

NLRP3 Inflammasome-Related Proteins Are Upregulated in the Putamen of Patients With Multiple System Atrophy

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Running head title: Activation of NLRP3 inflammasome in MSA patients

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Acknowledgments

Not applicable.

Conflict of interests

The authors declare no conflict of interest.

Funding

This work was supported by the MEXT KAKENHI Grant Numbers JP16K18383.

Abstract

Multiple system atrophy (MSA) is a neurodegenerative disease characterized by parkinsonism, ataxia, and autonomic dysfunction. Microglial infiltration is an important mediator in MSA. The nucleotide-binding domain, leucine-rich repeats-containing family, pyrin domain-containing-3 (NLRP3) inflammasome complex, comprising NLRP3, apoptotic speck protein containing a caspase recruitment domain (ASC), and cysteine aspartic acid protease 1 (Caspase 1), regulates microglial inflammation in several neurodegenerative diseases. However, its role in MSA remains unknown. This study aimed to investigate the role of the NLRP3 inflammasome in MSA.

Immunohistochemical staining of post-mortem brains from 11 cases of MSA, five of Parkinson's disease (PD), and six age-matched controls (CTL) were assessed. The relationships among α -synuclein deposition, microglial infiltration, and NLRP3 inflammasome-related proteins (NLRP3, ASC, and Caspase 1) were quantitatively analyzed. Double-labeling immunofluorescence staining confirmed colocalization of NLRP3 inflammasome-related proteins and Cluster of Differentiation 68 (CD68). We demonstrated that the density of microglia expressing NLRP3 inflammasome-related proteins was increased in the putamina of MSA cases and was significantly related to the deposition of phosphorylated α -synuclein (p-Syn)-positive glial cytoplasmic inclusions, tyrosine hydroxylase-positive fiber loss, and gliosis of glial fibrillary acidic protein-positive astrocytes. Our study suggests that the NLRP3 inflammasome is significantly upregulated and correlates with the neurodegenerative process in MSA.

Keywords: Inflammasome, microglia, multiple system atrophy, NLRP3, putamen

Introduction

Multiple system atrophy (MSA) is a neurodegenerative disease (1,2) which is characterized by parkinsonism, ataxia, and autonomic dysfunction (3). This clinical heterogeneity reflects varying pathological features including degeneration of the nigrostriatal system, cerebellum, and autonomic system.

There is currently no specific treatment for MSA. Dopaminergic therapy is sometimes used to activate the nigrostriatal system, but only up to 13% of patients have a sustained response (4,5). Compared to Parkinson's disease (PD) patients, the benefit of dopaminergic therapy does not continue as the disease progresses in MSA patients. Reasons for dopaminergic therapy failure in MSA are attributed to degeneration of postsynaptic neurons in the putamen, projective fiber loss from the nigra to putamen, and neuronal loss in the substantia nigra (6,7). To overcome these problems, the pathomechanism of degeneration in the putamen of MSA patients requires elucidation. In the putamina of MSA patients, there are glial cytoplasmic inclusions (GCIs), neuronal loss, microglial infiltration, and gliosis (8). GCIs are the hallmark of MSA pathology and its major component is α -synuclein in oligodendrocytes (9). However, the precise connection between GCI and neuronal degeneration is less clear. Additionally, microglial infiltration also acts as an important mediator in MSA in both disease initiation and progression (10,11). An *in vivo* study using positron emission tomography imaging (PET) demonstrated that microglial activation appears to parallel neurodegeneration in MSA patients (12). A neuropathological study using postmortem brains of MSA patients demonstrated that the degree of activated microglia correlated with the density of α -synuclein-positive GCI pathology (10). The increase in α -synuclein aggregation is correlated with microglial activation (13). Indeed, microglial activation is proposed to exaggerate neuronal loss and gliosis in affected brain areas in MSA (10,14). These pathological features imply that regulation of microglial activation may be a therapeutic target for MSA. However, the precise relationship between α -synuclein aggregation and microglial activation has not been elucidated.

Recently, the inflammasome has been reported to link aggregation of pathogenic proteins and

microglial activation in a variety of neurodegenerative disorders such as Alzheimer's disease (AD) and PD (15–17). The inflammasome comprises cytosolic caspase-activating protein complexes in macrophage lineage cells, and is responsible for protein cleavage, release of proinflammatory cytokines Interleukin 1 β (IL-1 β) and IL-18, and subsequent inflammatory processes (18). In particular, the nucleotide-binding domain, leucine-rich repeats-containing family, pyrin domain-containing-3 (NLRP3)-related inflammasome is related to the neurodegenerative processes of abnormal protein accumulation. These abnormal proteins, including β -amyloid and α -synuclein, stimulate invasive microglia (15,16,19). The NLRP3 inflammasome complex consists of NLRP3, apoptotic speck protein containing a caspase recruitment domain (ASC) and cysteine aspartic acid protease 1 (Caspase 1). Various signals through the Toll-like receptor/nuclear factor- κ B (TLR/NF- κ B) pathway recruit NLRP3 inflammasome-related proteins into the NLRP3 inflammasome complex, leading to the transcription of pro-IL-1 β and pro-IL-18. This results in the generation of mature cytokines IL-1 β and IL-18 (20). Regulation of the NLRP3 inflammasome may alleviate neuroinflammation and neurodegenerative processes in AD and PD models (21,22). However, our understanding of inflammasome activation and its relationship with GCIs, microglial infiltration, and gliosis in MSA patients is limited.

Here, we report the relationship between NLRP3 inflammasome-related proteins and microglial activation in the putamen of MSA patients.

Materials and Methods

Human brain tissue

Formalin-fixed human brain tissue samples from 11 patients with MSA (52–88 years old), five patients with PD (66–78 years old) and six age-matched controls (CTL) (62–86 years old) without significant α -synuclein disease and clinical history of parkinsonism were utilized in this study as shown in [Table 1](#). To obtain the corresponding portion from each case, the posterior putamen was obtained from coronal sections containing the globus pallidus. This study was approved by the ethics committee of Kyoto University (approval number R1038). Patient diagnosis was determined by clinical information and pathological examination.

Primary antibodies for immunohistochemistry

The primary antibodies used were as follows: Cluster of Differentiation 68 (CD68, mouse monoclonal [Cat No. M0814], DAKO, Glostrup, Denmark; 1:200), phosphorylated α -synuclein (p-Syn, mouse monoclonal [Cat No. 015-25191], WAKO, Tokyo, Japan; 1:2000), tyrosine hydroxylase (TH, mouse monoclonal [Cat No. T2928], Sigma-Aldrich Inc., St. Louis, Missouri, USA; 1:500), NLRP3 (rabbit polyclonal [Cat No. HPA012878], Sigma-Aldrich Inc., St. Louis, Missouri, USA; 1:500), cysteine aspartic acid protease 1 (Caspase 1, rabbit monoclonal [Cat No. ab108362], Abcam, Cambridge, UK; 1:1600), apoptotic speck protein containing a caspase recruitment domain (ASC, rabbit polyclonal [Cat No. ADI-905-173], Enzo Life Sciences, Inc., Farmingdale, NY; 1:400), glial fibrillary acidic protein (GFAP, mouse monoclonal [Cat No. M0761], DAKO, Glostrup, Denmark; 1:3000), interleukin 1 β (IL-1 β , rabbit polyclonal [Cat No. ab9722], Abcam, Cambridge, UK; 1:400).

Single-labeling immunohistochemistry

For single-labeling immunohistochemistry (IHC), 6- μ m sections were deparaffinized, and antigens were retrieved by autoclaving for 20 min at 120°C using the Histofine deparaffinizing antigen retrieval buffer, pH 6 (Cat No. 415281; Nichirei, Tokyo, Japan), followed by overnight incubation at 4 °C with primary antibodies in phosphate-buffered saline (PBS) containing 3% bovine serum albumin. Single-labeling IHC was performed with antibodies against CD68, p-Syn (pretreated with formic acid for 5 min), GFAP (without antigen retrieval), TH, NLRP3, ASC, Caspase 1 and IL-1 β ; and conducted with the peroxidase polymer-based method using the Histofine Simple Stain MAX-PO MULTI (Cat No. 424154; Nichirei, Tokyo, Japan) and DAB Substrate Kit (Cat No. SK 4100; Vector Laboratories, Burlingame, CA, USA).

Double-labeling immunofluorescence staining

Sections from an MSA and CTL case were selected for the double-labeling immunofluorescence method. The sections were simultaneously incubated with primary

antibodies against CD68 and NLRP3, CD68 and ASC, and CD68 and Caspase 1 in 0.01 M PBS at 4°C overnight. After washing with 0.01 M PBS, the sections were reacted with secondary antibodies (Alexa Fluor 488-labeled goat anti-mouse or rabbit IgG and Alexa Fluor 546-labeled goat anti-mouse or rabbit IgG, 1:200; Molecular Probes, Eugene, OR, USA). After rinsing with 0.01 M PBS, the slides were cover slipped with Vectashield containing DAPI (Vector Laboratories)) and were then viewed with a FLUOVIEW FV-1000 confocal laser scanning microscope (Olympus, Tokyo, Japan).

Quantitative analysis

To determine the exact densities of pathological changes of GFAP, CD68, NLRP3, ASC, and Caspase 1, we took three pictures under 200x magnification for each slide to encompass the lateral part of the putamen from each case. In each picture, the number of immunopositive cells was counted manually by two experienced pathologists who were blinded to case diagnosis. The mean density of pathological changes was calculated per square millimeter as the average of the three pictures. In each case, the density of cells expressing inflammasome-related proteins was divided by CD68-positive microglial density to calculate the ratio of inflammasome-related protein-positive cells to CD68-positive microglia. TH-positive fiber densities and p-Syn-positive areas were automatically quantified using ImageJ software (version 1.50i, Wayne Rasband, NIH, USA).

Brain homogenates and supernatant fluid preparation

Frozen brain tissue was cut into small pieces and homogenized for 5 min by ultrasound in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 0.1% sodium deoxycholate, and 0.5% sodium deoxycholate), containing protease inhibitor cocktail (Roche). The homogenate (final concentration of the sample tissue in the homogenate was 5%) was centrifuged for 15 min at 1500 g. The supernatant was transferred to 1-mL disposable containers, which were stored at -20 °C for up to 4 weeks. Protein concentrations were determined using the Pierce TM BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's protocol.

Primary microglia lineage cell cultures

Mixed glial cell cultures were obtained from cerebral cortices of 1-2-day-old Sprague Dawley rats and prepared as previously described (23). After the cells reached confluency (about 10 days), the flasks were shaken for 1 h on an orbital shaker (218 rpm) at 37 °C to collect microglia used for the experiments. Lipopolysaccharide (LPS) was added at 1 µg/mL for 18 h. After treatment with LPS, microglia cells were lysed in RIPA buffer (20 mM HEPES-KOH pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet-P40, 1% sodium deoxycholate), containing protease inhibitor cocktail (Roche).

Western blotting

Western blot analyses of IL-1 β and β -actin were performed on lysates separated on 5-20% SDS-PAGE gel (Wako) under reducing conditions, which were then transferred on to Immuno-Blot PVDF membrane (Bio-Rad, Hercules, CA, USA). In each well, 15 µg of protein in 15 µL was loaded. The membrane was blocked with 3% nonfat milk and 0.1% Tween-20 in TBS for 1 h at room temperature and was then incubated with primary antibodies overnight at 4 °C, followed by secondary antibody incubation the next day for 1 h at room temperature. Protein bands were detected with AI600.

Enzyme-linked immunosorbent assay (ELISA)

The expression levels of IL-1 β were determined in brain homogenates and supernatants (200 µg of protein in 100 µL) by ELISA, using the commercially available Quantikine® kit (R&D Systems, Inc., Minneapolis, MN, USA) for human IL-1 β , according to the manufacturer's instructions.

Statistical analysis

SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) software was used for statistical analysis.

The results were analyzed using one-way analysis of variance (ANOVA), independent sample *t*-test and Pearson correlation coefficient analysis. Results are represented as mean $\pm$ standard deviation (SD). Statistical significance was defined as $p \leq 0.05$.

Results

Microscopic examination with immunohistochemistry

Immunohistochemistry with anti-p-Syn antibody demonstrated abnormal aggregations of α -synuclein in the putamen of MSA and PD patients. In MSA cases, p-Syn-immunopositive GCIs were frequently observed (Fig. 1C). PD cases showed sparse p-Syn-immunopositive Lewy neurites which were compatible with p-Syn deposition in axons from the substantia nigra to putamen (Fig. 1B). There was no abnormal p-Syn-positive deposition in CTL cases (Fig. 1A).

To evaluate presynaptic fibers in the nigrostriatal system, the density of TH-positive fibers in putamen was measured in each case. TH-immunopositive fibers were abundant throughout the putamen in CTL cases (Fig. 1D). The density of immunopositive fibers was reduced in PD cases but less severely than in MSA cases (Fig. 1E). The density of TH-immunopositive fibers was severely decreased in MSA cases (Fig. 1F).

Astrocytes were immunostained with anti-GFAP antibody to evaluate the degree of putamen gliosis. GFAP-immunopositive astrocytes were observed in abundance in the putamen of MSA cases, while astrocytes were rarely seen in PD and CTL cases (Fig. 1G-I). Morphologically, gemistocytic astrocytes were frequently seen in MSA cases. On the other hand, astrocytes in PD and CTL cases had small cell bodies, indicative of inactive astrocytes.

The density of cells immunopositive for inflammasome-related proteins was increased in the putamen of MSA patients

CD68-positive microglia were consistently observed in MSA cases (Fig. 2C). Morphologically, rounded amoeboid microglia were seen in abundance in MSA cases. A small number of CD68-positive microglia in PD (Fig. 2B) and CTL cases (Fig. 2A) were also observed. These observations were compatible with ramified microglia, which represent an inactive form of

microglia.

In sections containing the putamen of MSA cases, we found that the number of NLRP3/ASC/ Caspase 1-immunopositive cells was increased (Fig. 2F, I, L). Morphologically, most of the positive cells had small nuclei and small cell bodies which were identifiable as microglia. In MSA cases, these immunopositive microglia had relatively large, round cell bodies which were compatible with rounded amoeboid microglia. On the other hand, immunoreactive glial cells did not resemble astrocytes or oligodendrocytes. Occasionally, weak immunostaining in neurons was observed, but the intensity of immunoreactivity in neurons was weaker than that in microglia (data not shown). This phenomenon was observed in all MSA cases. On the other hand, only a small number of NLRP3/Caspase 1/ASC-immunopositive cells were observed in PD and CTL cases.

Double-labeling immunofluorescence staining

Double-labeling immunofluorescence staining and confocal imaging analysis showed significant activation of NLRP3, ASC, and Caspase 1; mostly localized in CD68-labeled microglia in the putamen from MSA cases compared to CTL cases (Fig. 3). Confocal images of double-label immunostaining with NLRP3 (Fig. 3A-D) /ASC (Fig. 3I-L) /Caspase 1 (Fig. 3Q-T) and CD68 antibodies are depicted. On the other hand, NLRP3/ASC/Caspase 1/CD68-labeled microglia were sparsely observed in CTL cases (Fig. 3E-H, M-P, U-X).

Statistical analysis of immunopositive structures

The density of immunopositive glial cells in the putamina was counted as described in the methods. The results of the statistical analyses of p-Syn burdens, GFAP-positive astrocytes, TH-positive fiber loss, and CD68 immunopositive microglia in the putamen are shown in Table 2 and Fig. 4. In post hoc least significance difference (LSD) test correction among three groups, a mean p-Syn-positive area in MSA cases (7.61%) was significantly higher than CTL cases (0.04%). No significant difference was observed between MSA and PD cases (1.08%) (Fig. 4A). Mean TH-positive area in MSA cases was decreased to 3.34%. We found a significant difference among MSA, PD, and CTL cases in comparison PD cases decreased to 12.50% and CTL cases to 34.00%,

respectively (Fig. 4B). Significantly higher numbers of CD68-positive microglial cells (Fig. 4D) and GFAP-positive astrocytes (Fig. 4C) were observed in MSA cases compared to PD and CTL cases, whereas there were no significant differences between PD and CTL cases. The results of ANOVA of p-Syn, TH, and GFAP immunoreactivity are summarized in Table 2.

The density and ratio of cells expressing inflammasome-related proteins per CD68-positive microglia are increased in MSA

The density of NLRP3/ASC/Caspase 1-immunopositive microglia was increased in all cases of MSA compared to PD and CTL cases (Fig. 5A-C). The results of ANOVA of NLRP3/ASC/Caspase 1 immunoreactivity are summarized in Table 2. To approximate the degree of microglia upregulated with inflammasome-related proteins, we also calculated the ratio of the densities of cells expressing inflammasome-related proteins to CD68-positive microglia (Fig. 5D-F). This ratio was increased in MSA cases (mean ratio of NLRP3 CD68, 14.76%; mean ratio of ASC/CD68, 48.54%; mean ratio of Caspase 1/CD68, 42.97%) compared to PD cases (mean ratio of NLRP3 CD68, 4.60%; mean ratio of ASC/CD68, 16.44%; mean ratio of Caspase1/CD68, 20.24%) or CTL cases (mean ratio of NLRP3/CD68, 4.52%; mean ratio of ASC/CD68, 23.85%; mean ratio of Caspase 1/CD68, 31.82%). This ratio of NLRP3 (Fig. 5D) and ASC (Fig. 5E) was significantly higher in MSA cases. The mean ratio of Caspase 1 (Fig. 5F) was higher in MSA than CTL and PD but was not statistically significant. Four of the 11 cases with MSA were clinically classified as having the parkinsonian subtype (MSA-P), and seven of the cases had the cerebellar subtype (MSA-C) (Table 1). No significant immunohistochemical differences were observed between the two subtypes.

Correlation analysis

There were strong positive correlations between the number of cells immunostained with CD68, GFAP, NLRP3 inflammasome-related proteins, and p-Syn, as shown in Table 3 and Fig. 6 when all cases were included. TH fiber loss was significantly related to these factors (Table 3, Fig. 6). When correlation analysis of these factors only performed within the MSA group, there was no

significant correlation (data not shown).

IL-1 β protein expression levels

To investigate IL-1 β expression levels in MSA and CTL cases ([Supplementary Table 1](#)), we performed western blotting, ELISA, and single-labeling immunohistochemistry of IL-1 β . Although LPS-treated primary microglia from rat were positive for IL-1 β in western blotting ([Supplementary Figure 1A](#)) and served as positive control, in the case of brain homogenates, we obtained several non-specific bands and did not find any differences in IL-1 β expression between MSA and control groups ([Supplementary Figure 1A](#)).

ELISA analysis showed that IL-1 β protein expression was higher in occipital lobe homogenates from MSA (1.8 ± 1.2 pg/mL) than from CTL cases (0.7 ± 0.3 pg/mL); however, these results were not statistically significant ([Supplementary Figure 1B](#)). Phosphorylated α -synuclein-positive GCIs were also observed in the occipital lobe in MSA cases ([Supplementary Figure 2A](#)).

Single-labeling immunohistochemistry for IL-1 β showed highly-colored background and non-specific staining in neurons and glial cells. Thus, it was hard to detect significant differences among the three groups ([Supplementary Figure 1C](#)). Single-labeling immunohistochemistry for IL-1 β using a meningitis case also showed high background and non-specific staining of neurons, inflammatory cells, and glial cells ([Supplementary Figure 2B](#)).

Discussion

In the present study, we reported that the number of NLRP3 inflammasome-related proteins (NLRP3, ASC, and Caspase 1)-immunopositive microglia was increased in the putamen of MSA patients. These proteins were abundantly upregulated in rounded amoeboid microglia which is known to be an active form of microglia (24). Our quantitative analysis showed that the density of cells expressing NLRP3 inflammasome-related proteins was significantly associated with the intensity of microglial infiltration. Moreover, the ratio of cells expressing NLRP3 inflammasome-related proteins tended to increase in the microglia of MSA patients. These pathological results

indicate that the NLRP3 inflammasome plays a crucial role in microglial activation and infiltration in MSA.

The density of α -synuclein-positive GCI has been associated with the density of microglia expressing NLRP3 inflammasome-related proteins. Previous studies using BV2 cultured cells (25) and human monocytes (15) have reported that α -synuclein induced microglial IL-1 β production. These findings suggest that microglial activation and IL-1 β pathway activation can be attributed to aggregated α -synuclein, rather than a nonspecific response to neuronal death.

Our results showed that the density of GFAP-positive astrocytes correlated with microglial activation and upregulation of NLRP3 inflammasome-related proteins. These results suggest that microglial activation and upregulation of NLRP3 inflammasome-related proteins are associated with neuronal loss and gliosis in the putamen of MSA patients, as neuronal injury and degeneration activate astrocytes and induce gliosis (10,13). In cultured cells, Claudio et al. reported that α -synuclein induced microglial IL-1 β release leading to neuronal death in co-cultures of microglia and neurons (25). Previous studies showed that pro-inflammatory factors released by activated microglia can trigger astrogliosis (26–28). On the other hand, microglia are also activated in response to astrocytic activation (29). Collectively, these findings suggest that microglia expressing NLRP3 inflammasome-related proteins and active astrocytes interact with each other in the putamen of MSA patients.

The degree of microglial infiltration and upregulation of inflammasome-related proteins were also related to TH-positive fiber loss. TH is the enzyme responsible for catalyzing the conversion of levodopa which is the precursor for dopamine (30). TH-positive fibers reflect projections from the midbrain to putamen (31). Previous reports indicate that activated microglia damage axons (32,33). Therefore, it is possible that increased microglial-induced impairment of TH-positive fibers reflects damage to projection fibers from the substantia nigra to the putamen in MSA patients. Axonal loss in the putamen may cause neuronal death in the substantia nigra in MSA.

On the whole, we propose that that abnormal α -synuclein activates microglial NLRP3 inflammasome leading to IL-1 β release, microglial infiltration, gliosis, axonal damage, and neuronal death in MSA. A plausible mechanism of NLRP3 inflammasome-mediated changes in

MSA is illustrated in Fig 7. Although a previous report showed increased IL-1 β expression levels in cerebrospinal fluid of MSA (34), we only obtained negative data for IL-1 β levels in MSA. Our ELISA results indicated that IL-1 β protein expression in MSA brain homogenates are higher than in CTL ones, although the results were not significant due to the large variation in IL-1 β expression levels in MSA cases and the small sample size. Limitations in our study are associated with the samples that we used. First, frozen samples were obtained from the occipital cortex, which is not a commonly affected site in MSA. Although GCIs were also seen in our MSA cases, more commonly affected sites, such as the basal ganglia and cerebellum, are more suitable for WB analysis. Second, we tested the commercially available primary antibodies against IL-1 β on primary cultured cells from rat but did not test them on samples expressing recombinant human IL-1 β . Thus, the examination of IL-1 β in MSA requires further studies, using a larger sample size of frozen human brain tissue and human recombinant proteins.

These neuropathological findings suggest that the NLRP3 inflammasome may be a therapeutic target for neurodegenerative diseases, including MSA. Recently, Claudio et al. reported that autophagy inducers (rapamycin and trehalose) alleviated α -synuclein-induced neuronal death in cultured cells through suppression of microglial pro-inflammatory mediators including IL-1 β (25). NLRP3 is activated in AD mouse models, and knock down of NLRP3 alleviated symptoms in AD mouse models (21). Inflammatory activation of microglia seems to play a pathogenic role in neurodegenerative diseases. Thus, we believe that regulation of microglial NLRP3-inflammasome activation could be a potential therapeutic target for neuronal survival and degeneration in MSA. Future studies using long-term co-cultured cells *or in vivo* models should examine how microglial NLRP3-inflammasome activation influences oligodendrocytes, astrocytes, and neurons over a longer time course.

In conclusion, our study suggests that the microglial NLRP3 inflammasome is associated with abnormal α -synuclein deposition, gliosis, and axonal loss in the putamen of MSA. These findings may provide novel therapeutic strategies to target excessive activation of the inflammasome in MSA.

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Figure legends

Fig. 1. Representative neuropathological findings in age-matched controls (CTL), Parkinson's disease (PD) and multiple system atrophy (MSA) cases. No phosphorylated α -synuclein (p-Syn)-

positive deposition in a CTL case (**A**). Lewy bodies and Lewy neurites in neurons in a PD case (**B**). Glial cytoplasmic inclusions (GCIs) and p-Syn-positive inclusion bodies in neurons in an MSA case (**C**). Tyrosine hydroxylase (TH)-positive fibers remain throughout the putamen in a CTL case (**D**). The number of TH-positive fibers was reduced in a PD case (**E**) and MSA case (**F**). Glial fibrillary acidic protein (GFAP)-positive astrocytes were observed in abundance in an MSA (**I**) case compared to PD (**H**) and CTL (**G**) cases. Scale bars 100 μ m.

Fig. 2. Representative immunohistochemical findings of microglia stained with Cluster of Differentiation 68 (CD68) and Nod-like receptor protein 3 (NLRP3) inflammasome-related antibodies. CD68-positive microglia in age-matched controls (CTL) (**A**) and Parkinson's disease (PD) (**B**) cases were fewer than those in a multiple system atrophy (MSA) case (**C**). The number of NLRP3/Apoptosis-associated speck-like molecule containing CARD domain (ASC)/cysteine aspartic acid protease 1 (Caspase 1)-immunopositive microglia was increased in an MSA case (**F**, **I**, **L**), while no significant changes in CTL (**D**, **G**, **J**) or PD case (**E**, **H**, **K**) were observed. Scale bars 100 μ m.

Fig. 3. Immunofluorescence staining of microglia in the putamen. Confocal microscopy and double immunofluorescence staining using antibodies to a microglia-specific marker, Cluster of Differentiation 68 (CD68) and Nod-like receptor protein 3 (NLRP3)/Apoptosis-associated speck-like molecule containing CARD domain (ASC)/cysteine aspartic acid protease 1 (Caspase 1) confirmed NLRP3 (**A-D**)/ASC (**I-L**)/Caspase 1 (**Q-T**)-immunoreactivity in CD68-positive microglia of an MSA case. Scale bars 20 μ m.

Fig. 4. Mean numbers of phosphorylated α -synuclein (p-Syn)-positive deposits (**A**), tyrosine hydroxylase (TH)-positive fibers (**B**), Cluster of Differentiation 68 (CD68)-positive microglia (**C**), glial fibrillary acidic protein (GFAP)-positive astrocytes (**D**) in control (CTL), Parkinson's disease (PD) and multiple system atrophy (MSA) cases. * $p < 0.05$; ** $p < 0.01$. P values were calculated by one-way analysis of variance (ANOVA) and post hoc least significance difference (LSD) tests.

Fig. 5. Nod-like receptor protein 3 (NLRP3)-positive microglia (**A**), Apoptosis-associated speck-like molecule containing CARD domain (ASC)-positive microglia (**B**), and cysteine aspartic acid protease 1 (Caspase 1)-positive microglia (**C**) in putamen of age-matched controls (CTL) cases,

Parkinson's disease (PD) cases, and multiple system atrophy (MSA) cases. The ratio of NLRP3-positive microglia/Cluster of Differentiation 68 (CD68)-positive microglia **(D)**, ASC positive-microglia/CD68-positive microglia **(E)** and Caspase 1-positive microglia/CD68-positive microglia **(F)** were evaluated to approximate the degree of microglial inflammasome-related protein upregulation. * $p < 0.05$; ** $p < 0.01$. P values were calculated by one-way analysis of variance (ANOVA) and post hoc least significance difference (LSD) tests.

Fig. 6. Representative scatter plots of correlation analysis in all cases are shown. **(A)** phosphorylated α -synuclein (p-Syn)-positive density versus Cluster of Differentiation 68 (CD68)-positive microglia count in the putamina. **(B)** p-Syn-positive density versus tyrosine hydroxylase (TH)-positive fiber density in the putamina. **(C)** CD68-positive microglia count versus TH-positive fibers in the putamina. **(D)** CD68-positive microglia count versus Nod-like receptor protein 3 (NLRP3)-positive microglia in the putamina. **(E)** CD68-positive microglia count versus Apoptosis-associated speck-like molecule containing CARD domain (ASC)-positive microglia in the putamina. **(F)** CD68-positive microglia count versus cysteine aspartic acid protease 1 (Caspase 1)-positive microglia in the putamina. **(G)** p-Syn-positive density count versus NLRP3-positive microglia in the putamina. **(H)** p-Syn-positive density versus ASC-positive microglia in the putamina. **(I)** p-Syn-positive density versus Caspase 1-positive microglia in the putamina. $\square$, age-matched control (CTL) group; $\triangle$, Parkinson's disease (PD) group; $\circ$, multiple system atrophy (MSA) group. * $p < 0.05$; ** $p < 0.01$. P values were calculated with Pearson correlation coefficient analysis.

Fig. 7. A hypothetical model linking Nod-like receptor protein 3 (NLRP3) inflammasome activation to multiple system atrophy (MSA) pathogenesis. Activated microglia in putamen accompanies glial cytoplasmic inclusions (GCIs) and promotes chronic neuroinflammation through upregulation and activation of the NLRP3 inflammasome. Based on previous reports, released proinflammatory cytokines including interleukin 1 β (IL-1 β) and IL18 may cause neuronal death, axonal loss, astrocytic activation, and gliosis. The elements which we used are download and modify from <http://smart.servier.com/>

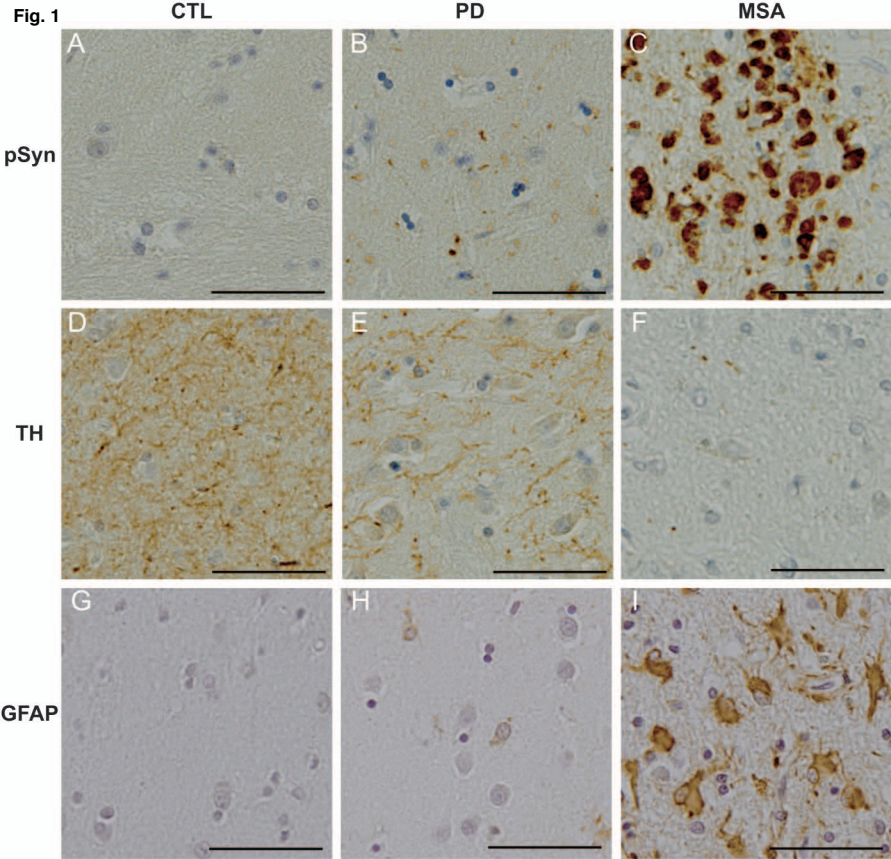
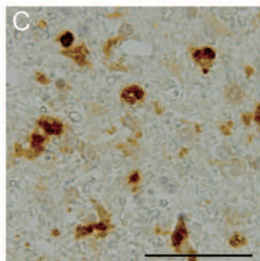
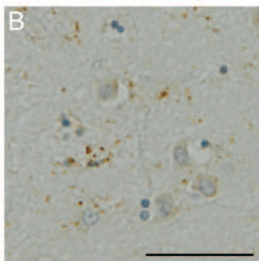
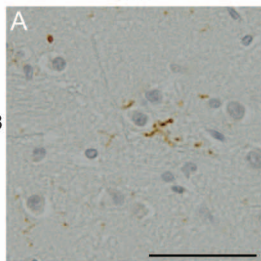
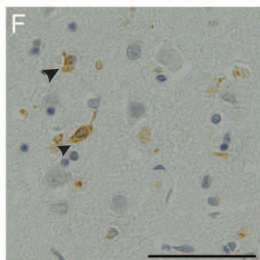
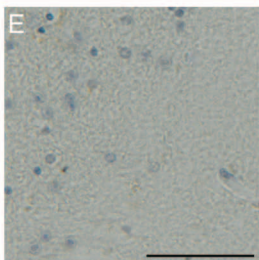
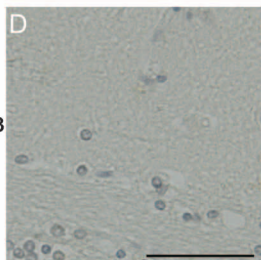


Fig. 2 CTL PD MSA

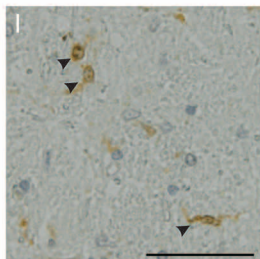
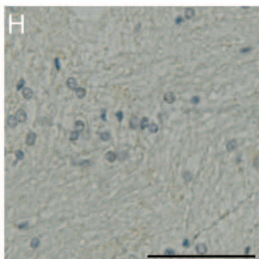
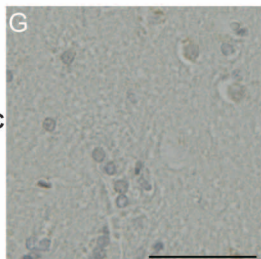
CD68



NLRP3



ASC



Caspase1

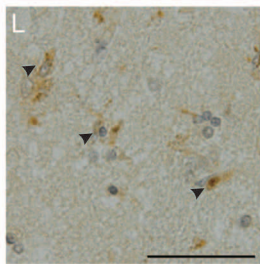
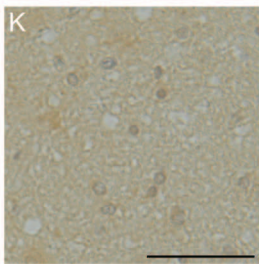
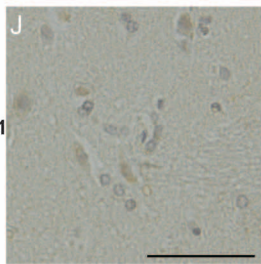
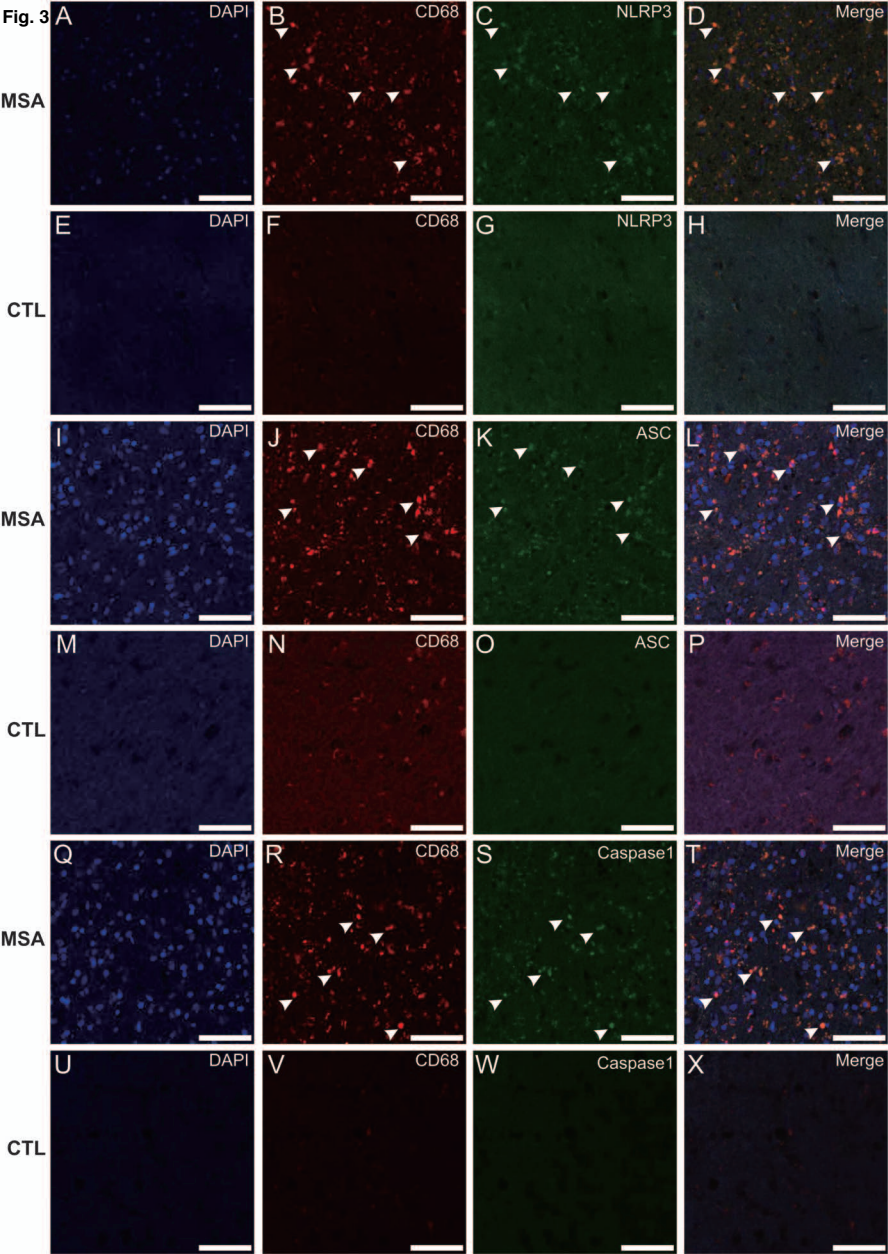


Fig. 3



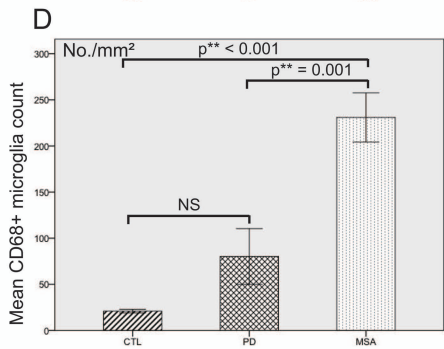
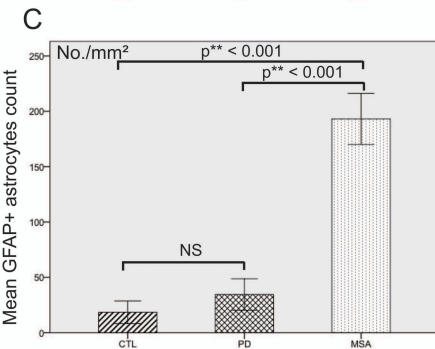
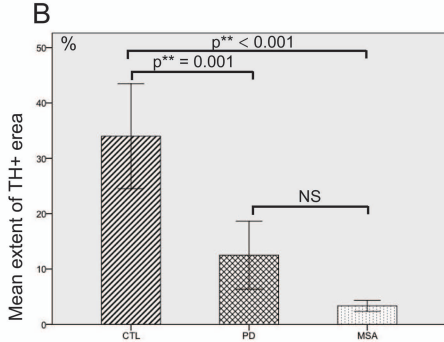
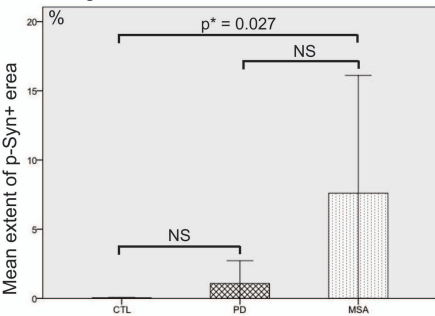
A Fig. 4

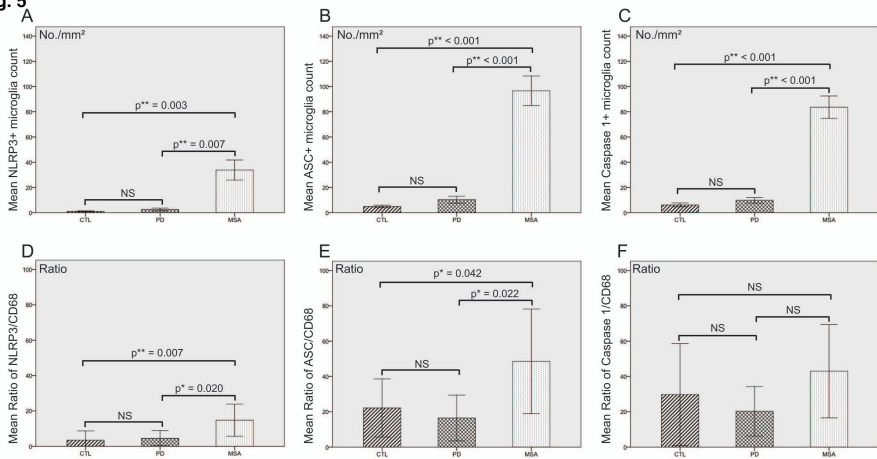
Fig. 5

Fig. 6

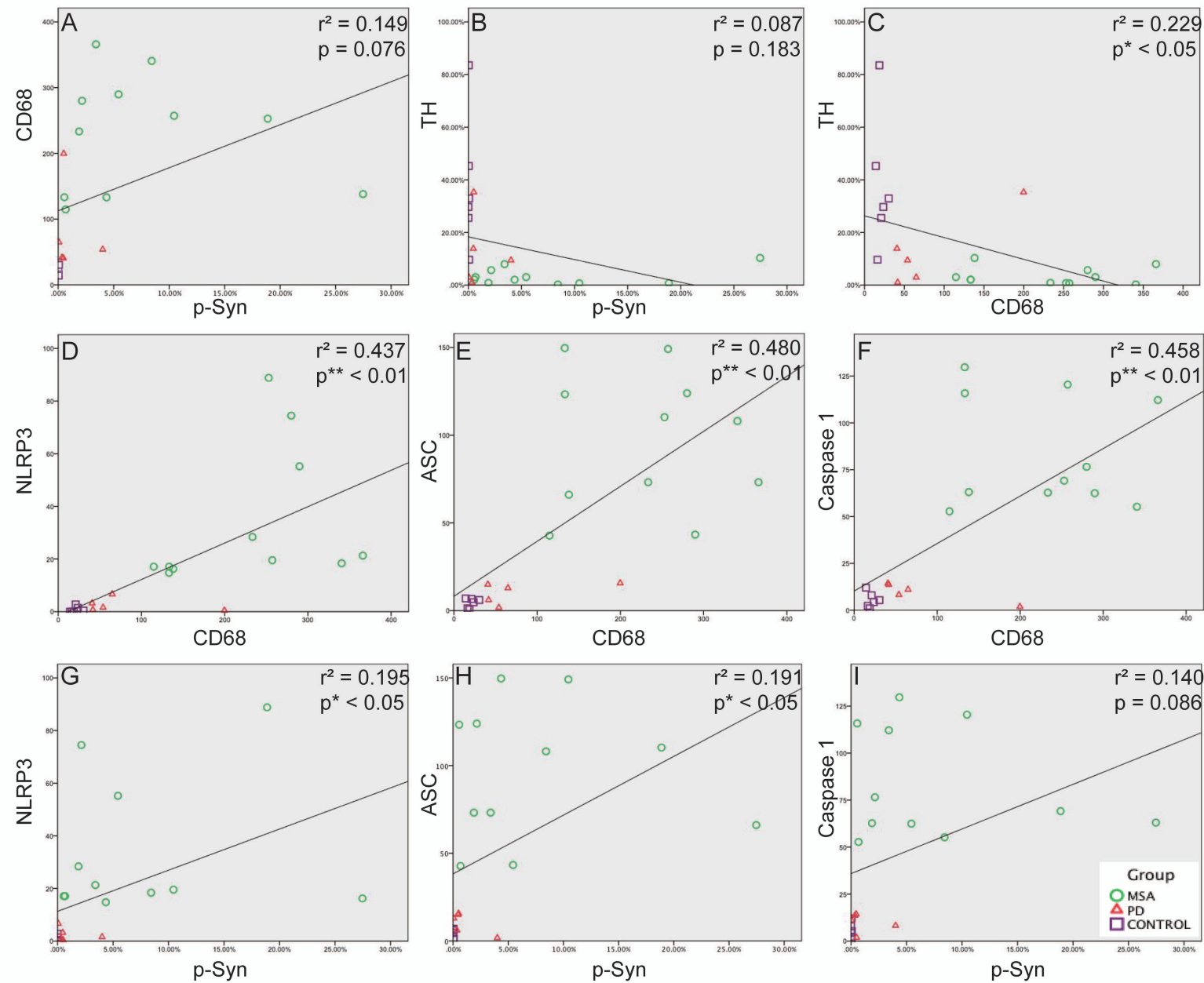
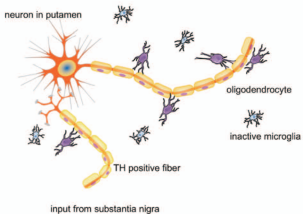
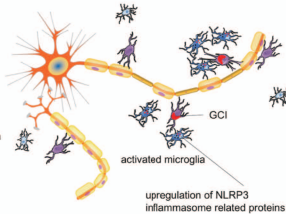


Fig. 7

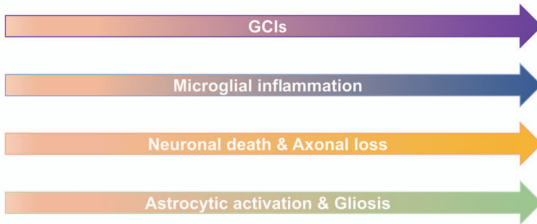
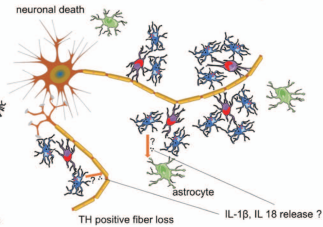
Normal Aging



MSA- early stage



MSA- advanced stage



↗ NLRP3 inflammasome related proteins (NLRP3, ASC, Caspase 1)

↗ IL-1 β , IL-18

Table 1. Case material studied

Case	Diagnosis	Age	Sex	Brain weight (g)	Duration of disease (years)	PMI (hours)
1	MSA-C	71	f	1180	4	8
2	MSA-C	72	f	990	NA	2
3	MSA-C	66	m	1360	3	3.5
4	MSA-C	78	m	1100	2	2
5	MSA-C	60	m	940	6	3
6	MSA-C	66	f	1020	7	1.5
7	MSA-C	76	m	1110	7	3
8	MSA-P	52	f	1300	4	2.5
9	MSA-P	72	f	1205	12	4
10	MSA-P	56	m	1105	2	3
11	MSA-P	88	f	1160	6	13
Sub-total*		68.8 ± 10.3		1133.6 ± 126.7	5.3 ± 3.0	4.0 ± 3.5
12	PD	69	m	1400	9	12
13	PD	66	m	1130	6	2
14	PD	67	m	900	17	3
15	PD	78	f	1090	11	12
16	PD	77	m	1220	11	2
Sub-total*		71.4 ± 5.7		1148.0 ± 182.9	10.8 ± 4.0	6.9 ± 4.8
17	FTLD-TDP	66	f	900		3
18	FTLD-TDP	69	m	1140		2.5
19	ALS	63	f	930		1.5
20	ALS	62	m	1290		1
21	GBS	62	m	1380		3
22	CI	86	f	940		4
Sub-total*		68.0 ± 9.2		1096.7 ± 205.2		2.5 ± 1.1
Total*		69.2 ± 8.9		1126.8 ± 156.3		4.2 ± 3.6

*Average ± standard deviation. Abbreviations: PMI, postmortem interval; MSA-C, multiple system atrophy-cerebellar; MSA-P, multiple system atrophy-parkinsonian; PD, Parkinson's disease; FTLD-TDP, frontotemporal lobar degeneration with TAR DNA binding protein 43kDa (TDP43) pathology;

ALS, amyotrophic lateral sclerosis; GBS, Guillain-Barré syndrome; CI, cerebral infarction.

Table 2. One-way analysis of variance (ANOVA) among MSA, PD and Control group and post hoc least significance difference (LSD) tests between the diagnostic groups

	One-way ANOVA	Post hoc LSD tests- P value		
	F value	MSA vs. PD	MSA vs. CTL	PD vs. CTL
p-Syn	3.62*	0.066	0.027	0.786
TH	10.885**	0.259	0.000	0.010
GFAP	21.653**	0.000	0.000	0.699
CD68	19.018**	0.001	0.000	0.185
NLRP3	7.718**	0.007	0.003	0.882
ASC	27.434**	0.000	0.000	0.738
Caspase 1	34.349**	0.000	0.000	0.745

MSA, multiple system atrophy; PD, Parkinson's disease; CTL, age-matched control; p-Syn, phosphorylated α -synuclein; TH, tyrosine hydroxylase; GFAP, glial fibrillary acidic protein; CD68, Cluster of Differentiation 68; NLRP3, Nod-like receptor protein 3; ASC, apoptosis-associated speck-like molecule containing CARD domain; Caspase 1, cysteine aspartic acid protease 1.

* $p < 0.05$; ** $p < 0.01$. P values were calculated by one-way ANOVA and post hoc LSD tests.

Table 3. Correlation analysis of microglia, astrocytes, and GCI burdens

	p-Syn	TH	GFAP	CD68	NLRP3	ASC	Caspase 1
p-Syn	1	-0.295	-0.476*	0.386	0.441*	0.437*	0.374
TH	-0.295	1	-0.532*	-0.479*	-0.416	-0.533*	-0.545**
GFAP	-0.476*	-0.532*	1	0.804**	0.579**	0.854**	0.837**
CD68	0.386	-0.479*	0.804**	1	0.661**	0.693**	0.677**
NLRP3	0.441*	-0.416	0.579**	0.661**	1	0.585**	0.488*
ASC	0.437*	-0.533*	0.854**	0.693**	0.585**	1	0.921**
Caspase 1	0.374	-0.545**	0.837**	0.677**	0.488*	0.921**	1

GCI, glial cytoplasmic inclusion; p-Syn, phosphorylated α -synuclein; TH, tyrosine hydroxylase; GFAP, glial fibrillary acidic protein; CD68, Cluster of Differentiation 68; NLRP3, Nod-like receptor protein 3; ASC, apoptosis-associated speck-like molecule containing CARD domain; Caspase 1, cysteine aspartic acid protease 1.

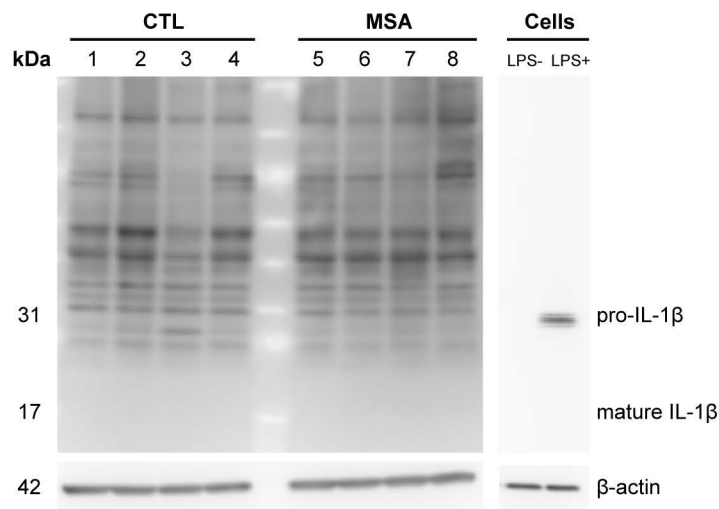
* $p < 0.05$; ** $p < 0.01$. P values were calculated with Pearson correlation coefficient analysis.

Supplementary Table 1. Demographics of all studied cases

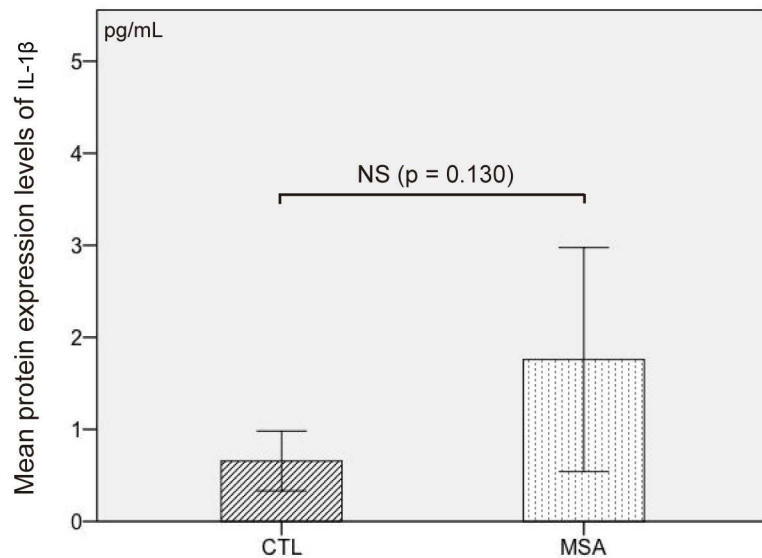
Case	Diagnosis	Age	Sex	Brain weight (g)	Duration of disease (years)	PMI (hours)
1	CI	84	m	1360		3
2	CI	62	m	1135		3
3	CI	87	f	1220		3.5
4	CI	76	m	1100		1.5
Sub-total*		77.3 ± 11.2		1203.8 ± 115.7		2.8 ± 0.9
5	MSA-C	66	f	1020	7	1.5
6	MSA-C	72	f	990	NA	2
7	MSA-C	66	m	1360	3	3.5
8	MSA-C	76	m	1110	7	3
Sub-total*		70.0 ± 4.9		1120.0 ± 167.9	5.7 ± 2.3	2.5 ± 0.9
Total*		73.6 ± 8.9		1161.9 ± 140.8		2.6 ± 0.8

*Average $\pm$ standard deviation. Abbreviations: PMI, postmortem interval; CI, cerebral infarction; MSA-C, multiple system atrophy-cerebellar.

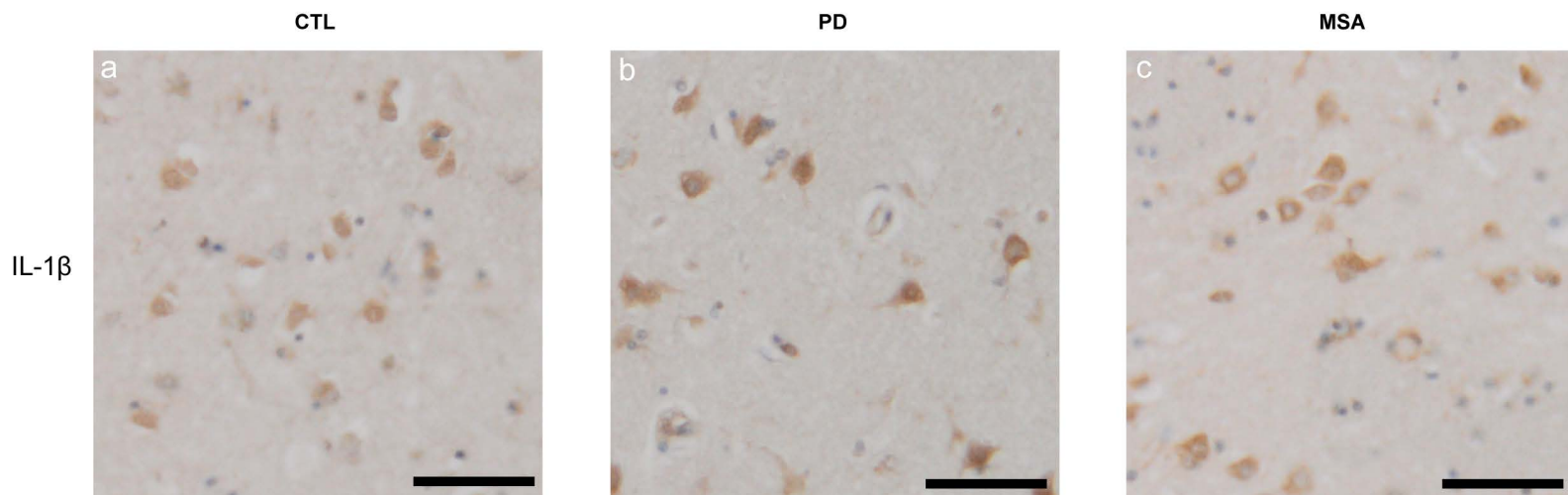
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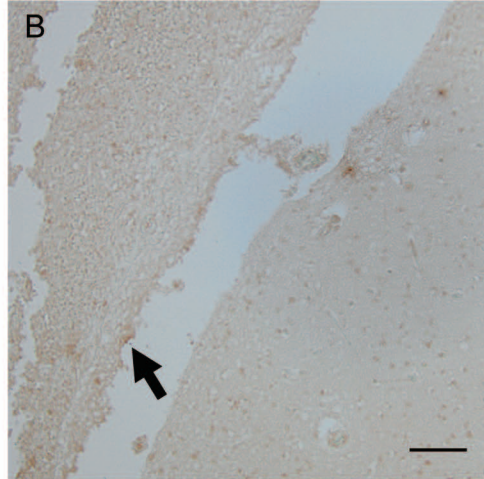
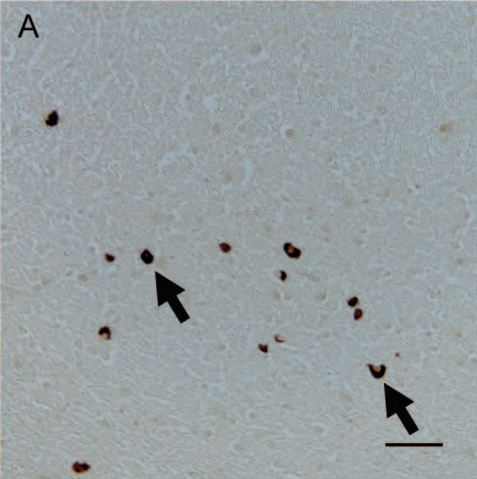
B



C



Supplemental Figure 1: Interleukin 1β (IL-1β) protein expression levels are increased in patients with multiple system atrophy (MSA). (A) Western blot analysis of IL-1β in brain homogenates from four age-matched controls (CTL) cases, four MSA cases, and LPS-untreated and LPS-treated primary microglia. Only pro-IL-1β could be detected in LPS-treated primary microglia. (B) Mean protein expression levels of IL-1β in brain homogenates from four CTL and four MSA cases. NS, not statistically significant. The P value was calculated by an independent sample t-test. (C) Representative neuropathological findings of IL-1β in CTL (a), Parkinson's disease (PD; b), and MSA (c) cases. IL-1β-positive neurons and microglia could be observed in all three cases. Scale bars 50 μm.



Supplemental Figure 2: Representative neuropathological findings. **(A)** phosphorylated α -synuclein-positive glial cytoplasmic inclusions (GCIs) in a sample of occipital cortex from a multiple system atrophy (MSA) case (66-year-old, female). The arrows indicate GCIs. Scale bar 50 μ m. **(B)** Interleukin 1 β positive neurons and macrophages could be readily observed in a bacterial meningitis case (79-year-old, male). The arrow indicates inflammatory infiltration in the meninges. Scale bar 100 μ m.