctate	patchy	patchy	clustered	clustered	patchy	punctate	punctate	punctate	punctate	punctate	punctate	punctate
M3	punctate	punctate	punctate	null	punctate	punctate	patchy	patchy	punctate	punctate	punctate	punctate	null	null	null	null
M4	punctate	punctate	null	punctate	punctate	punctate	patchy	clustered	patchy	clustered	punctate	punctate	punctate	punctate	punctate	punctate
M5	punctate	patchy	patchy	punctate	patchy	patchy	clustered	clustered	patchy	clustered	punctate	punctate	patchy	patchy	punctate	punctate
P1	punctate	punctate	punctate	punctate	punctate	null	punctate	punctate	punctate	punctate	punctate	punctate	punctate	punctate	punctate	punctate
P2	punctate	patchy	punctate	punctate	punctate	punctate	patchy	clustered	null	patchy	punctate	punctate	punctate	punctate	punctate	punctate
P3	null	punctate	punctate	punctate	punctate	punctate	punctate	punctate	punctate	punctate	punctate	null	null	null	null	null
P4	null	null	punctate	null	null	null	punctate	null	punctate	punctate	null	null	punctate	punctate	punctate	null
P5	patchy	null	punctate	punctate	-	-	clustered	patchy	punctate	punctate	punctate	punctate	punctate	punctate	punctate	punctate
C1	null	null	punctate	null	null	null	null	null	punctate	null	null	null	null	null	null	null
C2	null	null	null	null	null	null	null	null	null	null	null	null	null	null	null	null
C3	null	null	null	null	null	null	null	null	null	punctate	null	null	null	null	null	null
C4	null	punctate	null	null	null	punctate	null	null	null	punctate	null	null	null	null	null	null
C5	null	null	null	null	null	null	null	null	null	null	null	null	null	null	null	null
C6	null	null	null	null	null	null	null	null	null	null	null	punctate	punctate	null	null	null
C7	null	null	null	null	null	null	null	null	null	null	null	null	null	null	null	punctate
C8	null	null	null	null	null	null	null	null	null	null	null	punctate	null	null	null	null
C9	null	null	null	null	null	null	null	null	null	null	null	punctate	null	null	null	null

AS–PLA alpha-synuclein proximity ligation assay, *SNpc* substantia nigra pars compacta, *Pontine N* pontine nucleus, *ION* inferior olivary nucleus, *Dentate N* dentate nucleus of cerebellum, *Purkinje* Purkinje cell, *CA* cornu ammonis

Evaluation of AS–PLA

AS oligomers were examined on AS-PLA slides. First, the AS–PLA staining pattern of neurons was evaluated in cortex and nuclei and classified into four patterns (neuronal-clustered, neuronal-patchy, neuronal-punctate, and null) based on its appearance. The neuronal-clustered pattern was defined as AS–PLA signals present throughout neurons. When patchy signal was found within the neurons, the staining pattern was classified as neuronal-patchy. The neuronal-punctate pattern comprised dot-like AS–PLA signals in neurons.

In addition to the neuronal staining, diffuse-neuropil staining was observed in MSA cases. Therefore, to quantify the severity of AS oligomer accumulation in neuropils, in accordance with the method by Roberts et al. [33], PLA slides of each region were analyzed by two independent experimenters at 20 × magnification. The severity of the AS oligomer burden was scored on a scale from zero (no signal) to five (highest signal) using pre-made scoring plates (Online Resource, Fig. S1). Because the areas were relatively large, we eliminated bias by conducting AS–PLA scoring in the three most severely affected microscopic fields; average scores were recorded in the cortex and white matter (WM) of superior frontal gyrus, superior temporal gyrus, superior parietal lobule, lingual gyrus, cingulate gyrus, and parahippocampal gyrus. In other regions such as putamen, SNpc, pontine nucleus, inferior olivary nucleus, and dentate nucleus of cerebellum, the score of the most severely affected area was adopted because the areas were relatively small. In addition to the scoring as described above, the software ImageJ (National Institutes of Health, Bethesda, MD, USA) was used. We conducted the software analysis in the cortex and WM because their images were sufficiently large to fill all areas of 1360 × 1024 pixels at 20 × magnification. Three representative images of the cortex and WM of superior frontal gyrus, superior temporal gyrus, superior parietal lobule, lingual gyrus, cingulate gyrus, and parahippocampal gyrus were obtained with a Keyence BZ-9000 microscope (Keyence, Osaka, Japan) at 20 × magnification. Immunostaining quantification was performed using the ImageJ IHC Image Analysis Toolbox plugin. After the training procedure with Nova RED color, the images were analyzed with color detection and converted to 16-bit format. Then, the area of thresholded pixels was measured.

Western blotting

Brain tissues from frontal and parietal cortices of two patients with MSA, two patients with PD, and two control subjects were used; frontal and temporal cortices were used for one of the PD cases. They were stored at − 80 °C at the time of autopsy. Frozen brain samples were prepared for western blotting as previously described, with some modifications [8]. Briefly, brain tissue samples (250 mg) were

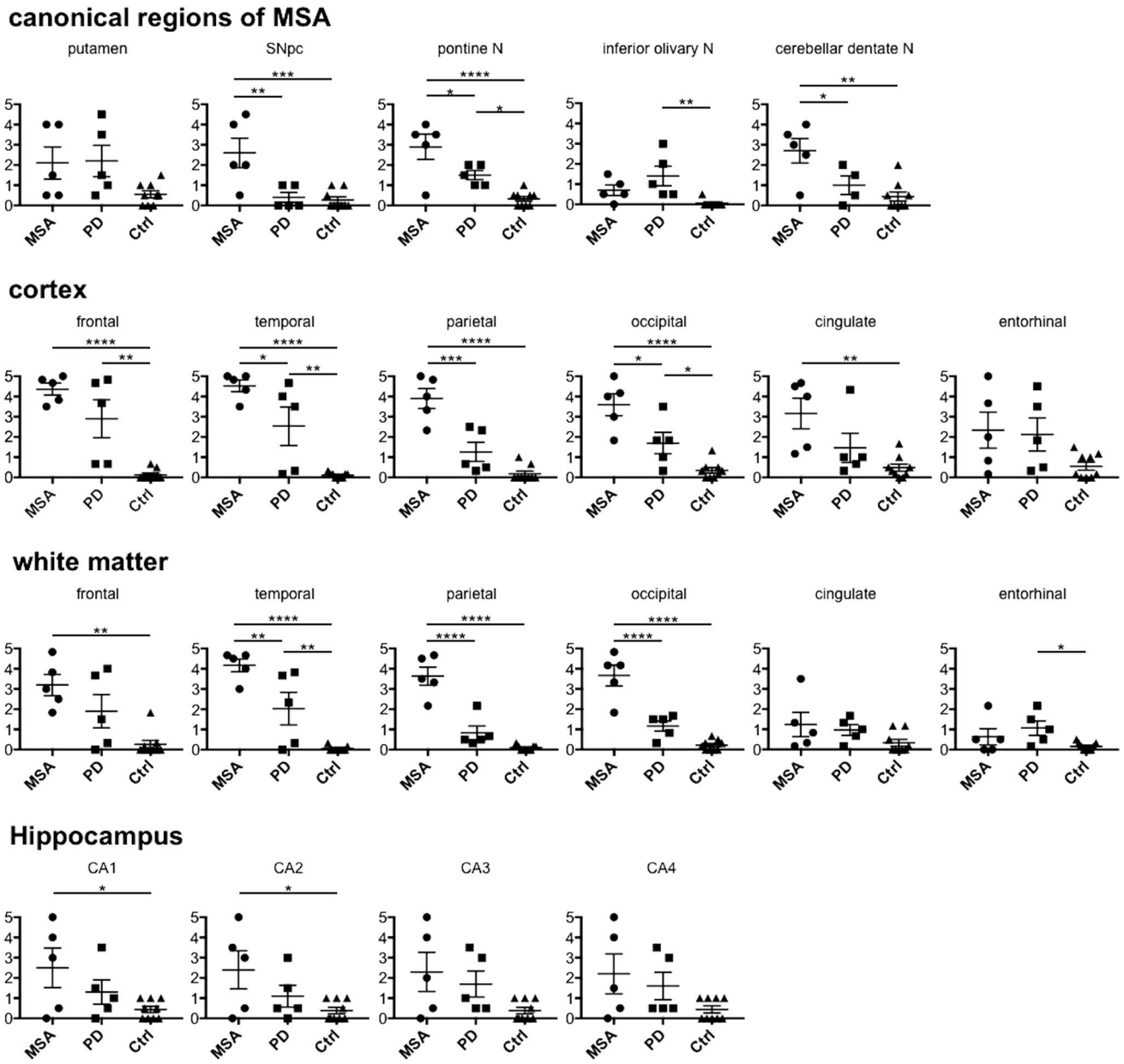

Fig. 2 Regional α-synuclein (AS) oligomer accumulation detected by the proximity ligation assay (PLA). Signal intensity in neuropils was evaluated on a scale from 0 to 5 by 2 independent experimenters, and then averaged. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$. *Ctrl* control group, *MSA* multiple system atrophy group, *N* nucleus, *PD* Parkinson's disease group, *SNpc* substantia nigra pars compacta, *CA* cornu ammonis

homogenized in 5 mL of homogenization buffer (10 mM Tris–HCl, pH 7.5, containing 0.8 M NaCl, 1 mM EDTA, and 1 mM dithiothreitol). Sarkosyl was added to the lysates (final concentration, 2%) and incubated at 37 °C for 30 min. Samples were centrifuged at 20,000×*g* at 25 °C for 10 min and the resultant supernatants were centrifuged at 100,000×*g* at 25 °C for 20 min. Final supernatants were used as the sarkosyl-soluble fraction. The pellets were washed with 500 µL of sterile saline, centrifuged at 100,000×*g* for 20 min and suspended in 100 µL of 30 mM Tris–HCl before use as the sarkosyl-insoluble fraction. Samples were electrophoresed on 4–20% gradient polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Burlington, MA, USA). After incubating with 0.4% paraformaldehyde, residual protein-binding sites were blocked with 3% (w/v) powdered skimmed milk in TBS containing 0.1% Tween 20 (TBS-T) at room temperature for 1 h. Membranes were then incubated with primary antibody (mouse monoclonal, syn211, Ab80627, Abcam) diluted in blocking solution at 4 °C overnight. Membranes

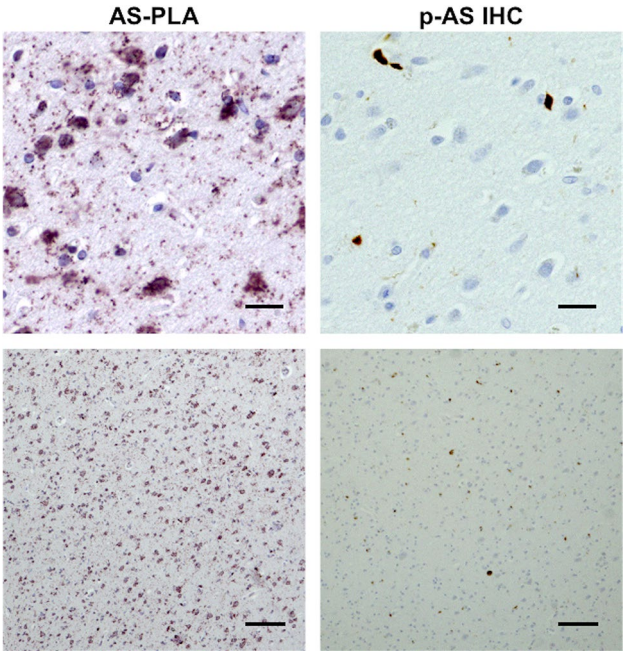

Fig. 3 Comparison of α-synuclein (AS) proximity ligation assay (AS–PLA) and phosphorylated (p)-AS immunohistochemistry (IHC) in multiple system atrophy brain samples. AS–PLA revealed abundant AS oligomer accumulation in neurons, whereas conventional p-AS IHC indicated the absence of neuronal inclusions and few glial cytoplasmic inclusions in the same region. Upper panels are higher magnification images of the lower panels. Scale bars 20 µm (upper panels), 100 µm (lower panels)

were subsequently washed three times in TBS-T and incubated with secondary antibody at room temperature for 1 h. Finally, membranes were washed three times in TBS-T and signal was visualized with ECL Prime (GE Healthcare, Little Chalfont, UK) using an ImageQuant LAS 4000mini instrument (Fujifilm, Tokyo, Japan).

Statistical analysis

Data analysis was performed using Prism (version 7.0d for Macintosh, GraphPad Software, La Jolla, CA, USA). PLA scores and IHC areas of cortex and white matter were analyzed using 1-way analysis of variance with Tukey's multiple comparison tests. The correlation of NL and AS–PLA score in canonical regions of MSA (putamen, substantia nigra, pontine nucleus, and inferior olivary nucleus) was analyzed using Spearman's correlation analysis. Statistical significance was defined by a p value < 0.05.

Results

Staining pattern of α-synuclein oligomers in neurons

We first analyzed the staining pattern in neurons of AS oligomers detected by AS–PLA. The staining pattern of AS oligomers in neurons was classified as neuronal-clustered staining, neuronal-patchy staining, neuronal-punctate staining, or no staining (Fig. 1a). The summary of AS–PLA staining patterns in neurons is indicated in Table 2 and the representative images of neurons are shown in Fig. 1b. In the MSA group, four of five cases showed neuronal-clustered staining in nine cortical regions (frontal and temporal cortex in M1, frontal and temporal cortex in M2, temporal and occipital cortex in M4, and frontal, temporal and occipital cortex in M5). Neuronal-clustered staining was not observed in PD or control cases, except in two regions of two PD cases with advanced disease, as in Braak stage six (temporal cortex in P2 and frontal cortex in P5).

In cerebellar Purkinje cells, AS–PLA revealed neuronal-clustered staining in one MSA case, neuronal-patchy staining in two MSA cases and neuronal-punctate staining in two MSA and three PD cases; no staining was observed in the remaining cases (Fig. 1b and Table 2). In contrast, no inclusions were observed in cerebellar Purkinje cells with p-AS IHC.

In the MSA cases, some neurons exhibited linear staining along apical dendrites (Fig. 1c), which was absent from the PD and control cases.

In canonical regions of MSA such as putamen, SNpc, pontine nucleus and inferior olivary nucleus, the AS–PLA signal was relatively weak. AS–PLA only showed neuronal-punctate staining in remaining neurons.

Distribution of neuropil α-synuclein oligomers

We next assessed the severity of neuropil AS–PLA staining of each region using pre-made scoring plates (Online Resource, Fig. S1). The results of AS–PLA scoring in each region are presented in Fig. 2. Compared with control patients, AS-PLA scores were significantly higher in SNpc, pontine nucleus, dentate nucleus of cerebellum, both cortex and WM of frontal, temporal, parietal, and occipital lobes; cingulate cortex; and CA1 and CA2 of the hippocampus in MSA patients. Additionally, scores were higher in SNpc, pontine nucleus, dentate nucleus of cerebellum, and both of cortex and WM in temporal, parietal and occipital lobes in patients with MSA than in patients with PD. We also conducted the quantification of AS–PLA staining areas in cerebral cortex and WM (Online Resource, Fig. S2). Compared with control subjects, AS–PLA-stained areas were

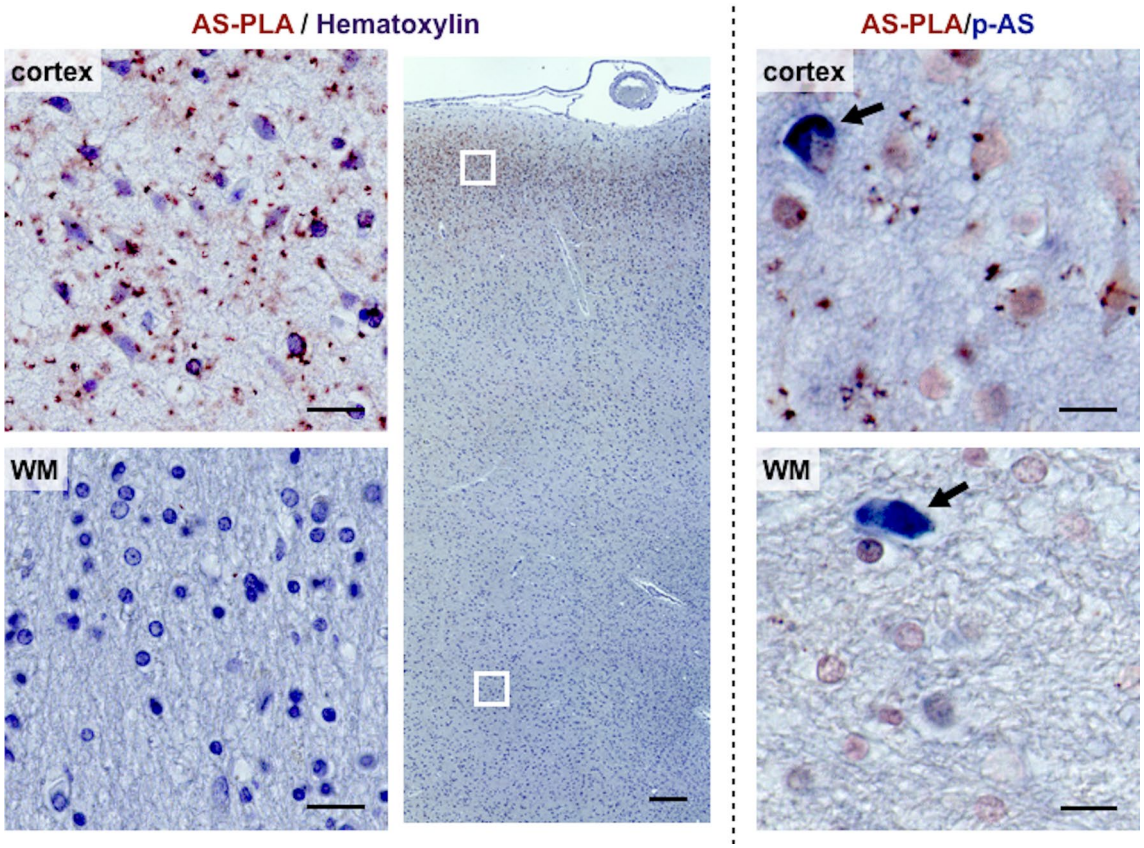

Fig. 4 Comparison of α-synuclein proximity ligation assay (AS–PLA) staining in the cortex and white matter (WM) of the cingulate gyrus in multiple system atrophy brain. Abundant AS oligomers were detected in the cortex, but not in the WM. The middle panel is lower magnification of cingulate gyrus ranging from the cortex to the WM. The left panels are the enlarged views of white squares in the middle panel. The right panels are images of double staining of AS–PLA and p-AS IHC. Few GCIs were detected in these areas. Scale bars 20 µm (left and right panels), 200 µm (middle panel)

significantly greater in frontal and cingulate cortex, and in both cortex and WM in temporal, parietal and occipital lobes in MSA patients. Moreover, compared with PD patients, AS–PLA stained areas were significantly greater in temporal and parietal cortex and parietal and occipital WM in MSA patients.

In the MSA group, abundant AS oligomers were observed in the cerebral cortex. Comparison of conventional p-AS IHC and AS–PLA using serial sections showed that p-AS IHC did not reveal any neuronal inclusions in the majority of AS–PLA positive neurons, although several GCIs were found in cortical areas (Fig. 3). These cortical regions also exhibited mild NL. In one MSA patient, although AS–PLA revealed abundant AS oligomers in neurons in the cingulate cortex, AS oligomers were rarely found in white matter (Fig. 4). No obvious neuronal or oligodendroglial loss was observed in this area, as indicated in the low-magnification image. Double staining of AS–PLA and p-AS IHC in this region revealed few GCIs.

We observed AS–PLA punctate staining in neurons of the hippocampus in four MSA cases. The signal was stronger in CA1 and CA2 than in CA3 or CA4 (Online Resource, Fig. S3).

In canonical regions of MSA, neuronal staining was relatively unremarkable and the severity of AS-PLA in neuropils was relatively low. We observed a significant inverse correlation of NL grade and AS–PLA score in these areas (Online Resource, Table S1 and Fig. S4). We also observed AS–PLA signals in the periphery of GCIs (Online Resource, Fig. S5).

Characterization of α-synuclein oligomers

To further characterize AS oligomers detected by AS–PLA, we conducted several experiments as follows. First, we performed proteinase K pretreatment on sections to reveal the firmness of aggregation of AS species in the form of AS oligomers or GCIs. We performed proteinase K treatment of sections of superior frontal gyrus from an MSA patient; nearly all AS–PLA signals in both neurons and neuropils disappeared after such treatment (Fig. 5). In contrast, GCIs detected by p-AS IHC were not reduced with proteinase K treatment. This finding indicates that AS oligomers detected

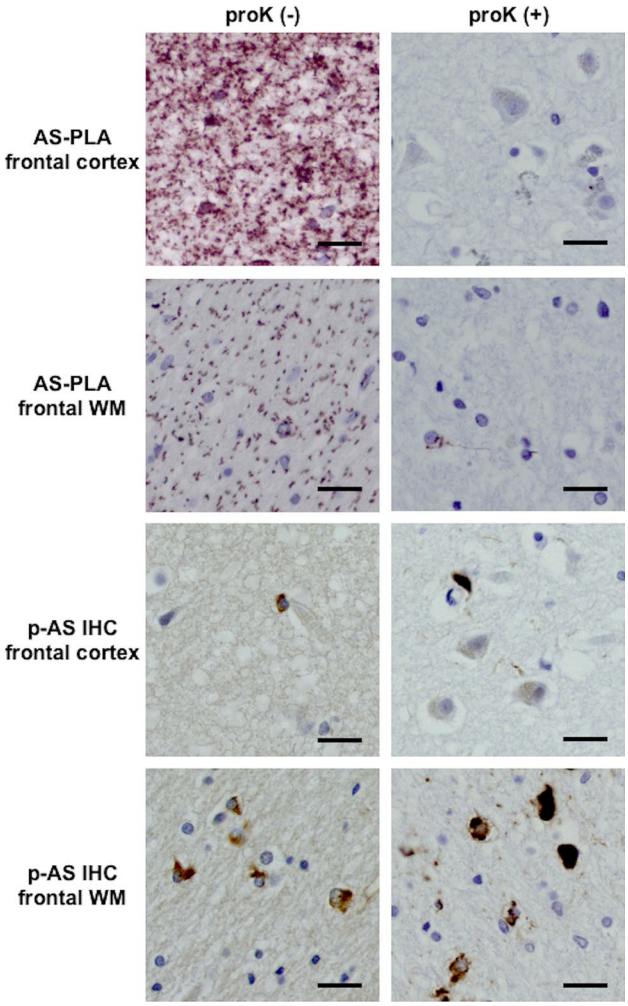

Fig. 5 Proteinase K pretreatment with AS–PLA or p-AS IHC. The left panels are images of AS–PLA or p-AS IHC without proteinase K and the right panels are images of AS–PLA or p-AS IHC with proteinase K. Most AS–PLA signals disappeared with proteinase K pretreatment, whereas p-AS IHC staining did not change. Scale bars 20 µm. *proK* proteinase K

by AS–PLA in this study had low resistance to proteinase K and suggests that they were early aggregations of AS. In contrast, GCIs had high resistance to proteinase K and were considered as late-stage aggregations of AS.

Next, to determine whether AS–PLA is associated with neurons or glia, we conducted double staining of AS-PLA and IHC of NeuN or Olig2 using serial sections of frontal gyrus from an MSA patient to observe the co-localization of AS oligomers with neurons or oligodendrocytes. In the cortex, most AS-PLA signals were co-localized with neurons (Online Resource, Fig. S6).

Moreover, we conducted western blot analysis to biochemically examine AS oligomers. Accumulation of high-molecular weight AS oligomers was detected in the sarkosyl-insoluble fraction of MSA samples (Fig. 6). The bands of AS oligomers were also observed in PD samples, whereas bands of higher molecular weight species were not detected in control cases. These results support the presence of AS oligomers in MSA brain samples.

The results of proteinase K pretreatment, double staining, and western blotting suggest that AS oligomers detected by AS–PLA in this study were early aggregations of AS, mostly colocalized with neurons, and were abundant in MSA brains.

Discussion

The distribution of AS oligomers in MSA brains, based on AS–PLA, has not been described previously. Our data indicated a wide distribution of AS oligomers in neurons as well as oligodendrocytes in patients with MSA. The observed pattern and distribution of AS oligomers were largely unique to MSA, as compared to PD and other neurological diseases.

The PLA method was originally developed to detect endogenous protein interactions, and showed good sensitivity and specificity [34]. This technique has recently been used to detect Aβ protofibrils in Alzheimer's disease [18] and AS oligomers in PD [33]. Roberts et al. used this approach to demonstrate the presence of AS oligomers in the cingulate cortex and the reticular formation of the medulla in PD, which was previously unrecognized. They conducted AS–PLA in the substantia nigra of an MSA patient and showed punctate staining at the periphery of GCIs. They found no obvious AS–PLA staining in normal brain. In our study, we did not identify significant AS–PLA signals in the control group, which represented ALS, LGMD, MyD, and cases without neurological diseases. Though the average age in the control group was approximately 10 years younger than that in the MSA and PD groups, an 80-year-old ALS patient (C4) also did not show significant accumulation of AS–PLA signals. Therefore, AS–PLA was unlikely to detect physiological AS. The results of western blotting, which indicated abundant presence of high-molecular-weight AS species in MSA brains, may support the AS–PLA staining findings in this study, as well as the findings of a previous report [33]. Dickson et al. also performed a western blotting analysis of brain samples from patients with MSA and found an abundance of high-molecular weight AS species in regions with few or no GCIs, such as the frontal cortex, temporal cortex, and hippocampus; moreover, there was no association between the number of GCIs detected and AS profiles [10]. In our study, we detected an abundance of AS oligomers in the frontal cortex, temporal cortex, and hippocampus using PLA. Therefore, we suggest that the wide distribution of AS oligomers bridges the gap between conventional IHC and western blotting analysis.

Emerging evidence suggests that AS oligomers are toxic and play a crucial role in the pathogenesis of

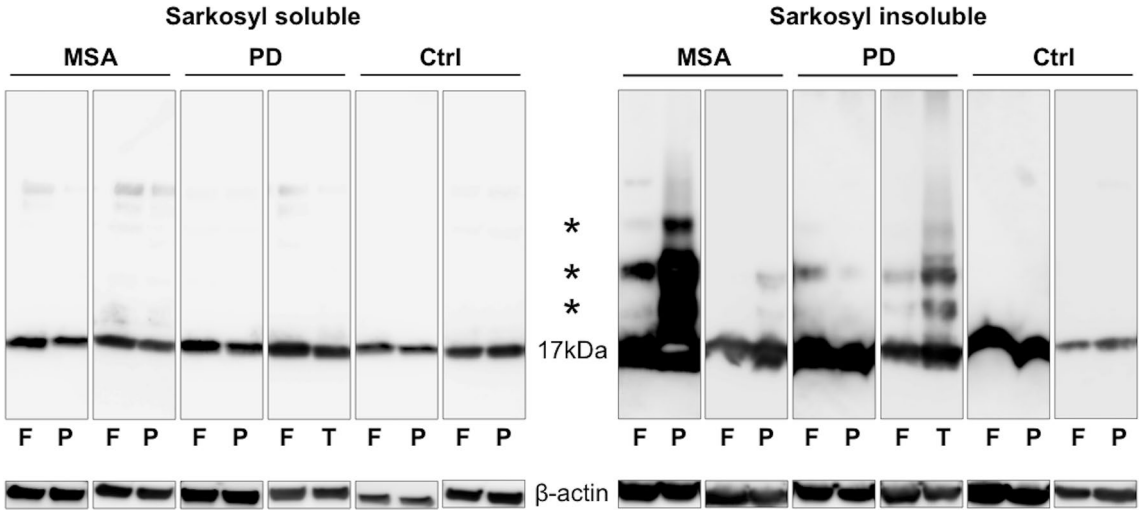

Fig. 6 α-Synuclein (AS) oligomers detected by western blotting. Brain sample homogenates were separated into sarkosyl-soluble and sarkosyl-insoluble fractions and AS oligomers (asterisks) were visualized in fractions from multiple system atrophy and Parkinson's disease patients' brain samples. *Ctrl* control group, *F* frontal cortex, *MSA* multiple system atrophy group, *P* parietal cortex, *PD* Parkinson's disease group, *T* temporal cortex

synucleinopathies. For example, mutations that decrease AS fibril formation and increase AS oligomer generation produce neuronal toxicity in the context of PD [19, 40]. Outeiro et al. showed that the stabilization of AS oligomers by bimolecular fluorescence complementation increased their toxicity, whereas eliminating AS oligomers reduced toxicity [27]. Various mechanisms have been proposed to underlie AS oligomer-induced toxicity, such as proteasomal impairment [12, 23], endoplasmic reticulum stress [6], mitochondrial dysfunction [31], and impairment of synaptic vesicle function [30]. Given evidence of its toxicity, our finding of AS oligomer accumulation in cortical neurons and cerebellar Purkinje cells in MSA brains has important implications for MSA pathogenesis. A previous study observed neuronal dysfunction throughout brain regions exhibiting minimal changes in MSA cases, despite the restriction of NL to the substantia nigra and locus coeruleus [24]. Although previous research has suggested that this neuronal dysfunction is caused by glial dysfunction, neuronal accumulation of AS oligomers, as detected by AS-PLA, suggests that oligomeric species may contribute to neuronal dysfunction in MSA in a more direct manner.

There was a significant inverse correlation of NL and AS–PLA signals in canonical regions of MSA such as putamen, SNpc, pontine nucleus, and inferior olivary nucleus. Accordingly, AS oligomer accumulation decreased with the progression of NL in a given area. This result can be interpreted as follows: AS–PLA detects the early aggregation of AS (e.g., AS oligomers) rather than fibrillized AS aggregates, which have a rich β-sheet structure (e.g., GCIs). Indeed, most AS-PLA signals disappeared with proteinase K pretreatment, whereas p-AS IHC staining was resistant to proteinase K. Moreover, there was no colocalization of AS–PLA signals and GCIs upon double staining of AS–PLA and p-AS IHC (Fig. 4). These results support the suggestion that AS oligomers detected by AS–PLA are early aggregations of AS. Our study used autopsied brain samples in patients with long disease duration (65–240 months). Therefore, AS–PLA signals may have decreased in regions affected earlier in the disease course. The highest numbers of AS oligomers were detected in neurons in the neocortex of patients with MSA. This finding is consistent with recent studies that suggested that cognitive impairment is more common in MSA than previously thought [4, 20]. Executive dysfunction is the most common cognitive symptom in MSA, and memory disturbance and visuospatial dysfunction have also been reported [35]. Although the underlying pathogenesis of cognitive impairment in MSA remains unclear, AS oligomers in the neocortex may play an important role in neuronal and subsequently cognitive dysfunction.

Another important finding of our study involved the temporal progression of lesions in MSA. The features of early-stage MSA are unknown. Although previous studies have focused on aggregation of fibrillized AS into NIs or GCIs, these inclusions appear to be pathological end-points rather than starting points. In the present study, we focused on AS oligomers to characterize the early pathogenesis of MSA. AS–PLA revealed abundant AS oligomers in the cortex of the cingulate gyrus, although no signal was evident in WM of the cingulate gyrus in one MSA case. This may be interpreted as suggesting that AS–PLA signals in this area disappeared before the time of death; however, because no

obvious neuronal or oligodendroglial loss was found in this area, pathological change in this area may have only been in the early phase. Moreover, the observation of double staining with AS–PLA and IHC of NeuN/Olig2 in the frontal gyrus indicated that most AS–PLA signals were co-localized with neurons. Based on these findings, we speculated that the earliest aggregations of AS oligomers may appear in neurons, rather than in oligodendrocytes.

In conclusion, we visualized AS oligomers in paraffin-embedded brain sections from patients with MSA using a PLA approach. The results indicated a wide distribution of AS oligomers in neurons of MSA brains, contrasting with a focus on oligodendrocyte involvement in previous studies. Considering the process of aggregation of AS and the toxicity of AS oligomers in neurons, our study suggests that AS oligomer-mediated toxicity is an early pathological event in MSA, advancing our understanding of MSA pathogenesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from the families of all individual participants included in the study.

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