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Pathological substrate of memory impairment in multiple system atrophy

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Short running title: Memory impairment in multiple system atrophy

Key points

- the MSA model developed memory impairment along with NCI-like structures in excitatory neurons of the hippocampus.
- α-Synuclein oligomers increased simultaneously in the hippocampus of the MSA model.
- Hippocampal dendritic spines of the MSA model also decreased in number, followed by suppression of long-term potentiation.
- Similar to the MSA model, human cases of MSA with memory impairment developed more NCIs in excitatory hippocampal neurons along with α-synuclein oligomers than those without.

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Abstract

Aims and methods: Synaptic dysfunction in Parkinson's disease is caused by propagation of pathogenic α-synuclein between neurons. Previously, in multiple system atrophy (MSA), pathologically characterised by ectopic deposition of abnormal α-synuclein predominantly in oligodendrocytes, we demonstrated that the occurrence of memory impairment was associated with the number of α-synuclein-positive neuronal cytoplasmic inclusions (NCIs) in the hippocampus. Here, using a mouse model of adult-onset MSA and human cases (MSA, N = 25; Parkinson's disease, N = 3, Alzheimer's disease, N = 2; normal controls, N = 11), we aimed to investigate how abnormal α-synuclein in the hippocampus can lead to memory impairment.

Results: In the MSA model, inducible human α-synuclein was first expressed in oligodendrocytes, and subsequently accumulated in the cytoplasm of excitatory hippocampal neurons (NCI-like structures) and their presynaptic nerve terminals with the development of memory impairment. α-Synuclein oligomers increased simultaneously in the hippocampus of the MSA model. Hippocampal dendritic spines also decreased in number, followed by suppression of long-term potentiation. Consistent with these findings obtained in the MSA model, post-mortem analysis of human MSA brain tissues showed that cases of MSA with memory impairment developed more NCIs in excitatory hippocampal neurons along with α-synuclein oligomers than those without.

Conclusions: our results provide new insights into the role of α-synuclein oligomers as a possible pathological cause of memory impairment in MSA.

Abbreviations

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor: AMPAR; CA: cornu ammonis region; CaMKII: Ca2+/calmodulin-dependent protein kinase II; Cre: Cre recombinase; DH: dentate hilus; DG: dentate gyrus; ER: oestrogen receptor; fEPSP: field excitatory postsynaptic potential; GCI: glial cytoplasmic inclusion; LTP: long-term potentiation; MI: memory impairment; MSA: multiple system atrophy; MSA-C: MSA cerebellar ataxia; MSA-P: MSA-parkinsonism; NC: normal cognition; NCI: neuronal cytoplasmic inclusion; NMDAR: N-methyl-D-aspartate receptor; OPC: olivopontocerebellar; OPCA: olivopontocerebellar atrophy; PLA: proximity ligation assay; Plp: proteolipid protein; SN: striatonigral; SND: striatonigral degeneration; TBS: tris-buffered saline.

Introduction

α-Synuclein is a 140-amino-acid protein present predominantly in presynaptic nerve terminals. It has been implicated in the regulation of presynaptic vesicle pooling, neurotransmitter release, endocytosis, mitochondrial activity and membrane lipid content [1-3]. Although α-synuclein is a highly soluble and abundant protein in the brain, under pathological conditions, it has a tendency to form oligomers and higher-order β-sheet rich aggregates, including protofibrils or fibrils [4,5]. α-Synuclein exhibits conformations, which vary in their pathogenic properties and ability to seed or propagate in synucleinopathies characterised by the presence of abnormal α-synuclein [i.e., Lewy body disease (Parkinson's disease and dementia with Lewy bodies) and multiple system atrophy (MSA)] [6-8]. Several lines of evidence have suggested that α-synuclein oligomers play a key role in the pathogenesis of synucleinopathies. In *in vitro* or *in vivo* models of Lewy body disease, α-synuclein oligomers cause synaptic dysfunction and suppress long-term potentiation (LTP), one of the major mechanisms of memory storage [9-13]. All of the Parkinson's disease-linked α-synuclein mutations discovered to date (e.g., A53T and A30P) have been shown to promote α-synuclein oligomerisation and/or fibril formation *in vitro* [14]. Injection of lentivirus with α-synuclein variants that form oligomers rather than fibrils has been shown to induce significant loss of dopaminergic neurons in the rat brain [15]. On the other hand, α-synuclein fibrils contribute to disease propagation or seeding [16]. Interestingly, Lewy body diseases

and MSA show structurally different α-synuclein fibrils, giving rise to distinct clinicopathological traits even within synucleinopathies [17-19]. Recently, Sekiya *et al.* have visualised α-synuclein oligomers in the brains of MSA cases. However, they did not compare the amount and distribution of α-synuclein oligomers in relation to clinical symptoms or clarify whether the accumulation of α-synuclein oligomers varied among cases [20]. Thus, little is known about how various α-synuclein conformers can contribute to the development of symptoms in human MSA cases.

MSA is an adult-onset, fatal neurodegenerative disease with a wide range of motor and non-motor symptoms, pathologically characterised by the ectopic appearance of abnormal α-synuclein in not only oligodendrocytes [i.e., glial cytoplasmic inclusions (GCIs)], but also neurons [i.e., neuronal cytoplasmic inclusions (NCIs)] [8, 21-23]. Despite the various symptoms that patients with MSA may develop, the disease is classified clinically into two subtypes: that with predominant parkinsonism (MSA-P), and that with predominant cerebellar ataxia (MSA-C), reflecting a pathological phenotype of striatonigral (SN) degeneration and olivopontocerebellar (OPC) atrophy, respectively [23]. Importantly, however, GCIs or NCIs are widely spread throughout the central nervous system beyond the SN and OPC systems, and their distribution can differ even among cases, thus explaining the clinical heterogeneity of MSA.

Unlike Lewy body diseases, cognitive impairment has been considered a rare manifestation of MSA. However, recent clinicopathological studies have shown that up to 37% of patients with autopsy-proven MSA can develop various types of cognitive impairment in life [24-29]. Koga *et al.* reported that 33 (32%) of 102 patients with pathologically proven MSA had cognitive impairment. In their study, processing speed (75%) was most commonly compromised, followed by executive function (69%) and memory (31%) [26]. In an earlier study, we also reviewed 148 consecutive patients with pathologically proven MSA [28]. Among them, 30 (20.3%) patients were documented to have developed cognitive impairment: 80% (24/30) of MSA patients with cognitive impairment had frontal subcortical dysfunction including executive dysfunction and 60% (18/30) had memory impairment (MI) [28]. Notably, MSA patients with MI developed NCIs in the dentate gyrus, cornu ammonis regions (CA) 1 - 4 and the entorhinal cortex more frequently than MSA patients with normal cognition (NC). Given the lack of difference in GCI load and neuronal loss between MSA patients with and without cognitive impairment [30], we concluded that the occurrence of memory impairment in individuals with MSA might be associated with the number of NCIs in the hippocampal formation [28]. However, the mechanism whereby abnormal α-synuclein in the hippocampus impairs memory in MSA patients still remains unknown.

To address this knowledge gap, we hypothesised that differences in the levels of α-synuclein oligomers and their deleterious effects on synaptic function and LTP could explain the occurrence of MI in MSA. To test this hypothesis, we generated an adult-onset mouse model of MSA in which inducible human α-synuclein is expressed with a Cre-loxP system [31]. This MSA model has several characteristics. First, human α-synuclein is induced exclusively in oligodendrocytes at least one week after tamoxifen injection [31]. This allows investigation of whether or not human α-synuclein expressed in oligodendrocytes propagates to neurons in the hippocampus over time, and if so, how neuronal functions could be compromised during the disease process. Second, human α-synuclein undergoes phosphorylation at Ser 129 and gains proteinase K resistance [31], two properties that represent key features of oligomeric or aggregated α-synuclein species. Interestingly, unlike human MSA cases, human α-synuclein is not positive for Gallyas-Braak silver stain even in MSA model mice with a long disease duration, suggesting no formation of α-synuclein fibrils in this model [31]. These features offer a unique opportunity to investigate the formation of α-synuclein oligomers and their contribution to the pathogenesis of MSA. In the present study, using this adult-onset mouse MSA model and human MSA cases, we aimed to investigate whether differences in the levels of α-synuclein oligomers between MSA patients with NC and MSA patients with MI can explain MI in MSA.

Materials and Methods

Definition of α-synuclein oligomer

α-Synuclein oligomer encompasses a wide spectrum of structure, molecular weight and morphology [16]. In the present study, α-synuclein oligomer was defined as soluble α-synuclein with a molecular weight of 25 to 180 kDa, which has not formed filamentous aggregates of α-synuclein.

MSA mouse model

All experiments in the present study were performed in accordance with the Guidelines for Animal Experimentation and approved by the Animal Research Committee of Hirosaki University (No. M19022). All animals were kept in temperature- and humidity-controlled rooms under a 12h:12h light:dark cycle, with illumination from 7:00 a.m. to 7:00 p.m. The mice were housed at 3-5 animals per cage with food and water provided *ad libitum*. In the present study, to generate the MSA mouse model, we crossed human α-synuclein-flox transgenic mice with the proteolipid protein (Plp)–Cre recombinase (Cre)/oestrogen receptor (ER) transgenic mice, in which Cre is under the control of the human Plp promoter and ER, as reported previously [31]. Immunohistochemistry using an antibody against human α-synuclein (syn211) confirmed that there was no immunoreactivity with human α-synuclein in oligodendrocytes of mice with or without α-synuclein-flox and Plp-Cre/ER before tamoxifen

injection (Supplementary Fig.1 a, b). Injection of tamoxifen (100 mg/kg, intraperitoneally) once per day for five days also did not induce human α-synuclein in mice without α-synuclein-flox and Plp-Cre/ER, mice with α-synuclein-flox alone, or mice with Plp-Cre/ER alone; mice with these genotypes injected with tamoxifen were used as controls (Supplementary Fig.1c-e). However, in mice with synuclein-flox and Plp-Cre/ER (MSA model), human α-synuclein was induced in oligodendrocytes after tamoxifen injection (Supplementary Fig.1f). The same results were also obtained with LB509 (data not shown). In addition, we performed BaseScope assay, showing that mRNA for human α-synuclein was expressed exclusively in oligodendrocytes after tamoxifen injection (Supplementary Fig.1g-j). Animal groups for analyses were evaluated four to seven weeks after injection in the present study. Otherwise, post week injection was documented in each analysis.

Human cases

In total, 41 autopsy cases were investigated; these included patients with MSA (N = 25), patients with Parkinson's disease (N = 3), patients with Alzheimer's disease (N = 2), and normal controls (N = 11).

Proximity ligation assay (PLA) detects two molecules in extreme proximity (less than 40 nm) [20,32]. To investigate alterations in the immunoreactivity of α-synuclein and the sensitivity of PLA signals to proteinase K treatment, we used cases of MSA (N = 3) and

age-matched cases of Parkinson's disease as disease controls (N = 3). For thioflavin S staining, we used cases of MSA (N = 3) and cases of Alzheimer's disease as positive controls (N = 2). These cases were obtained from the archive of the Department of Neuropathology, Hirosaki University Graduate School of Medicine. Demographic data are shown in Supplementary Table 1.

For PLA, we randomly selected five of MSA cases with MI and five of MSA cases with NC from the archive of the Queen Square Brain Bank for Neurological Disorders. For comparison, we selected five normal controls from the archive of the Department of Neuropathology, Hirosaki University Graduate School of Medicine. Each group was matched for gender, age at death, and concomitant disease pathologies (Alzheimer's disease and Parkinson's disease). In addition, MSA-MI and MSA-NC were matched for disease duration and the proportion of MSA pathological subtypes (SND, OPCA and mixed pathological subtype defined as equal involvement of the SN and OPC systems). Demographic data are shown in Supplementary Table 2.

For biochemical analyses, we investigated controls (N = 6), MSA cases with NC (N = 6) and MSA cases with MI (N = 6) obtained from the archive of the Department of Pathology, Brain Research Institute, Niigata University (Supplementary Table 3). Each group was matched for gender, and the proportion of and concomitant disease pathologies. MSA-

NC and MSA-MI were matched for age at death, disease duration and the proportion of the MSA pathological subtypes.

The brain donation programme and research protocols had received ethical approval from the NRES Committee London – Central, and tissue for research had been stored under a license issued by the Human Tissue Authority (No. 12198). Tissues of MSA cases were transported to the Department of Neuropathology, Hirosaki University Graduate School of Medicine, and utilised for research purposes under a license issued by the Queen Square Brain Bank for Neurological Disorders (EXTMA44-20). This study was also approved by the Institutional Ethics Committee of Hirosaki University Graduate School of Medicine (No. 2020-063; No. 2020-176; No. 2021-013).

Analyses

All methods for the present study are detailed in supplementary data; these include behavioural analysis, BaseScope assay, histological analysis, immunoelectron microscopy, proximity ligation assay, thioflavin S staining, semi-quantitative and quantitative analysis (supplementary Fig. 2a-d), immunoblotting and filter-trap analysis, spine density analysis, and electrophysiological analysis. Information about all antibodies used in the present study is also documented in supplementary data.

Statistical analysis

All statistical analyses in the present study were performed using SPSS 26.0 (SPSS Inc., USA). For comparison of two groups, the Shapiro-Wilk test, a test of normality, was first performed, followed by t-test for parametric data or Mann-Whitney test for non-parametric data. For comparison of more than two groups, we used the Shapiro-Wilk test, and then one-way analysis of variance followed by the Tukey or Games-Howell test for parametric data, or the Kruskal-Wallis test followed by the Dunn test for non-parametric data. For LTP analysis, the average of the last 15 min of the fEPSP slope was compared for statistical analysis. If data were normally distributed, they were presented with a column graph showing mean ± standard deviation. If not, they were presented with the box-and-whisker plot. Differences at $P < 0.05$ were considered to be significant.

Data availability

The raw data supporting the findings of the present study are available on request from the corresponding author.

Results

MSA model mice develop memory impairment after α-synuclein induction

First, to examine whether the MSA model mice developed memory impairment, we subjected 6 of them to an object-location memory task in comparison with 6 age-matched controls before, 1 week and 4 weeks after tamoxifen injection [33]. Mice normally show a spontaneous tendency to spend more time exploring a new object than a familiar one. We placed a mouse in a box containing two objects (A and B). We then moved object B and measured how long the mouse spent exploring the "new" object (B') 30 minutes after training (Fig. 1a). There was no difference in exploration rates between the control and MSA model groups before and 1 week after injection (Fig. 1b). However, MSA model mice 4 weeks after injection equally explored both objects (A and B'), and therefore had a lower exploration rate than the controls (Fig. 1b). Thus, the MSA model mice developed MI.

MSA model mice develop α-synuclein-positive NCI-like structures in the hippocampus

To investigate whether or not α-synuclein-positive NCI-like structures form in the hippocampal formation in the MSA mouse model, we performed immunohistochemistry using an antibody against human α-synuclein (LB509). Similar to human MSA cases, the MSA model mice had developed many α-synuclein-positive GCI-like structures by one week after human α-synuclein induction, as reported previously (data not shown) [31]. To a lesser

extent, NCI-like structures appeared in the central nervous system including the hippocampal formation [e.g., the dentate gyrus, CA1-3 or dentate hilus (corresponding to CA4 in human cases)] (Fig. 2b-d). In addition, human α-synuclein-positive dots were evident in the inner molecular layer of the dentate gyrus, to which excitatory mossy cells in the dentate hilus send their projections (Fig. 2b, c) [34]. These dots were also seen in the polymorphic layer of CA1-3, where pyramidal cells in CA1 receive inputs from CA3 through Schaffer collaterals or layer III of the entorhinal cortex, and CA2-3 pyramidal neurons receive inputs from dentate granule cells or layer II of the entorhinal cortex (Fig. 2d) [34]. We further investigated the immunoreactivity of human α-synuclein-positive structures in the MSA model. This revealed that NCI-like structures as well as human α-synuclein-positive dots were phosphorylated at Ser 129 and immunolabelled with antibodies against the N-terminus (amino acids 1-60), the non-amyloid-β component region (amino acids 61-95) and the C-terminus of α-synuclein (amino acids 96-140) (Supplementary Fig. 3a-h). Thus, these immunoprofiles of α-synuclein in the MSA model mimicked the staining patterns of NCIs in human MSA cases.

Next, using the approach described by Koga *et al*. [26], we semi-quantified NCI-like structures in the hippocampal formation before (N = 5), one week (N = 5) and four weeks (N = 5) after human α-synuclein induction. Whereas no NCI-like structures were evident in the hippocampal formation before induction (Fig. 2e-h), such structures were evident one week after induction (Fig. 2i-l) and had increased significantly by four weeks after induction in the

dentate hilus and CA1-3 (Fig. 2m-u). These observations confirmed that the MSA model mice developed MI along with the occurrence of NCI-like structures in the hippocampus.

Excitatory cells in the hippocampal formation of MSA model mice express human α-synuclein

We then studied the type(s) of neurons that expressed human α-synuclein. The hippocampal formation contains two types of neurons: excitatory (mossy cells or pyramidal neurons) and inhibitory (interneurons) [34]. For this purpose, we used MSA model mice four weeks after human α-synuclein induction (N = 5) and performed double immunofluorescence analysis using antibodies against human α-synuclein (LB509) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) 2, a marker of excitatory neurons [35], or parvalbumin, a marker of inhibitory neurons [36]. In neurons of the dentate hilus or CA1-3, human α-synuclein was co-localised with AMPAR2 (Fig. 3a-c), but only very rarely with parvalbumin (Fig. 3d-f). We counted the numbers of NCI-bearing excitatory and inhibitory neurons in the hippocampus. The proportion of NCI-like structures in excitatory neurons was significantly higher than that in inhibitory neurons (98% versus 4.7%, $P < 0.05$) (Fig. 3g). We further examined whether human α-synuclein-positive dots had accumulated in pre- or post-synaptic structures. This revealed that 26.4% of human α-synuclein-positive dots in the molecular layer were co-localised with synaptophysin-positive presynaptic vesicles and the

rest of human α-synuclein were found in the neuropile [37], whereas only 2% of human α-synuclein-positive dots were co-localised with drebrin-positive dendritic spines (Fig. 3h-n) (P <0.01) [38]. To confirm these findings, we performed immunoelectron microscopy analysis, which revealed gold labelling of human α-synuclein in the pre-synaptic structures of the inner molecular layer (Supplementary Fig. 4a, b). This confirmed that human α-synuclein is highly likely to accumulate in the somata of excitatory neurons and their presynaptic nerve terminals in the hippocampal formation of MSA model mice.

Accumulation of α-synuclein oligomers in the hippocampal formation of MSA model mice

In the present study, we hypothesised that an oligomeric form of α-synuclein contributes to the occurrence of MI in MSA. Conventional immunohistochemistry has not been reported to specify α-synuclein oligomer alone. However, two research groups have shown that α-synuclein oligomers can be visualised using the PLA, which detects inter-protein interactions *in situ* at distances of <40 nm [20,32]. This assay has been used to detect proteinase K-sensitive α-synuclein oligomers, and not α-synuclein fibrils [20]. Accordingly, we performed the PLA using an antibody against human α-synuclein (syn211). Consistent with the previous reports [20,32], almost all PLA signals in the MSA model disappeared after proteinase K treatment, similarly to human cases of MSA and Parkinson's disease, suggesting that these signals in MSA model mice can recapitulate the

early stage of (soluble) α-synuclein oligomers in human MSA cases (Supplementary Fig. 5a-o). We then performed the PLA to examine how human α-synuclein oligomers were formed in the hippocampus before and after human α-synuclein induction. Despite an absence of almost all signals before induction (Fig. 4a-d), by four weeks after induction the MSA model mice had developed human α-synuclein oligomers in the dentate gyrus, dentate hilus and CA1-3 (Fig. 4e-q). We were able to rule out the possibility that the PLA probes had accidentally reacted with two adjacent α-synuclein molecules in fibrils because no α-synuclein fibrils were formed in our MSA model (Supplementary Fig. 6a-f). Immunoelectron microscopic analysis further confirmed no α-synuclein fibrils in the MSA model even 58 weeks after induction (Supplementary Fig. 7a, b). Thus, these PLA signals must have been due to soluble α-synuclein oligomers. Demographic data of human cases for immunohistochemistry and proximity ligation assay with or without proteinase K treatment, and thioflavin S staining is shown in Supplementary Table 1.

While several commercially available anti α-synuclein antibodies are reported to show specific immunoreactivity for aggregated forms of α-synuclein, none of them can detect one specific conformer (α-synuclein oligomers or fibrils) under denatured conditions such as those in immunoblotting [39]. Previously, our group have performed a validation study of multiple a-synuclein conformation-specific antibodies, revealing that, under physiological conditions, clone 5G4 exhibits exceptional immunoreactivity with α-synuclein oligomers and

to a lesser extent with fibrils, but not with monomers [39]. Again, given that no α-synuclein fibrils formed in our MSA model (Supplementary Fig. 6 and 7), clone 5G4 is most likely to detect oligomeric α-synuclein under physiological conditions. To confirm the presence of α-synuclein oligomers in the hippocampal formation of MSA model mice, we performed immunoblotting and filter trap assay using the hippocampal formation of age-matched controls treated with tamoxifen (N = 3) and MSA model mice (N = 3). As reported previously [39], immunoblotting using clone 5G4 yielded non-specific bands with a molecular wight of 25 to 180 kDa in both groups (Fig. 4r asterisk). On the other hand, the filter trap assay revealed higher protein levels of human α-synuclein in the TBS-soluble faction, and those of 5G4-positive human α-synuclein in the TBS- and triton-soluble fractions from MSA model mice relative to the control group (Fig. 4s-w). No difference was seen in protein levels of 5G4-positive human α-synuclein in the sarkosyl and urea fractions between the two groups. These findings confirmed the presence of soluble α-synuclein oligomers in the hippocampus of MSA model mice.

MSA model mice have fewer dendritic spines and suppressed LTP

Because the object-location memory task in the present study demonstrated MI in the MSA model mice, we speculated that the model might also have post-synaptic alterations associated with MI. To investigate this possibility, we injected lucifer yellow into the

cytoplasm of dentate granule cells and CA3 pyramidal neurons of age-matched controls treated with tamoxifen and the MSA model mice, and then counted dendritic spines in relation to their distance from the neuronal soma (50-100, 100-150 and 150-200 μm) (Fig. 5a, b). As expected, the MSA model mice had fewer dendritic spines on the dendrites of dentate granule cells or CA3 pyramidal neurons than controls treated with tamoxifen (Fig. 5c, d). The numbers of neurons examined for this analysis are shown in Fig. 5c and d.

We then performed immunoblotting using the hippocampal formation of age-matched controls treated with tamoxifen (N = 3) and the MSA model mice (N = 3). For LTP induction, N-methyl-D-aspartate receptors (NMDARs) are first activated, followed by crucial phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKIIα) (Thr 286) and AMPAR1 (Ser 831) [40,41]. No difference in the protein level of NMDAR was found in the membrane fraction from the hippocampal formation between the controls and the MSA model mice (Fig. 6a, b). However, the MSA model showed significantly decreased expression of phosphorylated CaMKIIα (Thr 286) and AMPAR1 (Ser831) (Fig. 6c, d), indicating suppression of LTPs in the MSA model. To further confirm these findings, we recorded and compared the LTPs of dentate granule cells, and CA1 or 3 pyramidal neurons between age-matched controls treated with tamoxifen and the MSA model mice. Consistent with the findings of immunoblotting, the MSA model had a lower number of LTPs in neurons

of the dentate gyrus, CA1 and CA3 than the control group (Fig. 6e-g). The numbers of slices examined for LTPs are shown in Fig. 6.

MSA patients with MI have more α-synuclein oligomers in the hippocampal formation than MSA patients with NC

In our earlier study, we showed that MSA patients with MI showed more NCIs in the hippocampal formation than those with NC. To examine whether or not the mechanism of MI formation in the MSA model recapitulates that in human MSA cases, we performed double immunofluorescence analysis and the PLA using controls (N = 5), MSA patients with MI (N = 5) and those with NC (N = 5) from our cohort. As reported previously [28], MSA cases with MI developed NCIs in the hippocampal formation more frequently than those with NC (data not shown). Consistent with the findings obtained in the MSA model mice, double-immunofluorescence analysis using MSA cases with MI demonstrated co-localisation of α-synuclein (syn 211) with glutaminase, but not with parvalbumin (proportion of NCIs in excitatory neurons versus that in inhibitory neurons: 84.6% versus 0%, P <0.01) (Supplementary Fig. 8a-m). Thus, NCI-bearing neurons in the hippocampal formation of human MSA cases are excitatory. The PLA further revealed that in comparison to controls (Fig.7a-e), MSA patients with MI had more α-synuclein oligomers in the dentate gyrus and CA1-4 (Fig. 7k-t). More importantly, MSA cases with MI had more α-synuclein oligomers in

the dentate gyrus, CA1 and 4 than MSA cases with NC (Fig. 7f-q and t). Demographic data for human cases subjected to the PLA are shown in Supplementary Table 2.

Finally, with a view to validating the PLA findings in human MSA cases, we performed the filter trap assay using samples from the medial temporal regions of controls (N = 6), MSA patients with NC (N = 6) and those with MI (N = 6) from a different cohort. Consistent with results obtained from Fig. 7a-t, immunohistochemical analysis using syn211 and the PLA confirmed more NCIs and PLA signals in the dentate gyrus of MSA cases with MI compared to those with NC, respectively (data not shown). The controls showed almost no expression of 5G4-positive α-synuclein (Fig. 7u), whereas signals for 5G4-positive α-synuclein were increased in the TBS fraction of MSA cases with MI relative to controls and MSA cases with NC (Fig. 7v). On the other hand, both MSA-MI and -NC cases showed significantly increased levels of 5G4-positive α-synuclein in the sarkosyl fraction relative to controls (Fig. 7w). Together with the findings of our earlier study, the data suggested that human MSA cases with MI develop more soluble α-synuclein oligomers and NCIs in the hippocampus than MSA cases with NC. Total protein was based on Coomassie Brilliant Blue staining among the various cases (data not shown), and demographic data for human cases subjected to the filter trap assay are shown in Supplementary Table 3.

Discussion

The present study has provided new insights into the nature of the pathological substrate responsible for MI in MSA. Our earlier study had shown that the occurrence of MI in MSA was associated with the number of NCIs in the hippocampal formation [28]. In the present study, mice used in our model of adult-onset MSA, which is characterised by expression of inducible human α-synuclein monomers in oligodendrocytes, developed MI along with the accumulation of human α-synuclein – in the form of NCI-like structures – in the cytoplasm of excitatory neurons, as well as in presynaptic nerve terminals. Human α-synuclein monomers developed into oligomers in the hippocampal formation. Concomitantly, dendritic spines in the dentate granule cells or pyramidal neurons in the hippocampus decreased in number, followed by suppression of LTPs. Consistent with these findings, it was noted that in comparison to human MSA cases with NC, those with MI had more NCIs along with soluble α-synuclein oligomers in the hippocampal formation. Given that α-synuclein oligomers can cause synaptic dysfunction [9-13], the presence of α-synuclein oligomers would be highly likely to impair memory in human MSA cases, in a similar way to the MSA model. Abnormal α-synuclein is transported from presynaptic nerve terminals to neuronal somata, forming aggregates [42]. Thus, the number of NCIs may reflect the amount of α-synuclein oligomer in the hippocampal formation. The putative mechanism responsible for MI in MSA is shown in Fig. 8. However, unlike neurons harbouring NCI-like structures in

the MSA model, NCI-bearing neurons in human MSA may contain various stages of α-synuclein including oligomers and fibrils. Thus, the relative contributions of oligomers versus fibrils to the mechanism of memory impairment and α-synuclein prion propagation remain unknown. In addition, an oligomeric form of α-synuclein encompasses a wide range of structure, molecular weight and morphology, and therefore its pathological effects can vary even among α-synuclein oligomers [16]. We have not yet investigated an alternative hypothesis that neurons in MSA lose their function largely due to loss of oligodendroglial support, followed by the deleterious effects of α-synuclein oligomers on neurons. Thus, neuronal function may be affected in multiple ways rather than as a result of α-synuclein oligomers alone. In MSA, these pathological events may occur simultaneously in the brain. Further study will be needed to clarify which of these conformers and events lie at the basis of the pathomechanism.

The origin of abnormal α-synuclein in GCIs and NCIs is still open to discussion. Initial studies revealed no α-synuclein mRNA in oligodendrocytes in the brain of MSA patients and no difference of α-synuclein mRNA expression in the cerebrum, including GCI-rich white matter, between MSA cases and controls [43,44]. These findings suggest that oligodendrocytes may acquire abnormal α-synuclein from neurons. In contrast to these reports, we have demonstrated higher levels of α-synuclein mRNA expression in MSA oligodendrocytes than in control oligodendrocytes, whereas that in MSA neurons was slightly

lower than in controls [45]. Multiple research groups have shown that α-synuclein mRNA and protein are expressed in cultured oligodendrocytes from rodent as well as human brains [46-48]. In addition, GCIs can be widely distributed in the brain of patients with early-stage or preclinical MSA, even in regions with no obvious neuronal loss or NCI formation [49,50]. Baker *et al*. have also revealed that up to 40% of NCIs are immunoreactive for p25α, predominantly expressed in oligodendrocytes, suggesting that neurons in MSA may take in α-synuclein from oligodendrocytes [51]. Collectively, MSA may well be a primary oligodendrogliopathy and oligodendrocytes could be, at least in some cases, the primary source of α-synuclein for not only GCIs but also NCIs.

Although oligomeric α-synuclein was considered the most likely cause of MI in MSA, we have yet to understand what determines the induction of α-synuclein oligomers in the hippocampal formation and what regulates their stability or transition to higher-order aggregates and fibrils. Our earlier study of MSA cases with NC showed that the NCI burden in the hippocampal formation (dentate gyrus, CA1 and CA2) was correlated with disease duration [28]. In addition, there was an even stronger correlation between the NCI burden and disease duration in a mixed pathological subgroup, defined as having equal involvement of the SN and OPC systems [23,28]. This mixed pathological subgroup may be one in which α-synuclein pathology can easily spread over time. However, such a correlation was not observed in MSA cases with MI. In fact, in the mixed pathological subgroup, MSA cases

with MI developed even more hippocampal NCIs than MSA cases without MI. Consistent with the findings of Ando *et al.* [52], these results suggest that in MSA cases with MI, NCIs may form from an early stage of the disease. In the present study, we found that MSA cases with MI had more α-synuclein oligomers in addition to NCIs in the hippocampal formation. Taken together, these findings suggest that among MSA cases in the mixed pathological subgroup, some unknown traits may further accelerate the spread of α-synuclein oligomers early in the disease process.

Recent studies using pathologically proven cases of MSA have suggested a new clinicopathological variant of MSA with cognitive impairment [52,53]. Aoki *et al.* examined four patients with atypical MSA clinically consistent with frontotemporal dementia [53]. All had severe limbic α-synuclein pathology in addition to classical MSA pathology in the central nervous system, being referred to as the frontotemporal lobar degeneration (FTLD) α-synuclein variant [53]. We have also examined a case of atypical MSA with dementia in which the neuropathological features included marked atrophy of the frontal and temporal lobes including the limbic system [54]. The vast majority of dentate granule cells harboured Pick body-like NCIs [54]. Recently, Ando *et al.* have reviewed 146 consecutive, pathologically proven MSA cases, and noted an association between NCI load in the hippocampal formation and cognitive impairment [52]. On this basis, they classified MSA associated with cognitive impairment as 'hippocampal' MSA; the pathological features of

their cases appeared similar to those of our present cohort of MSA cases with MI. Based on the overlapping distributions of NCIs in the limbic as well as cortical areas, they postulated that both 'hippocampal' MSA and the FTLD α-synuclein variant might be part of the same spectrum [52]. In cases of 'hippocampal' MSA, concomitant Lewy body pathology was minimal and Alzheimer's disease-type pathology, including neurofibrillary tangles or senile plaques, was variable. However, these variants show striking differences in clinical and pathological features. The primary symptom of patients with FTLD α-synuclein is non-amnestic dementia. The severity of neuronal loss in the affected regions differs significantly between the variants. In addition, individuals with FTLD α-synuclein developed a great deal more NCIs in the limbic region than those with hippocampal MSA (i.e., MSA cases with MI in the present study). Together with the results of the present study, the available evidence suggests that individuals with FTLD α-synuclein may, for as yet identified reasons, overproduce pathogenic α-synuclein including oligomers, or other easily spreading aggregated forms of the protein, resulting in more NCIs and neuronal loss in the affected regions.

In conclusion, we have clarified differences in the distribution of α-synuclein species and the underlying pathology in cases of MSA with or without memory impairment. Different MSA clinical subtypes could provide new insights into the mechanism of toxicity

and the nature of pathogenic α-synuclein, thus suggesting future strategies for the development of diagnostics and therapies, even for specific disease subtypes.

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Author contributions

YM, TK, FM, KW designed the research project, performed the behavioural, pathological and biochemical analyses, and were responsible for writing the manuscript. KS and MT

performed the pathological analysis. SCF, KMS, YTA, CB, ZJ, MT, TL, AK, HL, AJL, JLH and TTW provided human tissues and supervised the study. FA, TK, HN, MT and HAL provided experimental materials and supervised the study. TS and HM performed BaseScope assay and supervised the study. SS and TF performed physiological analyses. HY provided Alzheimer's disease model. All authors revised the manuscript.

Conflicts of interest

Y.M., H.L. and T.T.W. are members of the Movement Disorder Society MSA criteria revision task force. All other authors have no competing interests to report.

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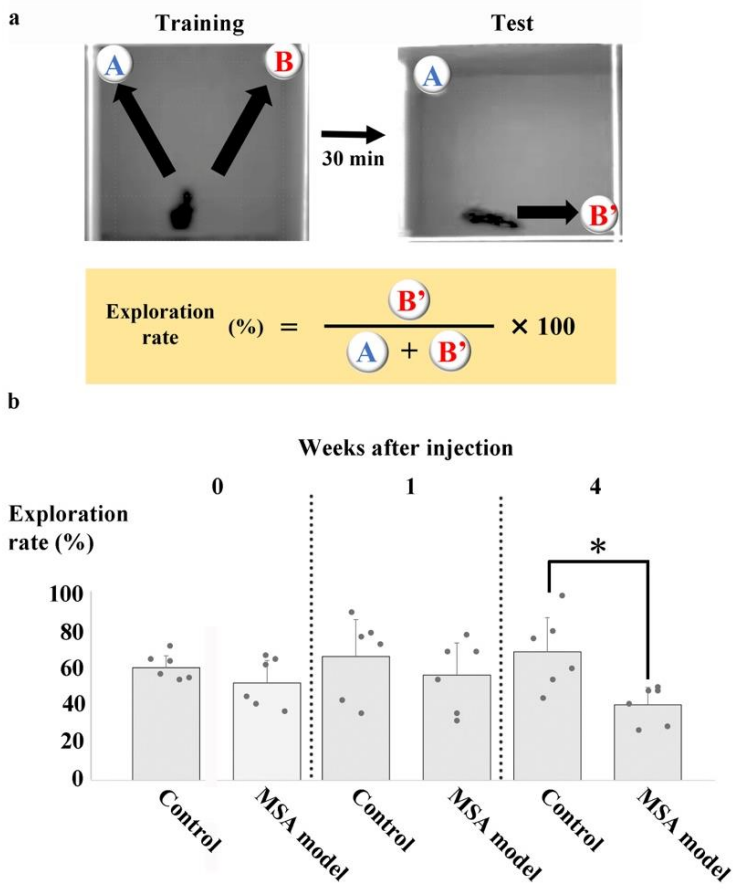

Fig. 1: Development of memory impairment (MI) in the mouse model of multiple system atrophy (MSA).

The MSA model mice express inducible human α-synuclein in oligodendrocytes after injection of tamoxifen once per day for five days (100 mg/kg, intraperitoneally). (a) Object-location memory task. Mice normally spend more time exploring a new object. A mouse is placed in a box containing objects A and B in the upper left (A) and upper right (B) corner, respectively. Thirty minutes after training, B is relocated to the lower right corner (B'). When a mouse has normal cognition, it explores B' more frequently. Thus, a higher exploration rate indicates better memory function. (b) Comparison of MI between age-matched controls (N = 6) and MSA model mice (N = 6) before, one week after and four weeks after tamoxifen injection. No difference is evident between controls and MSA model mice before and one week after injection. However, at four weeks after injection, MSA model mice explore the two objects equally, thereby exhibiting a lower exploration rate than controls (P <0.05), suggesting that in the MSA model, MI has developed by four weeks after injection. *P <0.05. Two types of controls were used: the proteolipid protein (Plp)–Cre recombinase (Cre)/oestrogen receptor (ER) alone, and α-synuclein-flox alone. Control is defined as a mouse that does not express human α-synuclein in the brain after tamoxifen injection. Data in (b) display mean ± standard deviation (SD) and analysed by two sample t-test.

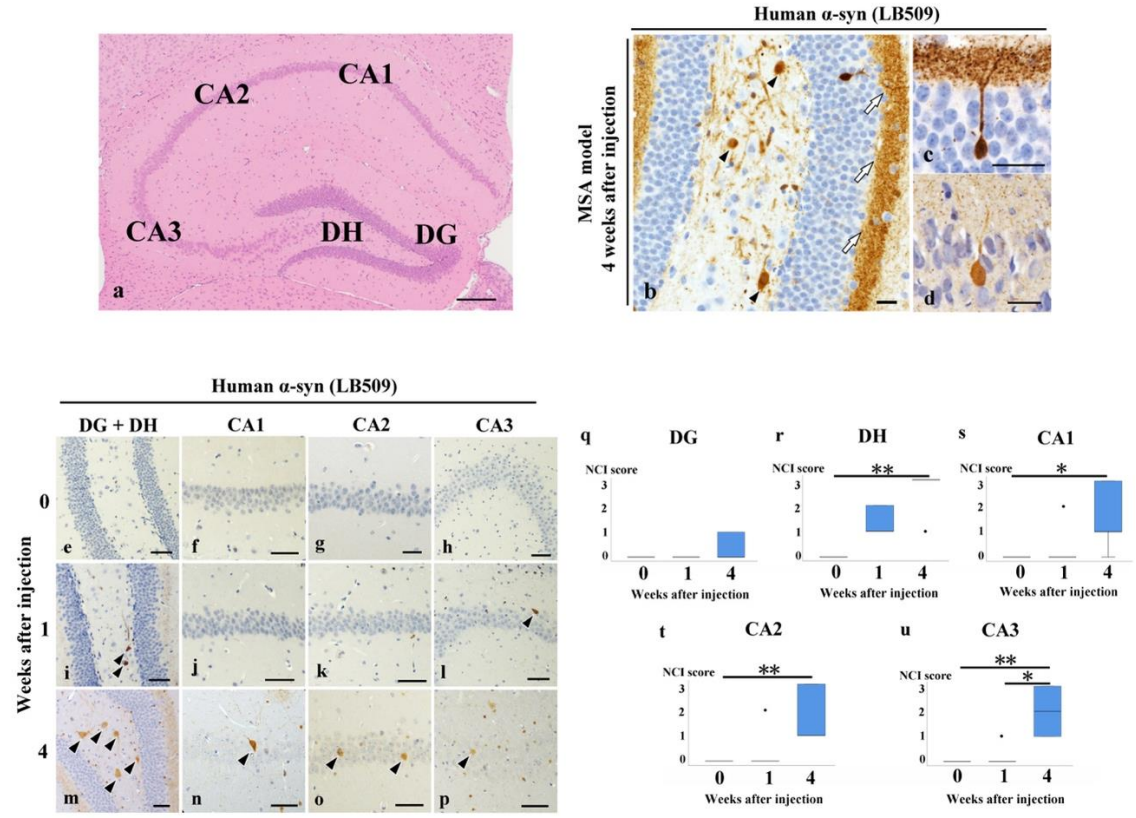

Fig. 2: Neuronal cytoplasmic inclusion (NCI)-like structures in the hippocampus of MSA model mice.

A coronal section of the mouse hippocampus shows the dentate hilus (DH), dentate gyrus (DG) and cornu ammonis (CA) region 1-3 (a). In the MSA model, human α-synuclein is induced in oligodendrocytes at least one week after tamoxifen injection. To a lesser extent, human α-synuclein-positive NCI-like structures (arrowheads) appear in DH (b), DG (c) and CA 3 (d) after human α-synuclein induction. Human α-synuclein-positive dots (white arrows) are also evident in the inner molecular layer of the dentate gyrus (b, c) and CA3 (d). Time course analysis shows that no human α-synuclein is expressed before induction (N = 5) (e-h), whereas human α-synuclein-positive NCI-like structures become evident one week after induction (arrowheads) (N = 5) (i-l) and have significantly increased in the DH and CA1-3 by four weeks after induction (arrowheads) (N = 5) (m-u). There is also a significant increase in NCI-like structures in CA3 region of the MSA model mice four weeks after induction compared to those one week after induction (u). The MSA model mice (b-d) are examined four weeks after injection. Human α-synuclein immunohistochemistry (LB509) (b-p). Data in (q-u) are presented in the box-and-whisker plot and analysed by the Kruskal-Wallis test followed by the Dunn test. Bars = 200 µm (a); 20 µm (b-d); 50 µm (e-p). * P <0.05; ** P <0.01.

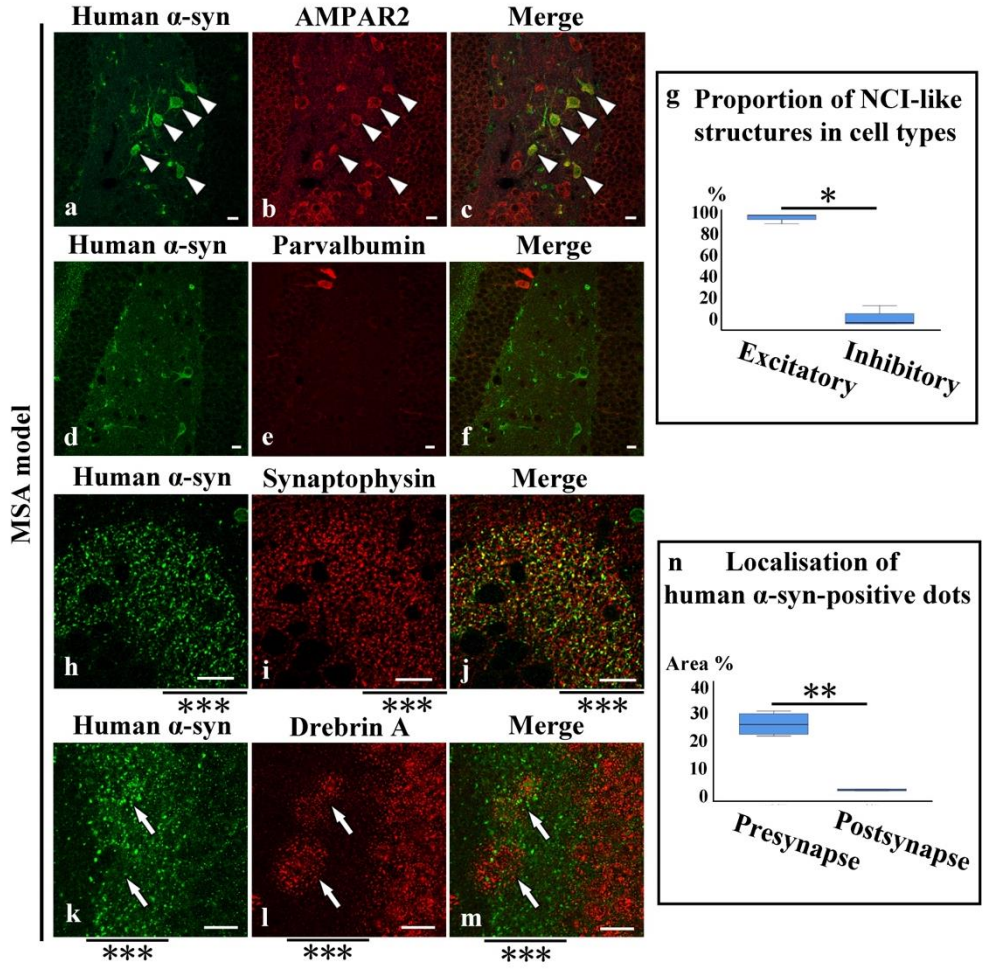

Fig. 3: Human α-synuclein is expressed in the cytoplasm and presynaptic terminals of excitatory neurons in the hippocampus of MSA model mice.

Double immunofluorescence analysis using MSA model mice (N = 5) shows that human α-synuclein is expressed in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) 2-poisitive excitatory neurons (white arrowheads) (a-c), but not in parvalbumin-positive interneurons (d-f). NCI-like structures are formed more frequently in excitatory hippocampal neurons than in inhibitory neurons (98% versus 4.7%, P <0.05) (g). Human α-synuclein-positive dots in the inner molecular layer of the dentate gyrus (***) are co-localised with some of the synaptophysin-positive presynaptic vesicles (h-j), but not with drebrin A-positive dendritic spines (white arrows) (k-m). 26.4% of human α-synuclein-positive dots in the molecular layer are co-localised with synaptophysin-positive presynaptic vesicles whereas only 2% of human α-synuclein-positive dots are co-localised with drebrin-positive dendritic spines (Fig. 3n) (P <0.01). Human α-synuclein (LB509) (a, d, h, k); AMPAR 2 (b); parvalbumin (e); synaptophysin (i); drebrin A (l). Data in (g-n) are presented in the box-and-whisker plot and analysed by Mann-Whitney test. Bars = 10 μm (a-f, h-m). * P <0.05; ** P <0.01

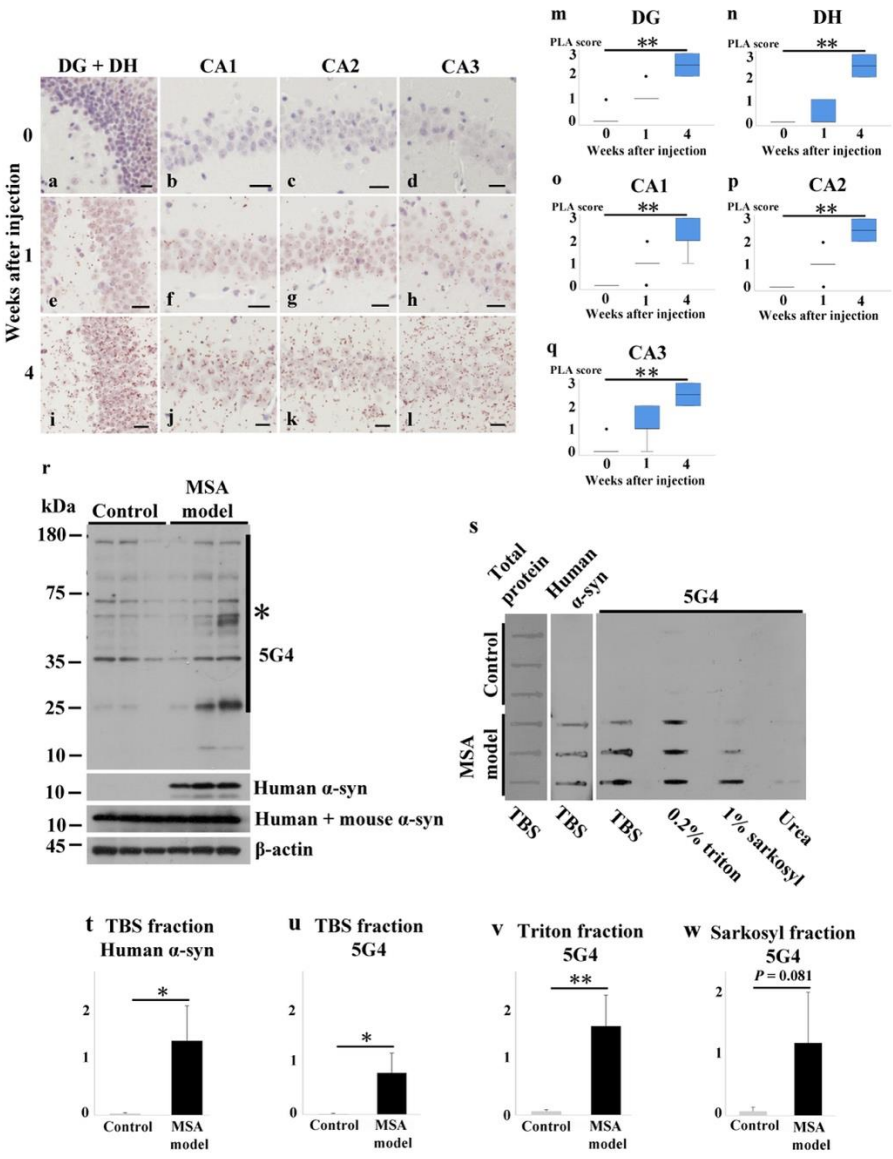

Fig. 4: Oligomeric α-synuclein in the hippocampus of MSA model mice.

Proximity ligation assay (PLA) for detection of α-synuclein oligomers using an antibody against human α-synuclein (syn211). No specific signals are evident in the hippocampus before human α-synuclein induction (N = 5) (a-d), whereas PLA signals (small red dots) appear in the hippocampus one week after induction (N = 5) (e-h). Four weeks after induction, the number of PLA signals in the whole area of the hippocampus has increased significantly (N = 5) (i-q). Immunoblotting shows no specific bands for α-synuclein oligomers (asterisk) (r). On the other hand, under physiological conditions including filter trap analysis, 5G4 shows strong immunoreactivity with aggregated forms of α-synuclein including oligomers, but not with monomers. Raw data for the filter trap assay using antibodies against human α-synuclein (syn211) and 5G4-positive human α-synuclein (s). The TBS fraction of the MSA model (N = 3) shows that expression levels of human (t) and 5G4-positive human α-synuclein (u) are increased to a greater degree than in age-matched controls

treated with tamoxifen (N = 3). In addition, the MSA model shows higher expression levels of 5G4-positive human α-synuclein in the 0.2% triton (v) fraction than those of controls. However, no difference is seen in the protein levels of 5G4-positive human α-synuclein in the sarkosyl (w) and urea fractions between the two groups. Expression levels of human or 5G4-positive α-synuclein in the TBS, triton and sarkosyl fractions were normalised to total protein levels of the corresponding fractions. Representative data of total protein levels in the TBS fraction are shown in (s). Data in (m-q) are presented in the box-and-whisker plot and analysed by the Kruskal-Wallis test followed by the Dunn test. Data in (t-w) display mean ± SD and analysed by two sample t-test. Bars = 20 μm.

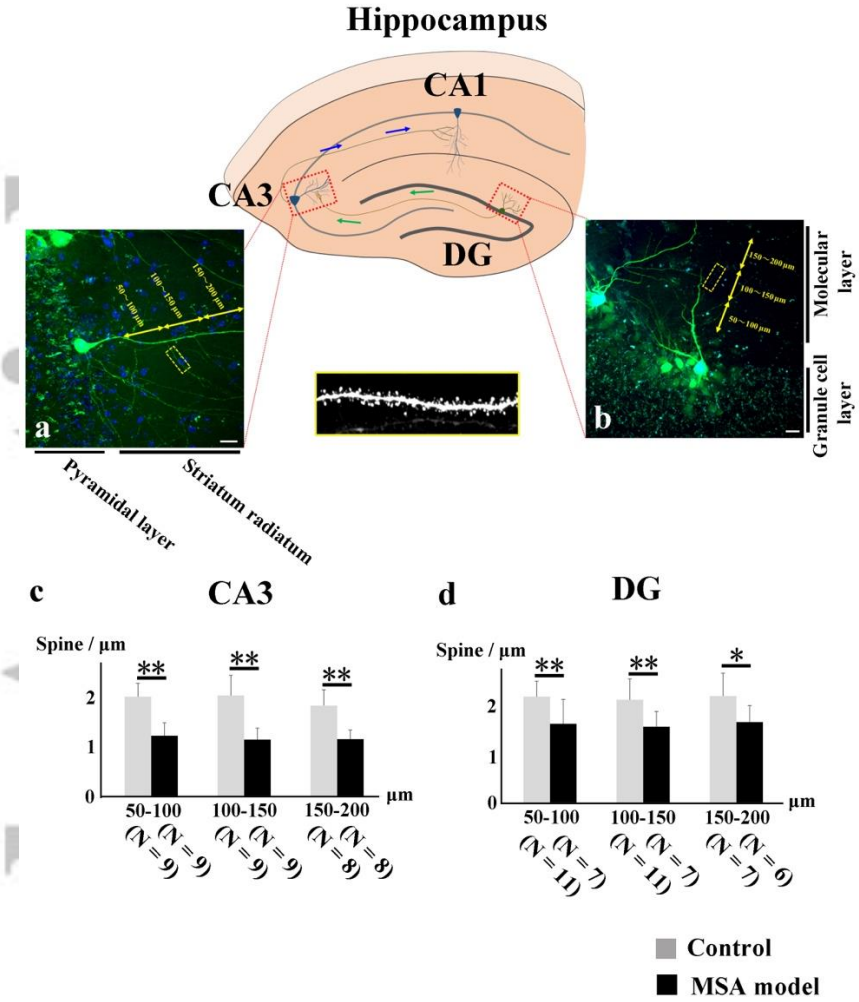

Fig. 5: Dendritic spines are decreased in number in MSA model mice.

Lucifer yellow is injected into CA3 pyramidal neurons (a) and DG cells (b) of age-matched controls treated with tamoxifen and MSA model mice. The MSA model mice have fewer dendritic spines in neurons of both regions than the controls (c, d). Ns in c and d indicate the numbers of cells examined for this analysis. Data in (c-d) display mean ± SD and analysed by two sample t-test. Bars = 100 µm.

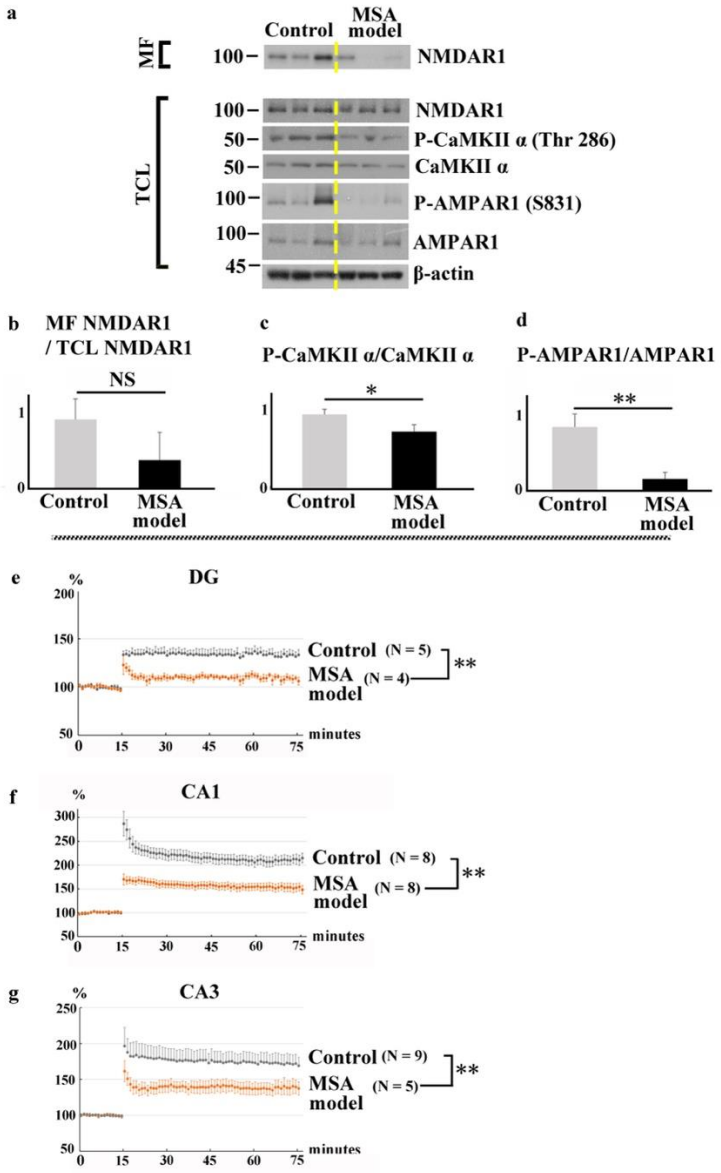

Fig. 6: Suppressed long-term potentiation (LTP) in MSA model mice.

Immunoblotting using age-matched controls treated with tamoxifen (N = 3) and MSA model mice (N = 3). Raw data for immunoblotting are shown in (a). Despite no difference in the levels of *N*-methyl-D-aspartate receptor (NMDAR) 1 in the membrane and total cell fractions between controls and MSA model mice (b), the latter fail to undergo phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Thr 286) (c) and AMPAR1 (Ser 831) (d), both of which are crucial for inducing LTP. Recording of LTPs in the hippocampus of age-matched controls treated with tamoxifen and MSA model mice shows that in the latter LTPs are suppressed in the DG, CA1 and CA3 (e-g). Ns in e-g indicate the numbers of slices examined for LTP analysis. Abbreviations: MF: membrane fraction; NS: not significant; TCL: total cell lysate. Data in (b-d) display mean ± SD. All data in Fig. 6 are analysed by two sample t-test. * P <0.05; ** P <0.01

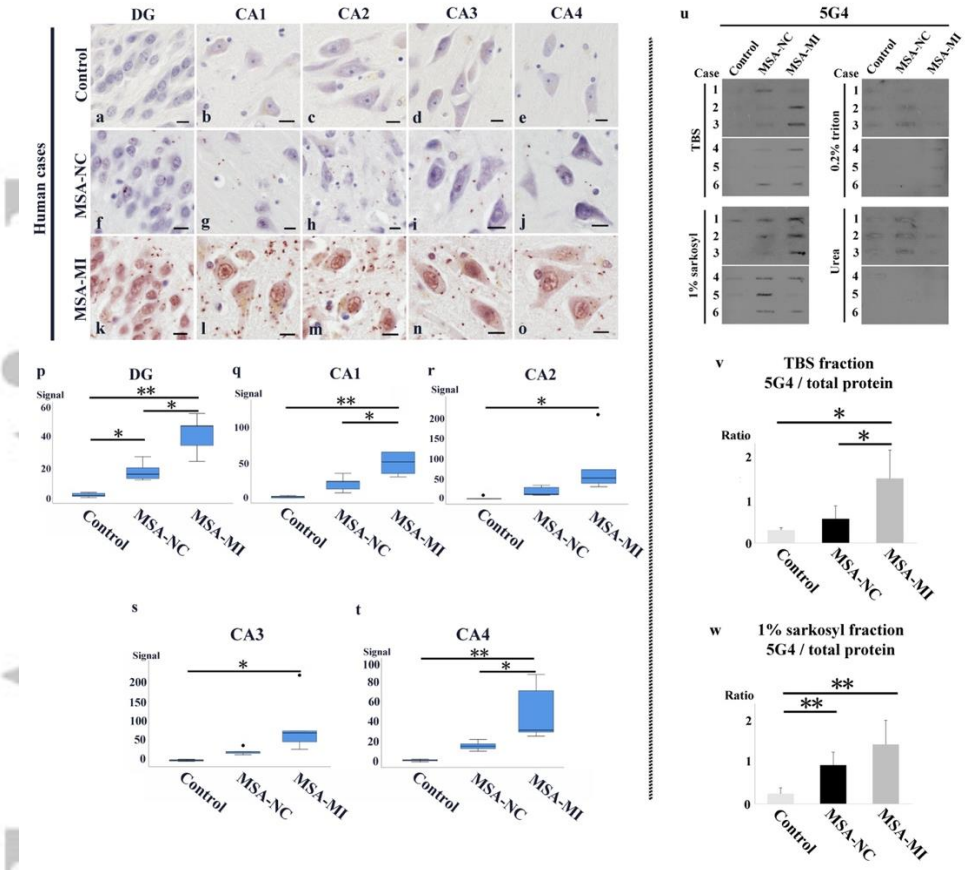

Fig. 7: Human cases of MSA with MI have more α-synuclein oligomers than those with NC.

PLA performed using the hippocampus of controls (N =5) (a-e), MSA cases with normal cognition (NC) (N = 5) (f-j) and MSA cases with MI (N = 5) (k-o). All PLA signals are counted in five randomly selected areas of each hippocampal sector (DG, CA1 to CA4). The average of PLA signals normalised to tissue area is calculated in each sector. MSA cases with NC have more PLA signals in the DG than the controls (p). MSA cases with MI have more PLA signals in the DG and CA1-4 than the controls (p-t). In addition, MSA cases with MI have more PLA signals in the DG, CA1 and 4 than MSA cases with NC (p, q, t).

Filter trap assay using the medial temporal regions of controls (N = 6), MSA cases with NC (N = 6) and MSA cases with MI (N = 6). Raw data for the filter trap assay are shown in (u). The TBS fraction of MSA cases with MI shows a higher level of 5G4-positive α-synuclein expression than those in the controls and MSA cases with NC (v). On the other hand, the levels of 5G4-positive α-synuclein in the 1% sarkosyl fraction are significantly higher in MSA cases with NC and MI than in controls (w). Total protein is based on Coomassie Brilliant Blue staining among the various cases. Data in (p-t) are presented in the box-and-whisker plot and analysed by the Kruskal-Wallis test followed by the Dunn test. Data in (v, w) display mean ± SD and analysed by one-way analysis of variance followed by the Tukey or Games-Howell test. Bars = 20 μm. * P <0.05; ** P <0.01

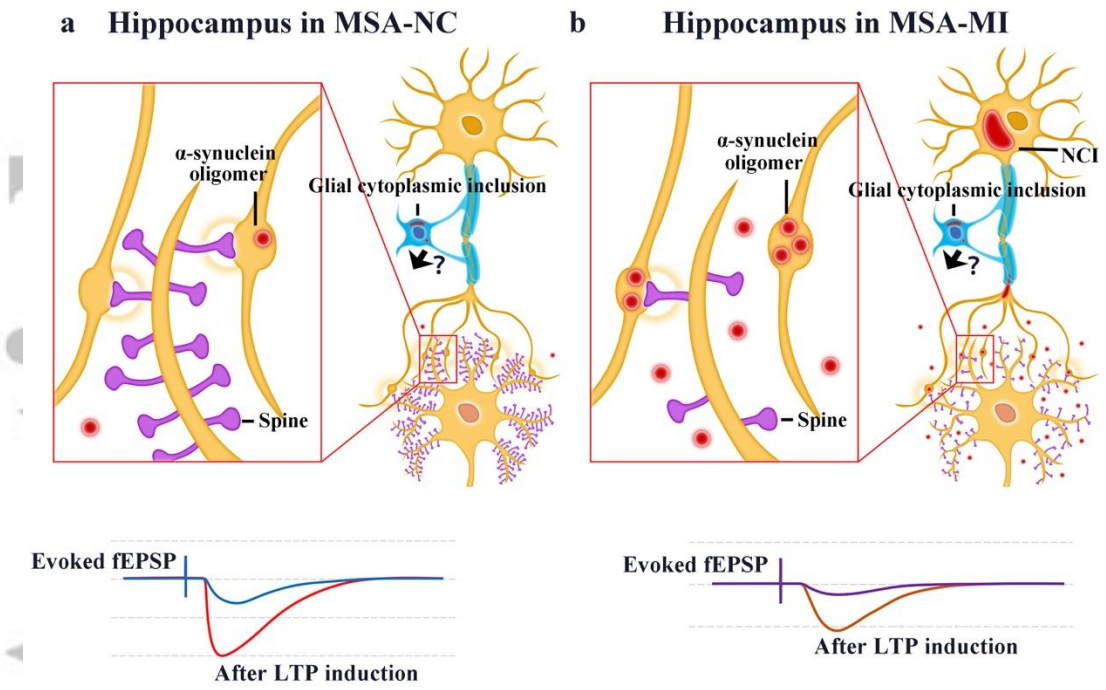

Fig. 8: Schematic view of the hippocampus in MSA cases with NC and MI.

In comparison to MSA cases with NC (a), MSA cases with MI have more α-synuclein oligomers in the hippocampus (b), subsequently developing NCIs. Excitatory neurons in the hippocampus have fewer dendritic spines in MSA cases with MI than in those with NC, resulting in suppressed LTP induction. Red dots represent α-synuclein oligomers, red inclusions in oligodendrocytes are glial cytoplasmic inclusions, the red inclusion in the cytoplasm is an NCI, and the purple mushrooms represent dendritic spines. Black arrows indicate a putative source of abnormal α-synuclein in MSA. fEPSP: field excitatory postsynaptic potential