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### Title

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### Permalink

<https://escholarship.org/uc/item/16q2x34d>

### Journal

Acta Neuropathologica, 135(1)

### ISSN

0001-6322

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### Publication Date

2018

### DOI

10.1007/s00401-017-1762-2

Peer reviewed

## **MSA prions exhibit remarkable stability and resistance to inactivation**

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**Acknowledgments:** We thank the Hunters Point animal facility staff for breeding and caring for the mice used in this study and Martin Ingelsson (Uppsala University) for providing control tissue. This work was supported by grants from the National Institutes of Health (AG002132 and AG031220), as well as by gifts from the Glenn Foundation and Daiichi Sankyo. The Massachusetts Alzheimer's Disease Research Center is supported by the National Institutes of Health (AG005134); the Parkinson's UK Brain Bank at Imperial College London is funded by Parkinson's UK, a charity registered in England and Wales (948776) and in Scotland (SC037554); and the Sydney Brain Bank is supported by Neuroscience Research Australia and the University of New South Wales. Glenda M. Halliday is a National Health and Medical Research Council of Australia Senior Principal Research Fellow (1079679).

**Key words (4–6):**  $\alpha$ -synuclein; neurodegeneration; propagation; proteinopathies; transmission models

## ABSTRACT

In multiple system atrophy (MSA), progressive neurodegeneration results from the protein  $\alpha$ -synuclein misfolding into a self-templating prion conformation that spreads throughout the brain. MSA prions are transmissible to transgenic (Tg) mice expressing mutated human  $\alpha$ -synuclein (TgM83<sup>+/-</sup>), inducing neurological disease following intracranial inoculation with brain homogenate from deceased patient samples. Noting the similarities between  $\alpha$ -synuclein prions and PrP<sup>Sc</sup> prions responsible for Creutzfeldt–Jakob disease (CJD), we investigated MSA transmission under conditions known to result in PrP<sup>Sc</sup> transmission. When peripherally exposed to MSA via the peritoneal cavity, hind leg muscle, and tongue, TgM83<sup>+/-</sup> mice developed neurological signs accompanied by  $\alpha$ -synuclein prions in the brain. Iatrogenic CJD, resulting from prion adherence to surgical steel instruments, has been investigated by incubating steel sutures in contaminated brain homogenate before implantation into mouse brain. Mice studied using this model develop disease, whereas wire incubated in control homogenate had no effect on the animals. Notably, formalin fixation does not inactivate  $\alpha$ -synuclein prions. Fixed MSA patient samples also transmitted disease to TgM83<sup>+/-</sup> mice, even after incubating in fixative for 244 months. Finally, at least 10% sarkosyl was found to be the concentration necessary to partially inactivate MSA prions. These results demonstrate the robustness of  $\alpha$ -synuclein prions to denaturing conditions. Moreover, they establish the parallel characteristics between PrP<sup>Sc</sup> and  $\alpha$ -synuclein prions, arguing that clinicians should exercise caution when working with materials that could contain  $\alpha$ -synuclein prions to prevent disease transmission.

## INTRODUCTION

Despite the steady accumulation of evidence arguing that most neurodegenerative diseases are caused by prions, most of these diseases have not transmitted neurological disease to rodents, with the exception of the PrP<sup>Sc</sup> diseases (reviewed in [21]). In contrast, brain homogenate from deceased multiple system atrophy (MSA) patients readily transmit neurodegeneration to transgenic mice expressing mutant (A53T)  $\alpha$ -synuclein (TgM83<sup>+/-</sup>) [51]. This finding provided the opportunity to determine if  $\alpha$ -synuclein adopts an alternative conformation that undergoes self-propagation, i.e., it becomes a prion. Testing this hypothesis, we found that 19 MSA patient samples from three continents transmitted diseases to the TgM83<sup>+/-</sup> mice, but six Parkinson's disease (PD) patient samples from two continents did not [40]. Moreover, using human embryonic kidney (HEK293T) cells expressing mutated  $\alpha$ -synuclein fused to yellow fluorescent protein ( $\alpha$ -syn140\*A53T-YFP), we also found that we could infect the cells with  $\alpha$ -synuclein prions isolated from MSA patient samples but not from PD patient samples [53]. The existence of two distinct strains of  $\alpha$ -synuclein prions in MSA and PD patients is consistent with work published by others demonstrating that  $\alpha$ -synuclein misfolding into distinct conformations produces differing physiological effects [5, 37].

To determine if these putative  $\alpha$ -synuclein prions exhibit properties similar to those observed for PrP<sup>Sc</sup>, we investigated the ability of MSA to transmit disease to TgM83<sup>+/-</sup> mice following peripheral inoculation. Using four MSA patient samples, we found both intraperitoneal (ip) and intramuscular (im) inoculations resulted in disease transmission. In addition, analogous to PrP<sup>Sc</sup>, stainless steel wires were incubated with



brain homogenate from MSA patients, and the contaminated wires transmitted disease to TgM83<sup>+/-</sup> mice following permanent insertion into the brain. We also investigated the resistance of  $\alpha$ -synuclein prions to formalin inactivation as well as to the denaturing detergent sarkosyl. MSA patient samples that were fixed in formalin up to 244 months (more than 20 years) transmitted disease to the inoculated mice. Furthermore, measuring prion infectivity in the  $\alpha$ -syn140\*A53T-YFP cells, up to 10% sarkosyl was needed to partially denature MSA prions.

MSA is a neurodegenerative disease resulting in the accumulation of glial cytoplasmic inclusions (GCIs) in oligodendrocytes [36]. With an incidence rate of 0.6 to 0.7 per 100,000 individuals annually [13, 45], MSA is characterized by rapidly progressing autonomic dysfunction and motor deficits presenting as either cerebellar symptoms or parkinsonism [22]. Despite the identification of GCIs as the neuropathological hallmark of MSA in 1989 [36],  $\alpha$ -synuclein was not recognized as the primary protein component until 1998 [44, 49, 50]. Nearly 20 years later,  $\alpha$ -synuclein was shown to misfold into a prion conformation capable of self-templating in MSA patient samples, and it is now recognized as the second neuropathogenic prion [40, 51].

Deep brain stimulation (DBS) has widely been used for the last 20 years to ameliorate motor symptoms, including tremors and rigidity, in PD patients. MSA patients, who are typically unresponsive to DBS [34], are most often misdiagnosed with PD [18, 24, 27, 35, 52] and may receive DBS as a treatment. In addition, DBS is increasingly being used to help patients with a number of disorders including major depression [1, 30], obsessive-compulsive disorder [14, 30], Tourette syndrome [10], and schizophrenia [9]. Accidental iatrogenic transmission of CJD previously occurred when

DBS depth electrodes were reused without proper decontamination [3, 20]. Given the growing use of DBS as a therapeutic intervention, the findings reported here demonstrate the need for further research into decontamination methods for surgical equipment that may become contaminated with  $\alpha$ -synuclein prions. These studies will be critical to minimize the risk of lateral transmission of MSA, particularly in light of the ability of  $\alpha$ -synuclein prions to transmit disease to the central nervous system (CNS) following peripheral exposure.

## RESULTS

### MSA prions transmit intraperitoneally

Previously, we used the TgM83<sup>+/-</sup> mouse model, which expresses human  $\alpha$ -synuclein with the familial A53T mutation under the prion protein (*Prnp*) promoter [19], to test the ability of MSA patient samples to transmit disease [40, 51, 53]. Following ic inoculation, brain homogenate from 14 MSA patient samples induced neurological disease and  $\alpha$ -synuclein neuropathology in this mouse model. Notably, while ic inoculation induced disease in both hemizygous and homozygous TgM83 animals, TgM83<sup>+/+</sup> mice develop spontaneous disease, whereas TgM83<sup>+/-</sup> mice do not [51]. In these hemizygous animals, inoculation with control patient samples had no effect [40]. Importantly, inoculation using  $\alpha$ -synuclein aggregates isolated from patient sample MSA14 following nucleic acid digestion with benzonase induced synucleinopathy in the TgM83<sup>+/-</sup> mice, demonstrating the transmission of neurological disease is a result of  $\alpha$ -synuclein prions present in the brains of MSA patients [53].

Given the observed similarities between  $\alpha$ -synuclein and PrP<sup>Sc</sup> prions, as well as the ability of PrP<sup>Sc</sup> to transmit disease via ip inoculation [26], we compared the transmissibility of four MSA patient samples (basal ganglia from MSA6 and substantia nigra including the surrounding midbrain from MSA12, MSA13, and MSA18) with one control patient sample (C2) following both ic (30  $\mu$ L 1% brain homogenate; Online Resource, Fig. S1) and ip (100  $\mu$ L 1% brain homogenate; Fig. 1a) inoculation. While ic inoculation of the four MSA samples transmitted disease to all inoculated TgM83<sup>+/-</sup> mice ( $P < 0.0001$ ), the MSA18 sample showed a reduced titer with a longer incubation period. This observation was consistent with ip inoculation, where all four cases again transmitted disease ( $P < 0.0001$ ), but MSA18 resulted in the longest incubation period and only transmitted disease to 2 of the 8 inoculated mice by 450 days post inoculation (dpi). Intraperitoneal inoculation using brain homogenate from patient C2 had no effect on the TgM83<sup>+/-</sup> mice by 450 dpi.

In earlier studies, we analyzed brain samples from TgM83<sup>+/-</sup> mice using the  $\alpha$ -syn140\*A53T-YFP cell assay, which expresses full-length human  $\alpha$ -synuclein with the A53T mutation fused to yellow fluorescent protein [53]. Infection in these cells is quantified by measuring the total fluorescence in the aggregates divided by the cell count ( $\times 10^3$  arbitrary units, A.U.). Infected cells emit a fluorescence measurement above  $10 \times 10^3$  A.U., whereas uninfected cells yield lower values. To confirm transmission of  $\alpha$ -synuclein prions from the MSA patient samples to the ip inoculated TgM83<sup>+/-</sup> mice, we tested the mouse brains in the  $\alpha$ -syn140\*A53T-YFP cell assay. The 10% brain homogenates were prepared from the frozen half-brains collected when the inoculated TgM83<sup>+/-</sup> mice developed disease (Fig. 1b). The  $\alpha$ -synuclein prions were

isolated using sodium phosphotungstic acid (PTA) following digestion of all nucleic acids by benzonase [42, 53], and the resulting protein pellets were incubated with the cells for 4 d prior to analysis. Following inoculation with one of the four MSA patient samples, brains from mice that developed neurological signs contained  $\alpha$ -synuclein prions (closed circles), whereas brains from mice that died as a result of other causes (triangles) did not. Most mice terminated at 450 dpi, including the C2-inoculated mice, did not contain  $\alpha$ -synuclein prions (open circles); however, one asymptomatic mouse inoculated with MSA12 contained  $\alpha$ -synuclein prions. Compared to C2-inoculated animals, brains from mice ip inoculated with MSA6, MSA12, and MSA13 contained significantly more  $\alpha$ -synuclein prions ( $P < 0.01$ ,  $P < 0.05$ , and  $P < 0.01$ , respectively).

Fixed half-brains collected from mice that tested positive for  $\alpha$ -synuclein prions, as well as C2-inoculated control animals, were processed and embedded in paraffin. Sections of 8  $\mu\text{m}$  thickness were cut and stained for phosphorylated  $\alpha$ -synuclein, astrogliosis (visualized using the glial fibrillary acidic protein, or GFAP, antibody), p62, and ubiquitin (Fig. 1c–e, S2). Phosphorylated  $\alpha$ -synuclein neuropathology was measured as the percent area of each brain region positive for immunostaining. Measurements were made in the hippocampus (HC), thalamus (Thal), hypothalamus (HTH), midbrain (Mid), and pons and were averaged from two adjacent sections from each animal. The Thal, HTH, Mid, and pons all contained phosphorylated  $\alpha$ -synuclein pathology following ip inoculation with all four MSA cases (Fig. 1c). MSA6 and MSA13 both induced significant pathological changes in the HC, but the magnitude of change compared to C2-inoculated mice was negligible. Notably, the  $\alpha$ -synuclein neuropathology was localized to neurons within the affected areas as a result of the

neuronal *Prnp* promoter used to express the transgene. The accumulation of phosphorylated  $\alpha$ -synuclein was accompanied by an increase in astrogliosis, as demonstrated in the brainstem (Fig. 1d, e). The two proteins p62 and ubiquitin are known to play a role in the degradation of aggregated protein in the proteinopathies and are often co-localized with  $\alpha$ -synuclein in the GCIs present in MSA patients [29, 32, 33]. Both p62 (Online Resource, Fig. S2a, b) and ubiquitin (Online Resource, Fig. S2c, d) co-localized with phosphorylated  $\alpha$ -synuclein in MSA-inoculated animals (merge shown in white in bottom panels of b, d; magnified view of co-localization shown from inset).

Finally, the frozen half-brains from two mice individually inoculated with each homogenate were tested for the presence of detergent-insoluble  $\alpha$ -synuclein. Protein aggregates were extracted from the 10% brain homogenate prepared from each mouse using 0.5% Nonidet P-40 (NP-40) and 0.5% sodium deoxycholate (DOC). The resulting protein pellets were resuspended in 1 $\times$  Dulbecco's phosphate-buffered saline (DPBS), and phosphorylated  $\alpha$ -synuclein was visualized by immunoblot (Fig. 1f). Consistent with the cell assay and neuropathology data, mice inoculated with the MSA patient samples developed phosphorylated  $\alpha$ -synuclein aggregates, but mice inoculated with C2 brain homogenate did not.

### **MSA prions transmit intramuscularly via the hind leg and tongue**

PrP<sup>Sc</sup> prions have been shown to transmit neurological disease to animals following a number of inoculation routes, including im inoculation into the hind leg and tongue [2, 47]. To test the ability of  $\alpha$ -synuclein prions to transmit neurological disease to mice following im exposure, we injected 5  $\mu$ L of a 1% brain homogenate from C2 and MSA6,

MSA12, MSA13, and MSA18 patient samples into TgM83<sup>+/-</sup> mice (Fig. 2, S3). Inoculation into the thigh transmitted neurological disease more efficiently than the tongue inoculation (Fig. 2a, b). However, inoculation of MSA patient samples via both routes transmitted disease (thigh,  $P < 0.0001$ ; tongue,  $P < 0.01$ ), whereas the C2 sample did not. To confirm the transmission of MSA, frozen half-brains from the inoculated mice were tested in the  $\alpha$ -syn140\*A53T-YFP cell assay (Fig. 2c, d). Mice inoculated into the thigh with MSA6, MSA12, and MSA13 samples contained significantly more  $\alpha$ -synuclein prions in their brains compared to C2-inoculated mice ( $P < 0.0001$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively). Although MSA18-inoculated mice were not significantly different from control-inoculated animals, all mice inoculated with MSA samples that showed neurological signs tested positive for  $\alpha$ -synuclein prions (closed circles). Healthy mice collected at 450 dpi tested negative for MSA prions (open circles). Only mice inoculated with MSA6 into the tongue contained significantly more  $\alpha$ -synuclein prions than the C2-inoculated mice ( $P < 0.01$ ), but a more thorough assessment of the data again shows a strong association between neurological symptoms and robust levels of  $\alpha$ -synuclein prions, as measured by the cell assay. Overall, the observed differences in the cell infectivity of the MSA patient samples after im inoculation into the thigh (Fig. 2a) and the tongue (Fig. 2b) are consistent with the infectivity differences seen between MSA18 and the other three MSA patient samples following ic inoculation (Online Resource, Fig. S1).

Phosphorylated  $\alpha$ -synuclein neuropathology was quantified in the brains of mice containing  $\alpha$ -synuclein prions following im inoculation, as well as C2-inoculated animals (Fig. 2e, f). Assessing the effect of im inoculation on  $\alpha$ -synuclein aggregation, we found

C2-inoculated mice contained no immunostaining, while the four MSA patient samples induced phosphorylated  $\alpha$ -synuclein aggregation in the Thal, HTH, Mid, and pons, regardless of whether mice were inoculated in the thigh (Fig. 2e) or the tongue (Fig. 2f). MSA13 and MSA6 induced significantly more phosphorylated  $\alpha$ -synuclein accumulation in the HC than the control mice following thigh or tongue inoculation, respectively, but the difference in magnitude was negligible. These increases in  $\alpha$ -synuclein deposition following im inoculation were accompanied by an increase in astrogliosis, which was not seen in C2-inoculated animals (Online Resource, Fig. S3a, b). Additionally,  $\alpha$ -synuclein co-localized with p62 and ubiquitin following im inoculation into both the thigh and the tongue (Online Resource, Fig. S3).

Frozen half-brains from these same animals were analyzed by immunoblot for the presence of detergent-insoluble  $\alpha$ -synuclein (Fig. 2g, h). Both routes of inoculation with MSA homogenate induced accumulation of insoluble phosphorylated  $\alpha$ -synuclein in the brains of TgM83<sup>+/-</sup> mice, but mice inoculated with the control sample did not contain  $\alpha$ -synuclein aggregates (Fig. 2g, h).

### **MSA prions adhere to stainless-steel wires**

Multiple studies have assessed the ability of PrP<sup>Sc</sup> prions to adhere to surgical stainless steel. After incubating steel suture in brain homogenate containing PrP<sup>Sc</sup> prions, both permanent and transient insertion of contaminated wire into the brain of an animal resulted in disease transmission [15, 38, 54, 55]. Due to cases of iatrogenic Creutzfeldt–Jakob disease (CJD) that resulted from improperly sterilized neurosurgical instruments after use on an asymptomatic CJD patient [21, 39, 46], this assay has been particularly

important for studying the conditions required for the denaturation of PrP<sup>Sc</sup>. To explore additional parallels in disease transmission between PrP<sup>Sc</sup> and  $\alpha$ -synuclein prions, we tested the ability of  $\alpha$ -synuclein prions in MSA patient samples to adhere to stainless-steel suture. 3-0 steel suture was cut into 4 mm pieces, which were incubated in 10% brain homogenate from C2 and MSA6, MSA12, MSA13, and MSA18 patient samples for 16 h at room temperature. The wires were washed in DPBS five times for 10 min each and were allowed to air-dry in a biosafety cabinet, as described [38]. One wire was then permanently implanted into the right hemisphere of each TgM83<sup>+/-</sup> mouse.

Wires incubated in C2 brain homogenate did not transmit disease to the mice, and the animals did not show any other deficits. In contrast, wires incubated in homogenate from MSA6, MSA12, and MSA13 samples transmitted neurological symptoms to TgM83<sup>+/-</sup> mice ( $P < 0.001$ ; Fig. 3a) with a substantially longer incubation period than observed after ic inoculation with the same samples (Online Resource, Fig. S1). Consistent with the lower titer observed for patient sample MSA18 in previous experiments, only one mouse implanted with wires incubated in MSA18 developed neurological signs. Testing the brains of all mice in the  $\alpha$ -syn140\*A53T-YFP cell assay showed that the mice implanted with wire incubated with MSA samples were infected with  $\alpha$ -synuclein prions (Fig. 3b). The number of  $\alpha$ -synuclein prions in the brains of mice implanted with MSA6- and MSA13-incubated wires was significantly greater compared to control animals ( $P < 0.01$  and  $P < 0.05$ , respectively). Brains from mice that tested positive for  $\alpha$ -synuclein prions also showed significant phosphorylated  $\alpha$ -synuclein accumulation in the Thal, HTH, Mid, and pons (Fig. 3c) accompanied by an increase in astrogliosis in comparison to control animals (Fig. 3d, e). The MSA-induced  $\alpha$ -synuclein



aggregates co-localized with both p62 and ubiquitin (Online Resource, Fig. S4). These brain samples were also assessed for the presence of detergent-insoluble  $\alpha$ -synuclein (Fig. 3f). Mice implanted with MSA-coated wire developed phosphorylated  $\alpha$ -synuclein aggregates, but C2-incubated wires did not induce  $\alpha$ -synuclein accumulation.

### **Formalin fixation does not inactivate MSA prions**

It is well established that formalin fixation does not inactivate PrP<sup>Sc</sup> prions. The earliest report examined sheep inoculated with a vaccine for the louping ill RNA virus but subsequently developed scrapie two years after vaccination [23]. The vaccine used 0.35% formalin to inactivate the RNA virus in brain homogenate from sheep that had contracted louping ill. However, one batch of the virus was made from sheep that had also developed scrapie; as the formalin did not inactivate PrP<sup>Sc</sup> prions in the homogenate, the sheep receiving the vaccine developed disease. To assess the ability of formalin to inactivate  $\alpha$ -synuclein prions in MSA patient samples, we homogenized formalin-fixed tissue from one control patient sample (C23; substantia nigra) and seven MSA patient samples (substantia nigra from MSA2 and MSA3; pons from MSA9, MSA10, MSA11, MSA27, and MSA28) and inoculated 30  $\mu$ L of a 1% homogenate into TgM83<sup>+/-</sup> mice. While mice inoculated with C23 did not develop neurological signs, mice inoculated with MSA patient samples developed disease ( $P < 0.0001$ ; Fig. 4a).

To confirm these neurological signs were a result of  $\alpha$ -synuclein prion transmission, we tested the brains from the MSA-inoculated mice in the  $\alpha$ -syn140\*A53T-YFP cell assay and found they contained significantly more  $\alpha$ -synuclein prions than mice inoculated with control patient tissue (MSA2:  $P < 0.0001$ ; MSA3,

MSA9, MSA27:  $P < 0.01$ ; MSA10, MSA28:  $P < 0.05$ ; MSA11:  $P < 0.05$ ; Fig. 4b). We assessed neuropathological accumulation of phosphorylated  $\alpha$ -synuclein in the brains of mice containing MSA prions (Online Resource, Fig. S5). Compared to C23-inoculated animals, MSA inoculation induced robust  $\alpha$ -synuclein immunostaining in the Thal, HTH, Mid, and pons (Online Resource, Fig. S5a). Six of the seven MSA patient samples also induced significant accumulation in the HC; however, the magnitude of change from the control was biologically insignificant. This neuropathology was accompanied by an increase in astrogliosis (Online Resource, Fig. S5b) and was co-localized with p62 and ubiquitin immunostaining as well (Online Resource, Fig. S5b, c). Detergent-insoluble, high molecular weight phosphorylated  $\alpha$ -synuclein aggregates were also detected in the brains of MSA-inoculated animals but were not detected in C23-inoculated mice, as shown by immunoblot (Online Resource, Fig. S5d).

Finally, we compared the length of time the MSA patient samples spent in formalin with the disease incubation times in TgM83<sup>+/-</sup> mice to assess if the amount of time a sample spent in fixative affected its infectivity, or titer (Fig. 4c). Comparing patient sample MSA28, which spent 16 months in formalin, with patient sample MSA27, which spent 244 months (or more than 20 years) in formalin, we observed no differences in incubation times, indicating that formalin does not inactivate  $\alpha$ -synuclein prions in MSA patients, regardless of the fixation time.

### **Extraction in 10% sarkosyl partially denatures MSA prions**

Following the discovery that formalin fixation does not inactivate  $\alpha$ -synuclein prions in MSA patient samples, we tested their resistance to denaturation by extraction with

increasing concentrations of sarkosyl. It is known that extracting brain homogenate in 2% sarkosyl during PTA precipitation increases the density of PrP<sup>Sc</sup> prions isolated from a sample [31]. Moreover, the addition of 2% sarkosyl to MSA patient samples also enables isolation of  $\alpha$ -synuclein prions using PTA [53]. However, the stability of  $\alpha$ -synuclein prions to higher concentrations of sarkosyl has been previously unexplored. Using two MSA patient samples (MSA10 and MSA13) and two mice individually inoculated with these samples, we altered the PTA precipitation protocol to extract  $\alpha$ -synuclein prions with increasing concentrations of sarkosyl, ranging from 2–20%. PTA was used to precipitate the remaining protein aggregates after extraction, and the isolated protein pellets were tested in the  $\alpha$ -syn140\*A53T–YFP cell assay (Fig. 5a). While all six samples showed at least partial resistance to denaturation in up to 8% sarkosyl, most of the infectivity measured in the cell assay was eliminated by extraction in 10% sarkosyl. Hypothesizing that increasing the detergent concentration resulted in the degradation of  $\alpha$ -synuclein prions into monomeric protein, we examined the total protein present in each sample by silver stain (Fig. 5b). In the lower dilution samples, a 15 kDa band coinciding with  $\alpha$ -synuclein monomer was absent, but 15 kDa bands became increasingly abundant for the two human and four mouse samples as the sarkosyl concentration increased (red arrows). To confirm the 15 kDa band was monomeric  $\alpha$ -synuclein, we analyzed the mouse samples via immunoblot using the MJFR1 total  $\alpha$ -synuclein antibody (Fig. 5c). A weak monomeric band at 15 kDa became increasingly more abundant as the sarkosyl concentration increased, starting at 10% sarkosyl, corresponding with the cell infection and silver stain data. These blots

confirmed that the denaturation of  $\alpha$ -synuclein prions in the samples resulted in an increase in monomeric protein.

## **DISCUSSION**

Increasing evidence indicates that in patients with MSA,  $\alpha$ -synuclein misfolds into a prion conformation and spreads throughout the brain causing progressive neurodegeneration. The biochemical parallels between  $\alpha$ -synuclein and PrP<sup>Sc</sup> led us to compare the transmissibility of the two prions by assessing the infectiousness of  $\alpha$ -synuclein prions in MSA patient samples using transmission paradigms previously reported for PrP<sup>Sc</sup>. While iatrogenic transmission of MSA has not been identified, the demonstrated ability of  $\alpha$ -synuclein prions to adhere to stainless-steel wires and resist formalin inactivation warrants investigation into the possibility of lateral disease transmission, particularly given the ability of  $\alpha$ -synuclein prions to affect the CNS after peripheral exposure.

Several studies have used wild-type and Tg mouse models to demonstrate peripheral transmission of proteinopathies giving rise to CNS disease [2, 26, 47] and protein accumulation [8, 12]. Along these lines, previous work using fibrillized recombinant  $\alpha$ -synuclein demonstrated that ip and intraglossal inoculation [6] and im inoculation into the hind leg [41] induced neurological signs and  $\alpha$ -synuclein pathology in TgM83 mice. Testing MSA brain homogenate for the ability to transmit disease following systemic exposure, we compared ic inoculation (30  $\mu$ L) with ip (100  $\mu$ L) and im (5  $\mu$ L) inoculations using 1% homogenate prepared from four MSA patient samples. While three of the patient samples produced similar incubation times following ic inoculation, patient sample MSA18 yielded a longer incubation time, likely due to

reduced titer. This difference in infectivity between MSA18 and the other MSA patient samples was seen across the three peripheral transmission paradigms tested. Both ip and im inoculation into the hind leg produced extended incubation times compared to ic inoculation, but the relatively longer im incubation times are likely attributable to the small volume used. Intraglossal inoculation was the least efficient transmission route, despite proximity to the CNS. This was consistent with data reported by Breid et al. who found that only one of five mice inoculated with recombinant  $\alpha$ -synuclein fibrils into the tongue developed disease [6].

Iatrogenic transmission of CJD has occurred from the incomplete sterilization of PrP<sup>Sc</sup> prions adhered to stainless-steel surgical instruments after use on an asymptomatic CJD patient [39, 46]. To model this transmission in animals, stainless-steel suture incubated in brain homogenate containing PrP<sup>Sc</sup> prions was permanently implanted into the brain [55]. After determining permanent insertion of the wires transmitted disease to the animals, transient insertion models were designed to better replicate surgical conditions. These studies found that PrP<sup>Sc</sup> transmission occurred even when wires were inserted into the brain for only 15 min [15]. Likewise, wires incubated in brain homogenate from aged TgAPP23 mice, a model for  $\beta$ -amyloid (A $\beta$ ) accumulation in Alzheimer's disease (AD), and implanted in young TgAPP23 mice induced plaque formation [11]. These results, along with ip transmission of A $\beta$  plaques to TgAPP23 mice [12], led the authors to suggest iatrogenic human transmissions may be concealed by longer incubation times than are seen with CJD patients [11]. To test the possibility of lateral  $\alpha$ -synuclein prion transmission via contaminated neurosurgical instruments, stainless-steel wires were incubated with brain homogenate from four MSA

patient samples and one control sample. Mice implanted with MSA-incubated wires developed neurological signs, while control wires implanted in TgM83<sup>+/-</sup> mice had no effect. Notably, these studies were performed using permanently implanted wires; studies testing the transmissibility of MSA prions following transient insertion have not been performed.

After using peripheral inoculations and wire implants to transmit disease, we tested brain samples from the TgM83<sup>+/-</sup> mice in the  $\alpha$ -syn140\*A53T-YFP cell assay [53] to confirm the apparent neurological signs were caused by  $\alpha$ -synuclein prions. Brain samples from control-inoculated mice terminated 450 dpi did not infect the cells. However, brain homogenate from MSA-inoculated animals induced robust  $\alpha$ -synuclein aggregation, demonstrating that both peripheral and wire transmissions resulted in  $\alpha$ -synuclein prion propagation in TgM83<sup>+/-</sup> mice. Brains from sick animals also contained detergent-insoluble  $\alpha$ -synuclein aggregates and phosphorylated  $\alpha$ -synuclein neuropathology that co-localized with p62 and ubiquitin. Notably, regardless of exposure method, inoculation with MSA samples induced neuropathology in the same brain regions (Thal, HTH, Mid, and pons). These findings could be explained either by the specific  $\alpha$ -synuclein prion strain in MSA patients inducing consistent pathology in the mice or by the neuron-specific promoter driving transgene expression in the model, neither of which was investigated here.

The unusual resistance of PrP<sup>Sc</sup> prions to formalin fixation [23], a method used to inactivate viruses for vaccine production, has prompted other groups to examine the effect of formalin on additional misfolded proteins, including A $\beta$  prions in AD patient samples [16]. Brain homogenate, prepared from two AD patient samples after fixation in

formalin for 2 years, inoculated into the hippocampus of TgAPP23 mice induced A $\beta$  plaque deposition within 4 months. Resistance to formalin inactivation was also demonstrated for  $\alpha$ -synuclein prions using Tg mouse samples. Formalin-fixed brains from spontaneously symptomatic mice expressing human  $\alpha$ -synuclein with the familial A30P mutation were shown to transmit disease to younger Tg mice [43]. Here, we found that seven formalin-fixed MSA patient samples also transmitted synucleinopathy to TgM83<sup>+/-</sup> mice, but inoculation with a fixed control sample had no effect. We compared the incubation times in the animal bioassay with the length of time five of the samples spent in formalin and found no correlation; remarkably, disease transmission time using a sample that spent 16 months in formalin was almost identical to disease transmission using a sample that spent 244 months in formalin. These data indicate that more than 20 years in fixative does not reduce the titer of  $\alpha$ -synuclein prions in an MSA sample any more than approximately one year in fixative.

Given the robust stability of  $\alpha$ -synuclein prions, it is critical to identify methods for denaturing and inactivating the protein to prevent human-to-human transmission. Work by two groups has measured residual protein following exposure to denaturing conditions using two different methods. In the first method, Thomzig et al. used a Western blot to show recoverable protein levels from a stainless-steel grid coated with human  $\alpha$ -synuclein after exposure to several harsh alkaline treatments [48]. In the second, Bousset et al. labelled synthetic  $\alpha$ -synuclein fibrils with a fluorescent tag prior to spotting the fibrils onto a number of laboratory surfaces, including glass and steel. After washing with various solutions, the residual protein was measured by fluorescence [4]. In both reports, the authors aimed to reduce detectable  $\alpha$ -synuclein but did not

differentiate between the infective and non-infective species. Previous work testing conditions for PrP<sup>Sc</sup> inactivation has demonstrated variability in results dependent upon prion strain and assay method(s) used (reviewed in [21]). Given the convergence between PrP<sup>Sc</sup> and  $\alpha$ -synuclein prions, it is critical that future studies investigate these disinfection methods in animal bioassays that directly measure transmissibility of MSA prions following decontamination. Work by our group and others highlighting the stability of  $\alpha$ -synuclein prions argue that validated protocols are not only needed in cleaning and decontaminating surgical instruments but also in decontaminating research equipment to prevent contamination across studies. For the inoculations reported here, single use syringes were discarded after each injection to prevent contamination across inoculum.

The diagnostic accuracy of MSA among movement disorder patients has ranged from 29% to 86% [18, 24, 27, 35, 52], and the majority of misdiagnosed pathologically confirmed MSA cases are clinically diagnosed as PD. While PD patients often see amelioration of symptoms following DBS treatment, the effects of DBS are not sustained in MSA patients, and it is therefore not a recommended treatment [34]. Concern about decontaminating neurosurgical instruments used for DBS in PD patients, or misdiagnosed MSA patients, has increased in light of recent data suggesting lateral transmission of A $\beta$  pathology. Reanalysis of tissue from iatrogenic CJD patients who developed disease after receiving contaminated human growth hormone or dura mater grafts showed some patients also developed A $\beta$  plaques earlier than age-matched controls [17, 25, 28]. Our finding that 10% sarkosyl is needed to at least partially denature  $\alpha$ -synuclein prions in MSA patient samples highlights the difficulty of disinfecting contaminated materials and suggests, analogous to PrP<sup>Sc</sup> prions, most



commonly used methods for disinfection will be ineffective. On account of the stability of  $\alpha$ -synuclein prions and their ability to transmit disease through peripheral exposure routes, additional care should be utilized when decontaminating instruments used during DBS procedures in PD patients until a diagnostic test for MSA is available.

The convergence of the neuropathogenic prions found in MSA and CJD patients, including their resistance to formalin and detergents and their ability to transmit disease through peripheral routes of exposure, emphasizes the need for further research to prevent any possible iatrogenic transmission of MSA. Future studies to establish successful methods for handling and sterilizing contaminated neurosurgical instruments and laboratory equipment should measure the remaining activity of  $\alpha$ -synuclein prions in sensitive animal and cellular bioassays. These studies should be carried out using preparations from MSA patient samples, rather than synthetic fibrils, to ensure the methods developed are effective against the  $\alpha$ -synuclein prion strain responsible for disease. In addition, improved diagnostic testing for MSA patients is needed to help prevent iatrogenic transmission. Importantly, currently available data indicate health workers and researchers should use caution when decontaminating equipment and working with materials potentially contaminated with MSA prions.

## **MATERIALS AND METHODS**

### **Experimental design**

The overall objective of this study was to assess the transmissibility and stability of  $\alpha$ -synuclein prions in MSA patient samples using animal and cellular bioassays to measure protein activity. A subsequent objective was to develop models of MSA

transmission to be used for measuring  $\alpha$ -synuclein prion inactivation. Based on several decades of prion transmission experiments, we have determined that 8 animals per inoculation group provide a sufficient sample size to achieve at least 80% power with an alpha of 0.05. This sample size accounts for some early animal loss following inoculation, which can occasionally occur if a mouse recovers poorly from the procedure. In all studies reported here, 8 mice were inoculated with each inoculum. At least four individual MSA patient samples were tested for each transmission route investigated. Two human and four mouse samples were used in the sarkosyl denaturation experiments. While ic inoculation with MSA transmits disease in ~120 days [40], experiments were terminated 450 dpi to account for extended incubation periods resulting from the indirect routes of exposure tested. Transmission of MSA prions was determined by testing the mouse brains in the  $\alpha$ -syn140\*A53T-YFP cell assay. After assessing the dynamic range of the assay, we defined infection with a measurement above  $10 \times 10^3$  A.U. Mice that tested positive for  $\alpha$ -synuclein prions were then assessed neuropathologically and biochemically for phosphorylated  $\alpha$ -synuclein aggregates, as were control-inoculated mice. No outliers were excluded.

### **Human tissue samples**

Frozen brain tissue samples from neuropathologically confirmed cases of MSA were provided by the Massachusetts Alzheimer's Disease Research Center, the Parkinson's UK Tissue Bank at Imperial College, and the Sydney Brain Bank. Control patient tissue was provided by Dr. Martin Ingelsson (Uppsala University). Demographic information about samples used are included in Online Resource, Table S1.

## **Mice**

Animals were maintained in an AAALAC-accredited facility in compliance with the Guide for the Care and Use of Laboratory Animals. All procedures used in this study were approved by the University of California, San Francisco, Institutional Animal Care and Use Committee. All animals were maintained under standard environmental conditions with a 12:12-h light:dark cycle and free access to food and water. To generate the hemizygous TgM83<sup>+/-</sup> mice used in this study, homozygous TgM83<sup>+/+</sup> mice [19], which express human  $\alpha$ -synuclein with the A53T mutation on a B6;C3 background, were purchased from the Jackson Laboratory (USA) and were bred with B6C3F1 mice.

## **Inoculations**

Fresh frozen human tissue was used to create a 10% (wt/vol) homogenate using calcium- and magnesium-free 1× DPBS using the Omni Tissue Homogenizer (Omni International). For inoculation studies, the homogenate was diluted to 1% using 5% (wt/vol) bovine serum albumin in 1× DPBS. Fixed frozen tissue was homogenized to use as inoculum under the same conditions. Prior to homogenization, the fixed tissue was incubated in sterile 1× DPBS for 2 h at room temperature with shaking to remove residual formaldehyde.

Ten-week-old mice were anesthetized with isoflurane prior to inoculation.

Inoculations were performed using 1% brain homogenate inoculated into the right parietal lobe (30  $\mu$ L), peritoneal cavity (100  $\mu$ L), right hind thigh (5  $\mu$ L), or tongue (5  $\mu$ L). Transmission studies using stainless-steel wires contaminated with MSA prions were carried out as previously reported [38]. Following inoculation, all mice were assessed

twice a week for the onset of neurological signs based on standard diagnostic criteria for prion disease [7] and were euthanized within 2 d of demonstrated CNS dysfunction. Following euthanasia, the brain was removed and bisected down the midline. The left hemisphere was frozen for biochemical analysis, and the right hemisphere was fixed in formalin for neuropathological assessment.

### **Alpha-synuclein prion precipitation**

A 10% (wt/vol) brain homogenate was prepared using frozen human tissue in calcium- and magnesium-free 1× DPBS using the Omni Tissue Homogenizer with disposable soft tissue tips (Omni International). Aggregated protein was isolated from the patient samples using sodium PTA (Sigma) as described [42, 53]. Isolated protein pellets were diluted 1:10 in 1× DPBS before testing in the cell aggregation assay.

### **Cell aggregation assay**

HEK293T cells expressing  $\alpha$ -syn140\*A53T-YFP were previously reported, and culture and assay conditions were used as described [53]. Briefly,  $\alpha$ -syn140\*A53T-YFP cells were cultured and plated in 1× Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (Thermo Fisher), 50 units/mL penicillin, and 50  $\mu$ g/mL streptomycin (Thermo Fisher). Cells were maintained in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C.

The  $\alpha$ -syn140\*A53T-YFP cells were plated at a density of 2,500 cells/well in a 384-well plate with black polystyrene walls (Greiner) with 0.012  $\mu$ g/well of Hoechst 33342. Plates were returned to the incubator for 2–4 h. Lipofectamine 2000 (1.5% final volume; Thermo Fisher) was incubated with each sample for 1.5 h at room temperature

prior to adding OptiMEM (78.5% final volume; Thermo Fisher). Samples were then added to the  $\alpha$ -syn140\*A53T–YFP cells in six replicate wells. After the plates were incubated for 4 d at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>, individual plates were imaged using the IN Cell Analyzer 6000 (GE Healthcare). DAPI and FITC channels were used to collect two images from five different regions in each well; these images were analyzed with the IN Cell Developer software using an algorithm designed to detect intracellular aggregates in living cells, quantified as total fluorescence per cell ( $\times 10^3$ , arbitrary units, A.U.).

### **Statistical analysis**

Data are presented as mean  $\pm$  standard deviation. Statistical analysis comparing the onset of neurological symptoms in mice was compared using a log-rank (Mantel–Cox) test to assess the distribution of survival curves. Statistical analysis of data collected from the  $\alpha$ -syn140\*A53T–YFP cell assay was analyzed using a one-way ANOVA with a Dunnett multiple comparison post hoc test. Mouse neuropathology was analyzed using a two-tailed Student's *t*-test with unequal variance to compare MSA-inoculated mice with control-inoculated mice by brain region. Significance was determined with a P value < 0.05.

## **FUNDING**

This work was supported by grants from the National Institutes of Health (AG002132 and AG031220), as well as by gifts from the Glenn Foundation and Daiichi Sankyo. The Massachusetts Alzheimer's Disease Research Center is supported by the National Institutes of Health (AG005134); the Parkinson's UK Brain Bank at Imperial College London is funded by Parkinson's UK, a charity registered in England and Wales (948776) and in Scotland (SC037554); and the Sydney Brain Bank is supported by Neuroscience Research Australia and the University of New South Wales. Glenda M. Halliday is a National Health and Medical Research Council of Australia Senior Principal Research Fellow (1079679).

## **AUTHOR CONTRIBUTIONS**

A.L.W., S.H.O., and S.B.P. designed research; A.L.W., S.A.K., S.P., Y.F., and A.O. performed research; A.A., D.A.M., G.M.H., L.T.M., and S.M.G. contributed new reagents/analytic tools; A.L.W., S.A.K., S.P., S.H.O., and S.B.P. analyzed data; and A.L.W., S.H.O., and S.B.P. wrote the paper.

## **COMPLIANCE WITH ETHICAL STANDARDS**

### **Conflict of interest**

The Institute for Neurodegenerative Diseases has a research collaboration with Daiichi Sankyo (Tokyo, Japan). S.B.P. is the chair of the Scientific Advisory Board of Alzheon, Inc., which has not contributed financial or any other support to these studies.

## **Ethical approval**

Animals were maintained in an AAALAC-accredited facility in compliance with the Guide for the Care and Use of Laboratory Animals. All procedures used in this study were approved by the University of California, San Francisco, Institutional Animal Care and Use Committee.

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## FIGURE LEGENDS

**Fig. 1. Intraperitoneal transmission of MSA prions.** TgM83<sup>+/-</sup> mice received intraperitoneal (ip) injection of 1% brain homogenate from one control and four MSA patient samples. (a) Kaplan–Meier plot shows the onset of neurological signs. Upticks indicate mice that did not die from synucleinopathy. (b) Alpha-synuclein prions were measured from frozen half-brains in the  $\alpha$ -syn140\*A53T–YFP cell assay ( $\times 10^3$  A.U.). Closed circles indicate mice containing prions (above dotted line), while triangles indicate mice that died from other causes. Open circles indicate mice terminated at 450 days post inoculation (dpi). Mice inoculated with MSA6 ( $P < 0.01$ ), MSA12 ( $P < 0.05$ ), and MSA13 ( $P < 0.01$ ) contained significantly more  $\alpha$ -synuclein prions than controls. (c) Quantification of phosphorylated  $\alpha$ -synuclein neuropathology in C2-inoculated mice compared to mice positive for synucleinopathy. The percent area containing pathology in the thalamus (Thal), hypothalamus (HTH), midbrain (Mid), and pons was increased in MSA-inoculated mice. Measurable staining was detected in the hippocampus (HC) from MSA6- and MSA-13 inoculated mice. Data shown as mean  $\pm$  standard deviation. (d, e) Representative images of phosphorylated  $\alpha$ -synuclein (EP1536Y; green) and glial fibrillary acidic protein (GFAP; red) staining in the brainstem of C2 (d) and MSA6 (e) inoculated mice. Nuclei in blue. Scale bar = 200  $\mu$ m. (f) Detergent-insoluble phosphorylated  $\alpha$ -synuclein aggregates were visualized by immunoblot. \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\*\* =  $P < 0.0001$ .

**Fig. 2. Intramuscular transmission of MSA prions.** TgM83<sup>+/-</sup> mice received intramuscular injection of 1% brain homogenate from one control and four MSA patient samples. (a, b) Kaplan–Meier plots show the onset of neurological signs following right

thigh (a) and tongue (b) inoculation. Upticks indicate mice that did not die from synucleinopathy. (c, d) Frozen half-brains from all mice were tested in the  $\alpha$ -syn140\*A53T-YFP cell assay ( $\times 10^3$  A.U.). Closed circles indicate mice containing prions (above dotted line), while triangles indicate mice that died from other causes. Open circles indicate mice terminated at 450 dpi. (c) Mice inoculated into the thigh with MSA6 ( $P < 0.0001$ ), MSA12 ( $P < 0.01$ ), and MSA13 ( $P < 0.001$ ) and (d) into the tongue with MSA6 ( $P < 0.01$ ) contained significantly more  $\alpha$ -synuclein prions than C2-inoculated mice. (e, f) Quantification of phosphorylated  $\alpha$ -synuclein immunostaining in the HC, Thal, HTH, Mid, and pons of inoculated mice. MSA inoculation into the thigh (e) and tongue (f) induced  $\alpha$ -synuclein pathology, but C2 inoculation had no effect. Data shown as mean  $\pm$  standard deviation. (g, h) Detergent-insoluble phosphorylated  $\alpha$ -synuclein was detected in the brains of mice inoculated with MSA in the thigh (g) and the tongue (h) but was not detected in C2-inoculated animals. \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ , \*\*\*\* =  $P < 0.0001$ .

**Fig. 3. MSA prions adhere to stainless-steel surgical wires.** Stainless-steel wires (4 mm) were incubated in 10% brain homogenate from one control and four MSA samples for 16 h at room temperature. The wires were washed in DPBS (5  $\times$  10 min), dried, and implanted into the right hemisphere of TgM83<sup>+/-</sup> mice. (a) Kaplan–Meier plot shows the onset of neurological signs following wire implantation. Upticks indicate mice that did not die from synucleinopathy. (b) Frozen half-brains from all mice were tested in the  $\alpha$ -syn140\*A53T-YFP cell assay ( $\times 10^3$  A.U.). Closed circles indicate mice containing prions (above dotted line), while triangles indicate mice that died from other causes. Open circles show mice terminated at 450 dpi. Wires incubated with MSA6 and MSA13

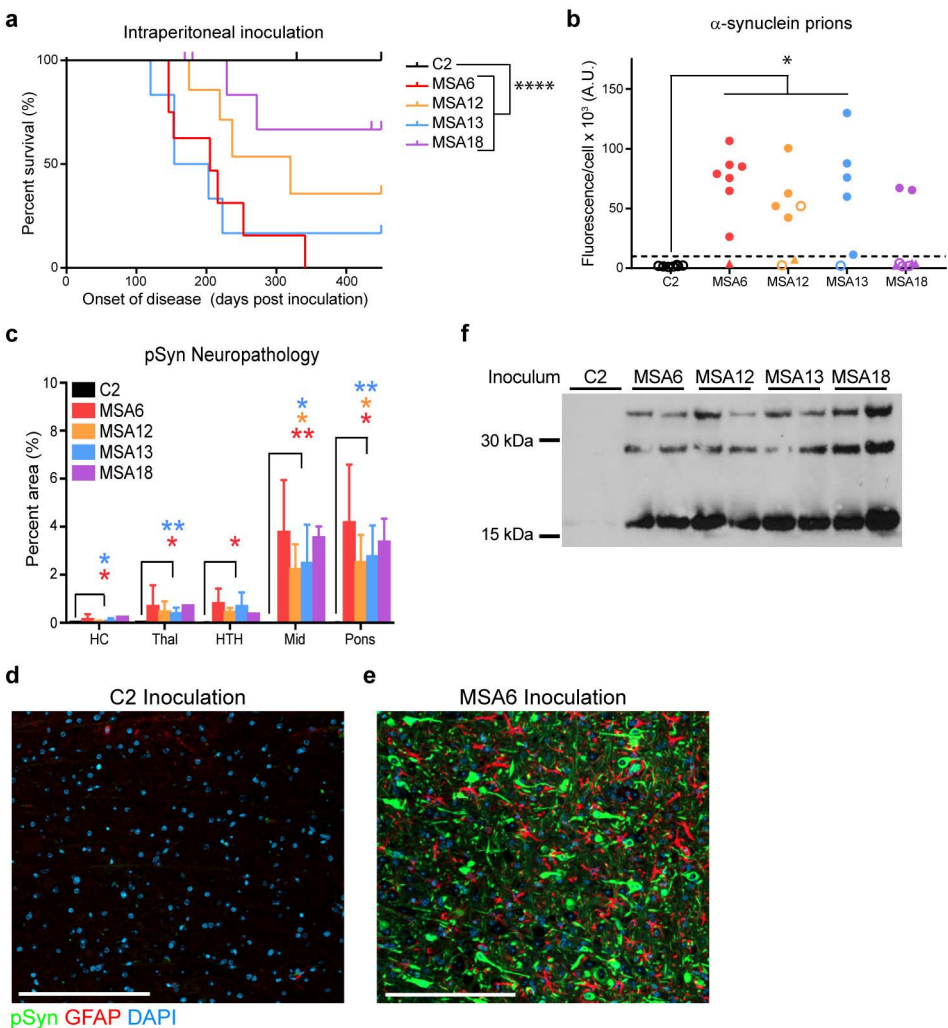


transmitted significantly more  $\alpha$ -synuclein prions than the control ( $P < 0.01$  and  $P < 0.05$ , respectively). (c) Quantification of phosphorylated  $\alpha$ -synuclein immunostaining. Wire incubation in C2 homogenate did not induce  $\alpha$ -synuclein aggregation, but MSA incubation induced accumulation in the Thal, HTH, Mid, and pons. Data shown as mean  $\pm$  standard deviation. (d, e) Representative images of phosphorylated  $\alpha$ -synuclein (green) and GFAP (red) immunostaining in the brainstem of mice implanted with C2 (d) and MSA6 (e) incubated wires. Nuclei in blue. Scale bar = 200  $\mu$ m. (f) Detergent-insoluble phosphorylated  $\alpha$ -synuclein aggregates were visualized by immunoblot. \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ .

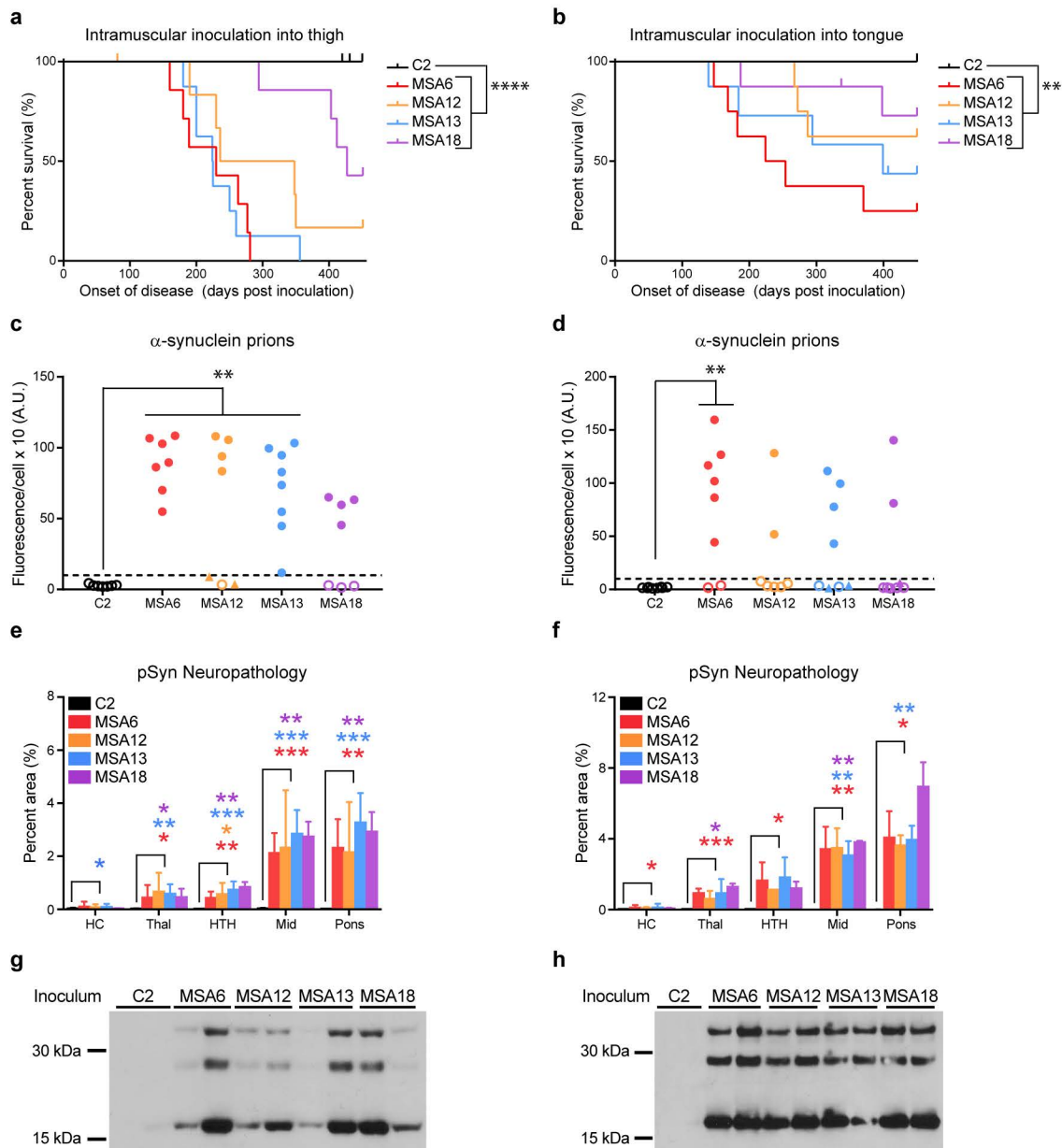
**Fig. 4. Formalin fixation does not inactivate MSA prions.** Formalin-fixed tissue from one control and seven MSA patient samples was homogenized in DPBS, and 30  $\mu$ L of a 1% homogenate was inoculated intracranially into TgM83<sup>+/-</sup> mice. (a) Kaplan–Meier plot shows onset of neurological signs in TgM83<sup>+/-</sup> mice following inoculation. Upticks indicate mice that died for reasons other than synucleinopathy. (b) Frozen half-brains from TgM83<sup>+/-</sup> mice were tested in the  $\alpha$ -syn140\*A53T–YFP cell assay ( $\times 10^3$  A.U.). Brains from the mice inoculated with all seven formalin-fixed MSA samples contained  $\alpha$ -synuclein prions (closed circles above dotted line), whereas control mice terminated at 450 dpi (open circles) and animals that died from other causes (triangles) did not. Transmission of  $\alpha$ -synuclein prions from the formalin-fixed MSA samples was significant compared to C23-inoculated mice (MSA2,  $P < 0.0001$ ; MSA3, MSA9, and MSA27,  $P < 0.01$ ; MSA10 and MSA28,  $P < 0.001$ ; MSA11,  $P < 0.05$ ). (c) Plot comparing the length of time that five of the MSA samples spent in formalin versus the incubation time in TgM83<sup>+/-</sup> mice. Fixation times ranged from

16 months (MSA28) to 244 months (MSA27), but the infectivity remained constant. Incubation times plotted as mean  $\pm$  standard deviation. \* =  $P < 0.05$ , \*\*\*\* =  $P < 0.0001$ .

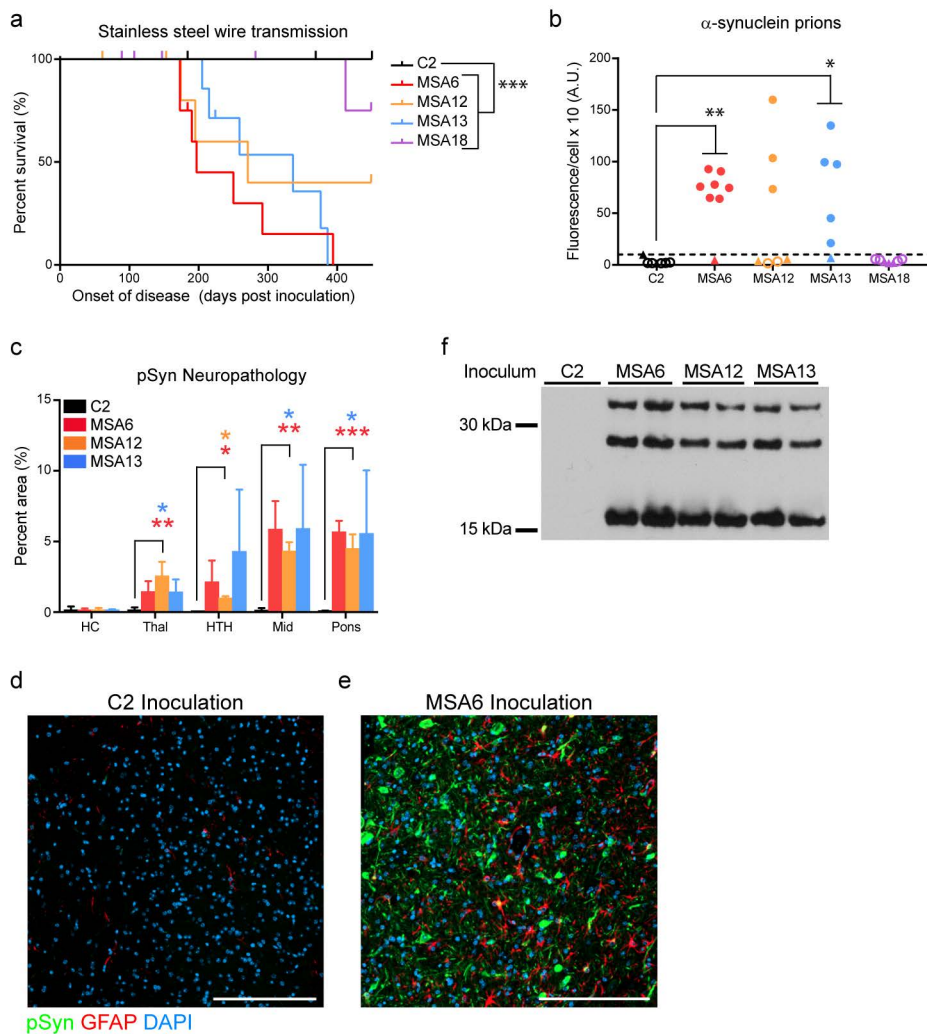
**Fig. 5. Stability of MSA prions to denaturation with sarkosyl.** Ten percent brain homogenates from two MSA patient samples (MSA10 and MSA13) and two mice inoculated with each sample were extracted using increasing concentrations of sarkosyl (2–20%). Residual protein aggregates were collected using PTA precipitation. **(a)** Protein pellets were diluted in DPBS and tested in the  $\alpha$ -syn140\*A53T–YFP cell assay. The titration curve shows an inflection point between 8% and 10% sarkosyl, with infectivity significantly reduced following extraction in 10% sarkosyl. **(b)** Silver stains of protein pellets from all six samples tested reveal a 15 kDa band, which corresponds to the molecular weight of  $\alpha$ -synuclein. This band is not present in the lower sarkosyl concentrations but becomes more abundant as the sarkosyl concentration increases (red arrows). **(c)** Immunoblot using the total  $\alpha$ -synuclein antibody MJFR1 from the four mouse samples shows a faint monomeric band in the lower sarkosyl concentrations, but it becomes increasingly more abundant starting at 10% sarkosyl.



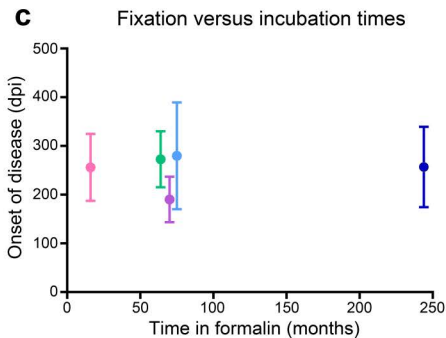
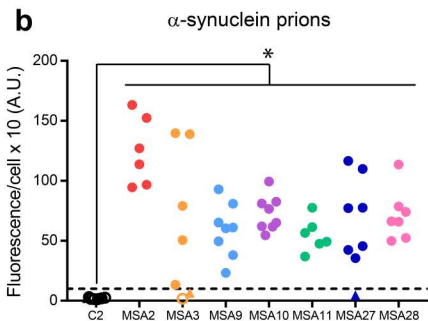
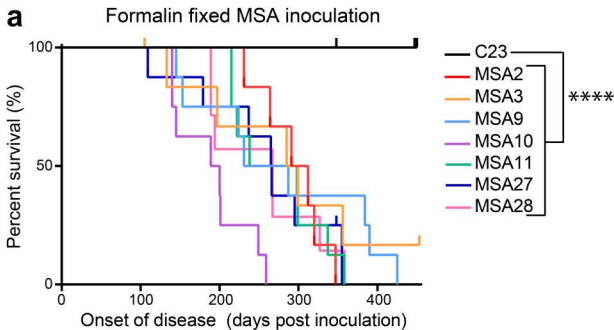
**Figure 1**

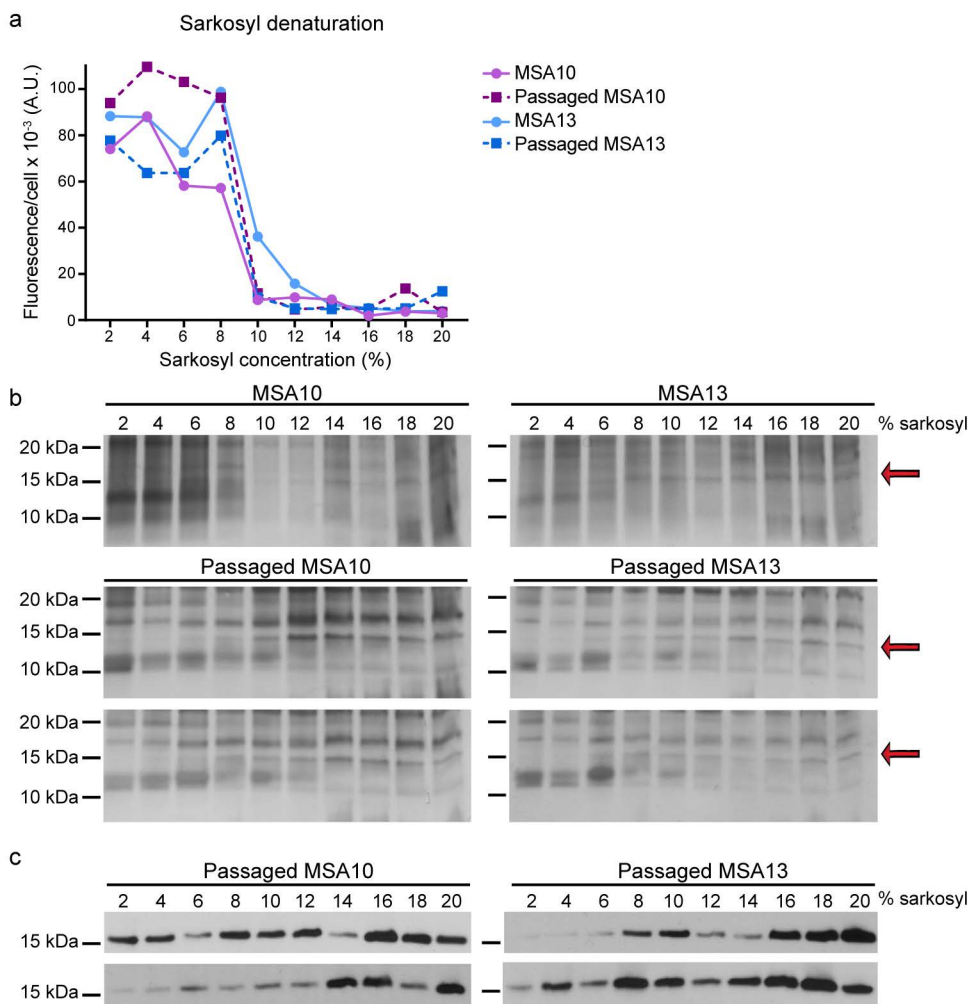


**Figure 2**



**Figure 3**





**Figure 5**

## ONLINE RESOURCE

### **Supplementary Material: MSA prions exhibit remarkable stability and resistance to inactivation**

#### **Acta Neuropathologica**

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## **MATERIALS AND METHODS**

### **Human patient neuropathology**

Patient samples obtained from the Massachusetts Alzheimer's Disease Research Center (ADRC) Brain Bank were assessed to confirm the diagnosis of multiple system atrophy (MSA). Fresh brains were dissected down the midline with one half fixed in 10% (vol/vol) neutral buffered formalin and coronally sectioned and the other half coronally sectioned before rapid freezing. The fixed tissue was evaluated histologically using a set of blocked regions representative of a variety of neurodegenerative diseases. All blocks were stained with Luxol fast blue (LFB) and hematoxylin and eosin (H&E). Selected blocks were used for immunohistochemical staining for  $\alpha$ -synuclein,  $\beta$ -amyloid, and phosphorylated tau. A confirmed MSA diagnosis required the presence of GCIs [2].

Tissue received from the Parkinson's UK Brain Bank were assessed neuropathologically following bisection of the brain with one hemisphere fixed in 10% buffered formalin and the other hemisphere sliced coronally, photographed on a grid, and rapidly frozen. Fixed blocks from 20 key brain regions were stained with H&E and LFB. To diagnose and stage disease, appropriate blocks were stained with antibodies against  $\alpha$ -synuclein,  $\beta$ -amyloid, tau, and p62. The MSA diagnosis was based on  $\alpha$ -synuclein inclusions in oligodendrocytes [1].

Human samples provided by the Sydney Brain Bank were bisected with one hemisphere randomly designated for fresh dissection and the other for fixation in 15% (vol/vol) buffered formalin [39% (vol/vol) aqueous formaldehyde solution] for 2 weeks before sectioning. Standard neuropathological assessment was done using H&E-stained sections, and a modified Bielschowsky silver stain was used to identify Alzheimer-type pathologies. Immunohistochemical staining for phosphorylated  $\alpha$ -synuclein (BD Biosciences USA; 1:7,000), phosphorylated tau (AT8, Thermo Scientific USA; 1:1,000), and  $\beta$ -amyloid (Dako Denmark; 1,500) was performed.

### **Immunohistochemistry and neuropathology**

Mouse brains were fixed in 10% (vol/vol) formalin and cut into four sections prior to processing through graded alcohols, clearing with xylene, infiltrating with paraffin, and embedding. Thin slices (8  $\mu$ m) were cut, collected on slides, deparaffinized, and exposed to heat-mediated antigen retrieval with citrate buffer (0.1 M, pH 6) for 20 min. Slides were stained using a Thermo Fisher 480S Autostainer with 30 min blocking in 10% (vol/vol) normal goat serum and incubation with primary antibody (2 h) and secondary antibody (2 h). Primary antibodies used include EP1536Y (pS129; 1:1,000; Abcam), glial fibrillary acidic protein (GFAP; 1:500; Abcam), p62 (Anti-SQSTM1; 1:1,000; Abcam), and ubiquitin (Ubi1; 1:5,000; Millipore). Secondary antibodies conjugated to AlexaFluor 488, 568, or 647 (Thermo Fisher) were used. Slides were then imaged using the Zeiss AxioScan.Z1.

Digital images of each slide were analyzed using the Zen Analysis software package (Zeiss). For each antibody, a pixel intensity threshold was determined for EP1536Y and applied to all slides. Regions of interest were drawn around the hippocampus, thalamus, hypothalamus, midbrain, and pons, and the percentage of pixels positive for staining in each region was determined. Two consecutive sections were cut and analyzed from each mouse, and the average percent area was calculated.

### **Detergent extraction**

Using a 10% (wt/vol) brain homogenate, samples were diluted to a final concentration of 1 mg/mL total protein in 1× detergent buffer [0.5% (vol/vol) Nonidet P-40 (NP-40), 0.5% (wt/vol) sodium deoxycholate (DOC) in Dulbecco's phosphate-buffered saline (DPBS)] and were incubated on ice for 15 min. Following a 1,000 × *g* centrifugation for 5 min, the supernatants were removed and the pellets were resuspended in 1× NuPAGE lithium dodecyl sulfate (LDS) loading buffer and boiled for 10 min. Samples were loaded onto a 10% Bolt Bis-Tris gel (Thermo Fisher), and SDS-PAGE was performed using the MES buffer system. Protein was transferred to nitrocellulose membranes using a semi-dry transfer. Membranes were blocked using blocking buffer [5% wt/vol nonfat milk in 1× Tris-buffered saline containing 0.05% (vol/vol) Tween 20 (TBST)] for 1 h at room temperature and then incubated in EP1536Y (1:4,000; Abcam) primary antibody overnight at 4 °C. Membranes were washed three times with 1× TBST before incubating with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; Bio-Rad) diluted in blocking buffer for 1 h at room temperature. After washing blots three times in 1× TBST, they were developed using the enhanced chemiluminescent detection system (GE Healthcare) for exposure to X-ray film.

### **Sarkosyl denaturation**

A 10% (wt/vol) brain homogenate was incubated in 0.5% (vol/vol) benzoyl peroxide (Sigma) with a final concentration of 2, 4, 6, 8, 10, 12, 14, 16, 18, or 20% (vol/vol) sarkosyl for 2 h at 37 °C with constant agitation (1,000 rpm) in an orbital shaker. Sodium phosphotungstic acid (PTA) (dissolved in double-distilled H<sub>2</sub>O, pH 7) was added to a final concentration of 2% (vol/vol), and samples were then incubated overnight in the same conditions. Samples were centrifuged at 16,000 × *g* for 30 min at room temperature. After removing the supernatant, pellets were resuspended in the same concentration of sarkosyl initially used. Sodium PTA was added to a final concentration of 2% (vol/vol), and the samples were again incubated at 37 °C with shaking for 1 h. After a second centrifugation step, the supernatant was removed and the final pellet was resuspended in DPBS using 10% of the initial starting volume.

### **Silver staining**

Samples were diluted in DPBS 1:5 with 1× NuPAGE LDS sample buffer and 1× NuPAGE Reducing Agent and boiled for 10 min. Samples were loaded onto a 10% Novex Bis-Tris gel (Thermo Fisher). SDS-PAGE was performed using the MES buffer system, and the gel was incubated in fixative [50% methanol and 12% glacial acetic acid (vol/vol)] overnight. After washing the gel for 30 min in sodium dodecyl sulfate (SDS) removal buffer [10% ethanol and 5% glacial acetic acid (vol/vol)], it was incubated in Farmer's solution [4.5 mM potassium ferricyanide, 19 mM sodium thiosulfate, and 4.7 mM sodium carbonate] for 2 min. The gel was washed three times for 5 min in double-distilled H<sub>2</sub>O and incubated in 12 mM silver nitrate for 20 min. Following a 40-s rinse in milliQ H<sub>2</sub>O, the gel was developed in 0.3 M sodium carbonate with 0.0005% (vol/vol) formaldehyde and incubated in stop solution [10% (vol/vol) glacial acetic acid] for 30 min before imaging. The gel was imaged using the AlphaImager (Alpha Innotech). All chemicals were purchased from Sigma.

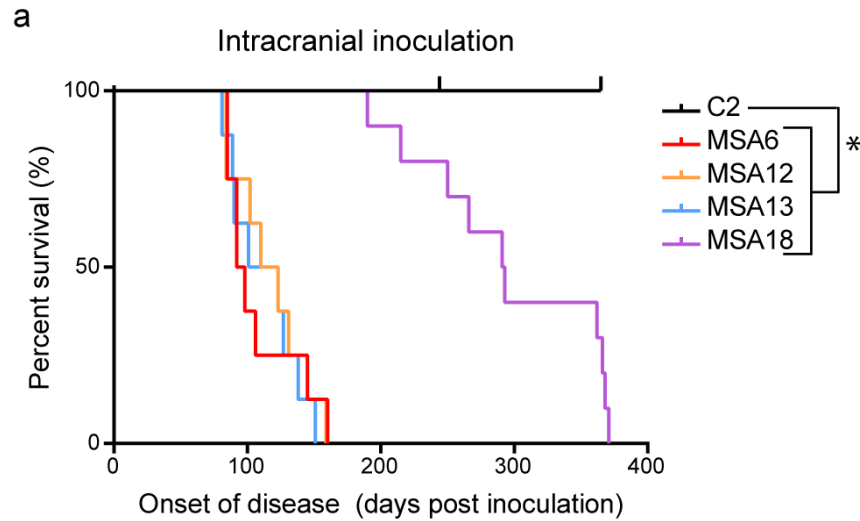
### **Immunoblotting**

Sarkosyl denaturation samples were diluted 1:1.7 with 1× NuPAGE LDS sample buffer and 1× NuPAGE Reducing Agent and boiled for 10 min. Samples were loaded onto a 10% Novex Bis-

Tris gel (Thermo Fisher). SDS-PAGE was performed using the MES buffer system. Protein was transferred to a nitrocellulose membrane using a semi-dry transfer. Membranes were dried for 1 h before blocking in blocking buffer for 1 h at room temperature. Primary antibody incubation (MJFR1; 1:5000; Abcam) was in blocking buffer overnight at room temperature. Membranes were washed three times with 1× TBST before incubating with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:10,000; Bio-Rad) diluted in blocking buffer for 1 h at room temperature. After washing blots three times in 1× TBST, membranes were developed using the enhanced chemiluminescent detection system (GE Healthcare) for exposure to X-ray film.

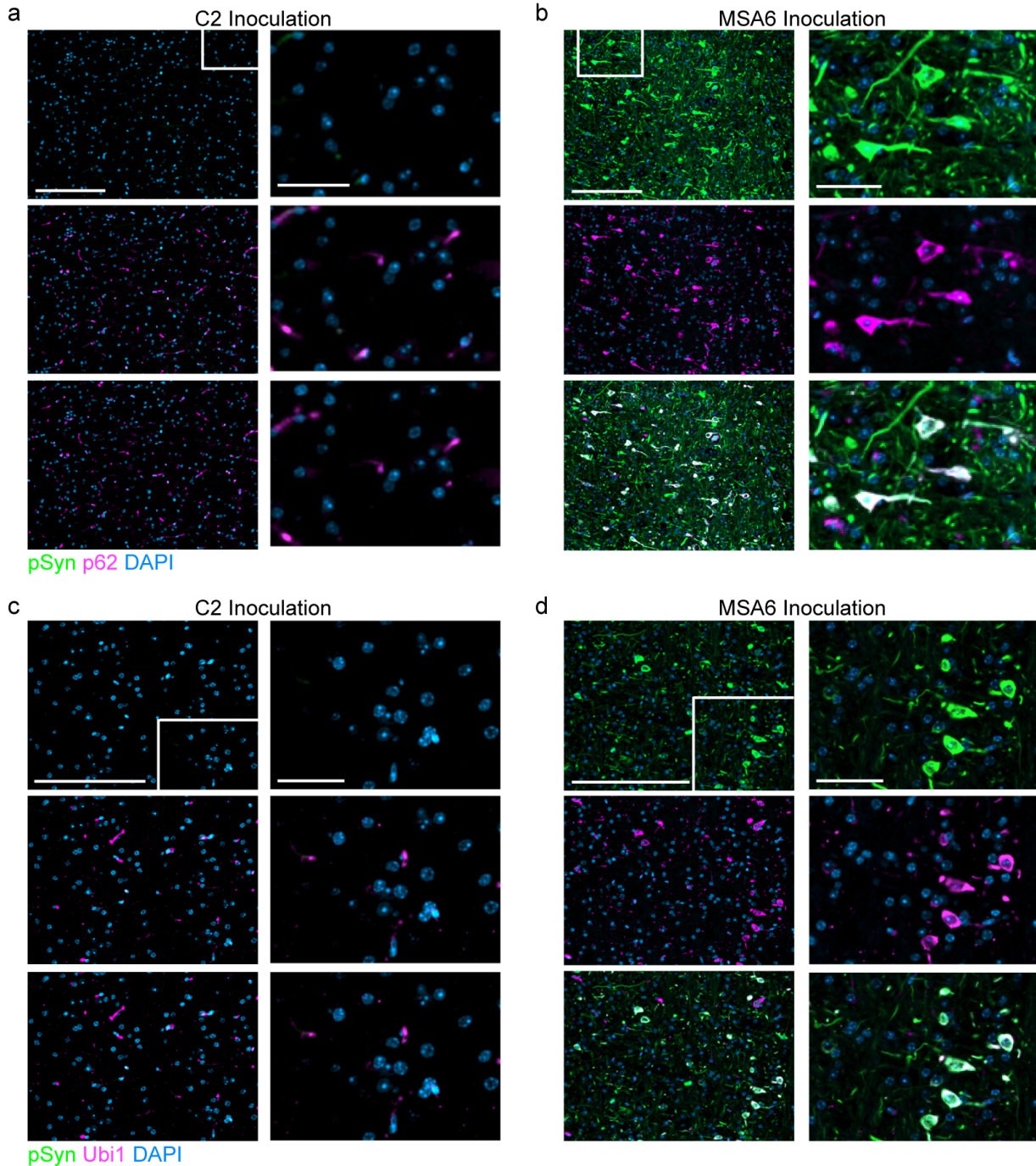
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- 3 Prusiner SB, Woerman AL, Rampersaud R, Watts JC, Berry DB, Patel S, Oehler A, Lowe JK, Kravitz SN, Geschwind D et al (2015) Evidence for  $\alpha$ -synuclein prions causing multiple system atrophy in humans with signs of Parkinson's disease. *Proc Natl Acad Sci USA* 112: E5308–E5317



## Figure S1

**Fig. S1. Intracranial MSA inoculations.** TgM83<sup>+/-</sup> mice were inoculated with 1% crude brain homogenate prepared from one control and four MSA patient samples. Kaplan–Meier plot shows the onset of neurological signs in days post inoculation (dpi; MSA6: 108 ± 29 dpi; MSA12: 117 ± 27 dpi; MSA13: 113 ± 26 dpi; MSA18: 297 ± 67 dpi). Mice inoculated with C2 samples were terminated at 450 dpi. Uptick represents mouse that died for reasons other than transmission of synucleinopathy. \* = P < 0.0001. Incubation times for MSA6, MSA12, and MSA13 were previously reported [3].

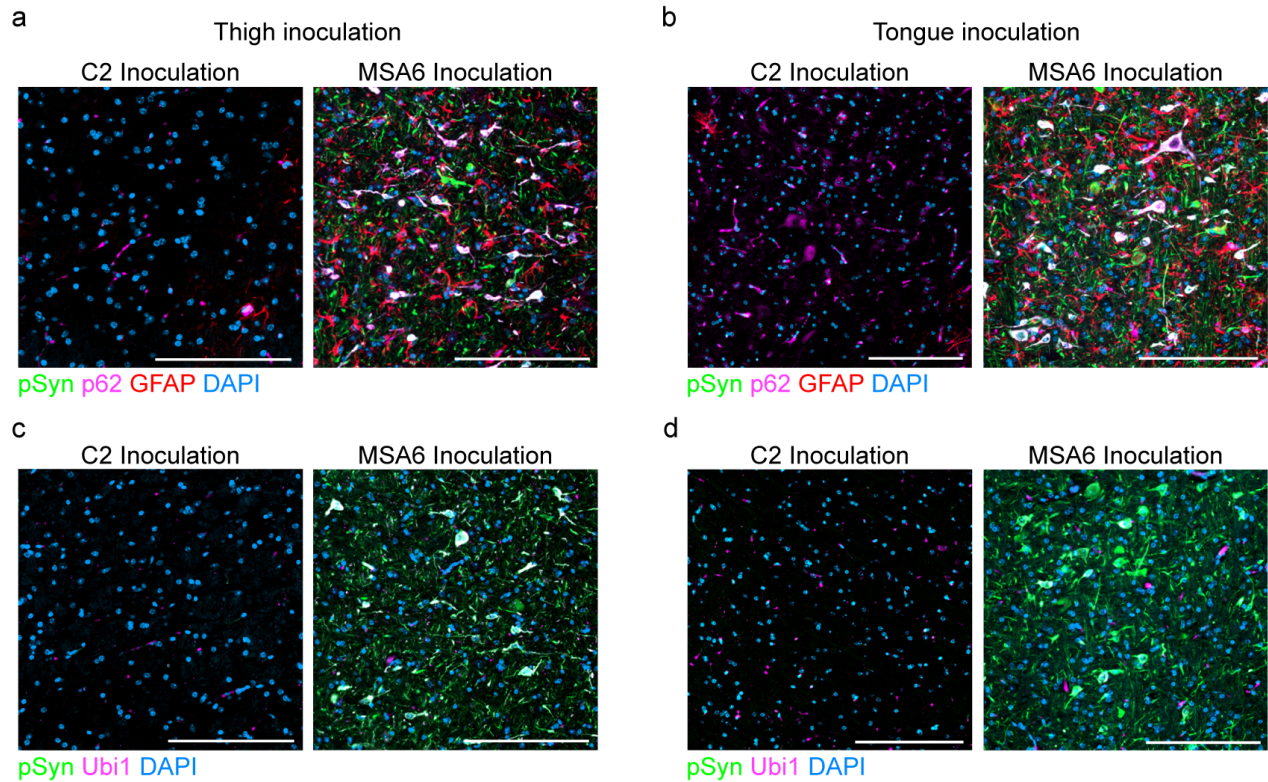


**Figure S2**

**Fig. S2. Intraperitoneal inoculation of MSA homogenate induces  $\alpha$ -synuclein neuropathology.** *TgM83<sup>+/-</sup>* mice were inoculated intraperitoneally with 1% crude brain homogenate prepared from one control patient sample (C2) and one MSA patient sample (MSA6). Formalin-fixed half-brains from control mice collected 450 dpi (**a, c**) and MSA-inoculated animals that developed synucleinopathy (**b, d**) were assessed for co-localization of

phosphorylated  $\alpha$ -synuclein with p62 and ubiquitin. Representative images of immunostaining shown here. **(a, b)** Phosphorylated  $\alpha$ -synuclein (green; top panels) and p62 (violet; middle panels) co-localized in the brainstem of MSA6-inoculated mice (white; bottom panels; **b**). Mice inoculated with C2 homogenate did not have staining in the brainstem (**a**). Magnified view from inset shown to the right. **(c, d)** Phosphorylated  $\alpha$ -synuclein (green; top panels) and ubiquitin (violet; middle panels) co-localized in the brainstem of MSA6-inoculated mice (white; bottom panels; **d**). Mice inoculated with C2 did not show immunostaining (**c**). Magnified view from inset shown to the right. Nuclei labeled with DAPI (blue). Scale bar in larger images = 200  $\mu$ m. Scale bar in magnified images = 50  $\mu$ m.

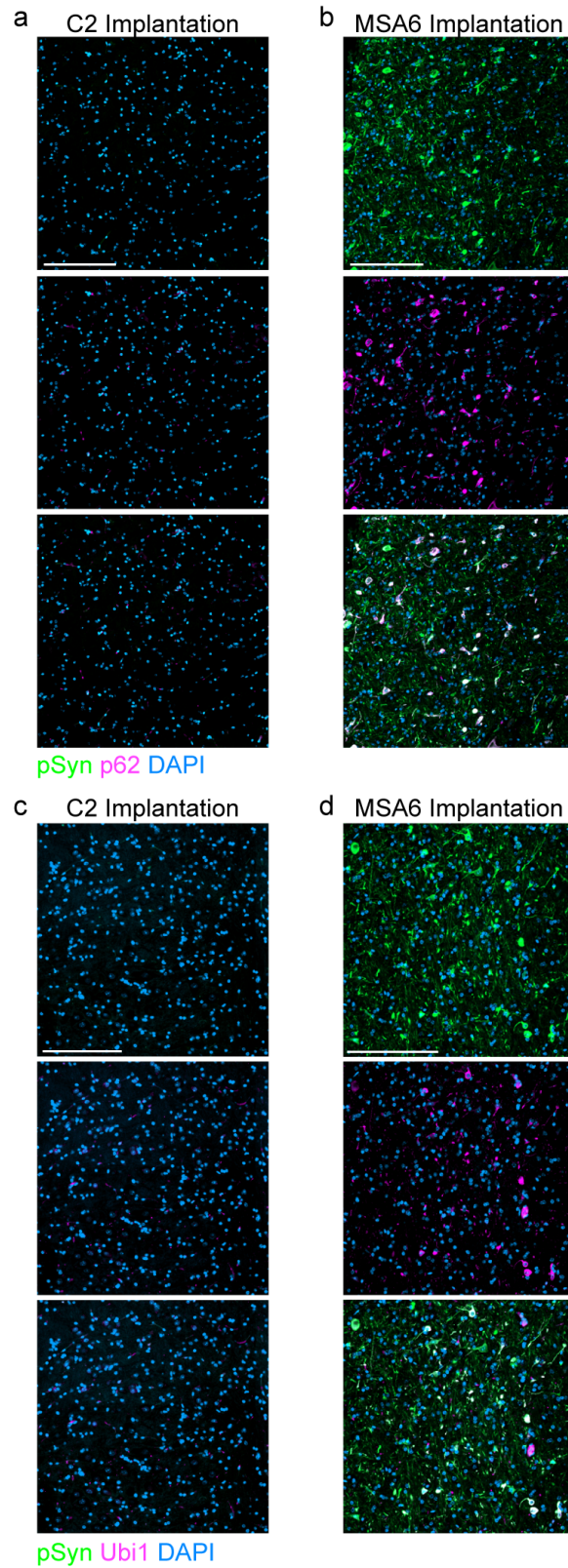




## Figure S3

**Fig. S3. Intramuscular inoculation of MSA homogenate induces  $\alpha$ -synuclein neuropathology.** Intramuscular inoculation into TgM83<sup>+/-</sup> mice was performed using 1% crude brain homogenate from one control patient sample (C2) and one MSA patient sample (MSA6) into the right hind leg (**a, c**) or the tongue (**b, d**). Formalin-fixed half brains from control mice collected 450 dpi and MSA-inoculated animals that developed synucleinopathy were assessed for phosphorylated  $\alpha$ -synuclein neuropathology (green). (**a, b**) Representative images from the brainstem of control- and MSA-inoculated mice show an increase in  $\alpha$ -synuclein aggregation and glial fibrillary acidic protein (GFAP; red). p62 (violet) co-localizes with  $\alpha$ -synuclein, shown in white. (**c, d**) Representative images from the brainstem of control- and MSA-inoculated mice show co-localization of  $\alpha$ -synuclein with ubiquitin (violet) in white. Nuclei labeled with DAPI (blue). Scale bar = 200  $\mu$ m.

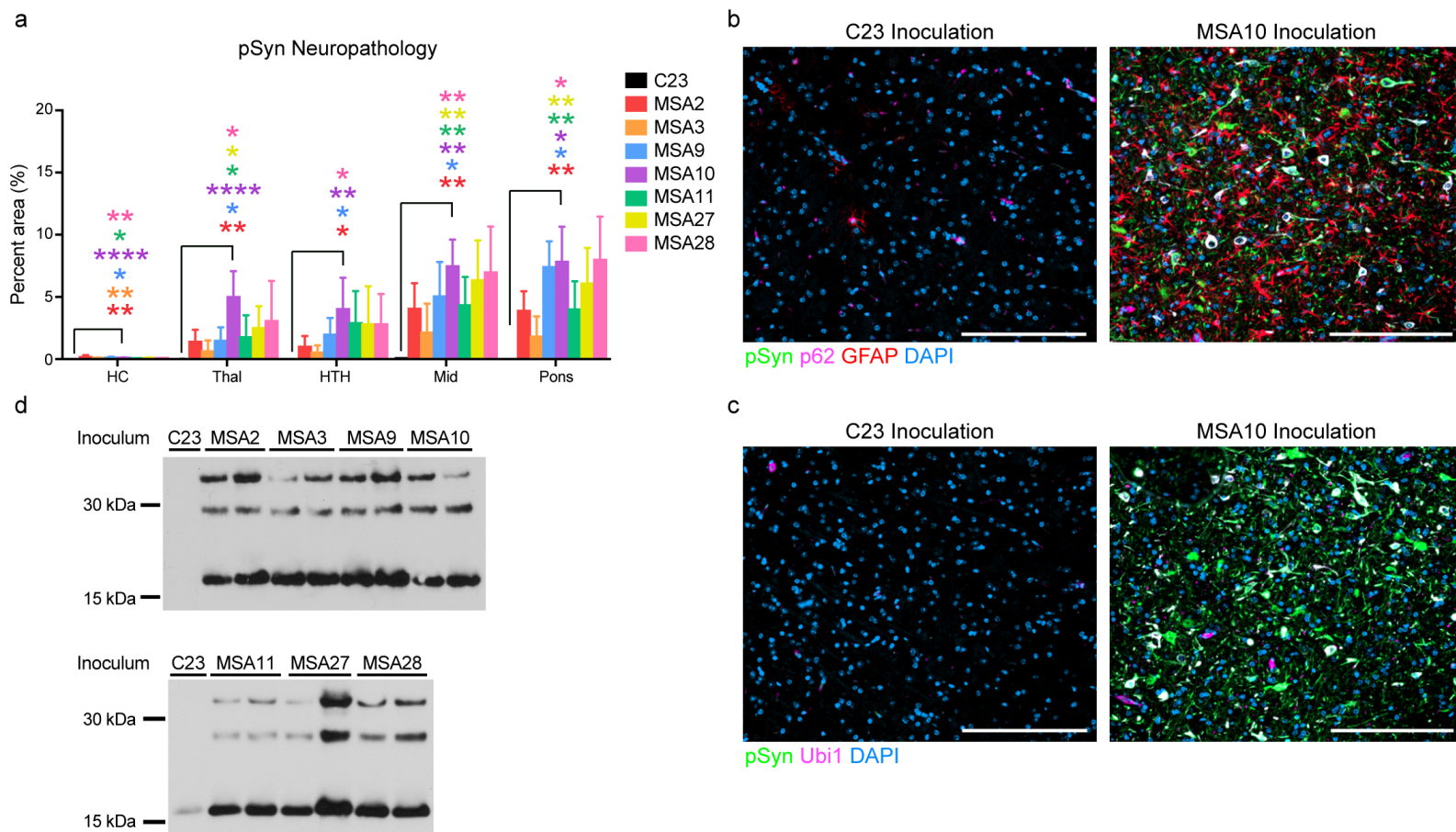




**Figure S4**

**Fig. S4. Wire transmission of MSA prions induces  $\alpha$ -synuclein neuropathology.** Stainless-steel suture was cut into 4 mm pieces, and the wires were incubated in C2 and MSA6 10% brain homogenate for 16 h. After washing for 10 min five times in DPBS, wires were dried and

implanted into the right hemisphere of TgM83<sup>+/-</sup> mice. Formalin-fixed half-brains from control mice collected 450 days post implantation (**a, c**) and animals implanted with MSA-incubated wires that developed synucleinopathy (**b, d**) were assessed for co-localization of phosphorylated  $\alpha$ -synuclein with p62 and ubiquitin. Representative images of immunostaining in the brainstem shown here. (**a, b**) Phosphorylated  $\alpha$ -synuclein (green; top panel) and p62 (violet; middle panel) co-localization is shown in white (bottom panel). Wire incubation with MSA6 induced co-localization in TgM83<sup>+/-</sup> mice, whereas C2 incubation did not. (**c, d**) Phosphorylated  $\alpha$ -synuclein (green; top panel) and ubiquitin (violet; middle panel) co-localization is shown in white (bottom panel). Wire incubation with C2 did not induce co-localization, whereas MSA6 incubation did. Nuclei labeled with DAPI (blue). Scale bar = 200  $\mu$ m.



## Figure S5

**Fig. S5. Inoculation of formalin-fixed MSA homogenate induces aggregation of phosphorylated  $\alpha$ -synuclein.** Brain homogenate was prepared from one formalin-fixed control and seven MSA patient samples. TgM83<sup>+/-</sup> mice received intracranial inoculations of 30  $\mu$ L 1% brain homogenate. Formalin-fixed half-brains from mice containing  $\alpha$ -synuclein prions at the time of death were compared

with brains from control mice for the presence of aggregated phosphorylated  $\alpha$ -synuclein. **(a)** Quantification of aggregated  $\alpha$ -synuclein in the HC, Thal, HTH, Mid, and pons shows an increase in phosphorylated  $\alpha$ -synuclein in the Thal, HTH, Mid, and pons in MSA-inoculated mice compared to mice inoculated with the control sample. Data shown as mean  $\pm$  standard deviation. \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ , \*\*\*\* =  $P < 0.0001$ . **(b, c)** Representative images of phosphorylated  $\alpha$ -synuclein immunostaining in C23 (left panels) and MSA10 (right panels) inoculated animals. Phosphorylated  $\alpha$ -synuclein (green) co-localized with p62 (violet; **b**) and ubiquitin (violet; **c**) in MSA10-inoculated mice but not in C23-inoculated mice. Inoculation of MSA also resulted in an increase in astrogliosis (red; **b**). Nuclei labeled with DAPI (blue). Scale bar = 200  $\mu$ m. **(d)** Detergent-insoluble phosphorylated  $\alpha$ -synuclein aggregates were visualized by immunoblot.

**Table S1. Patient sample information.**

<b>Patient</b>	<b>Disease</b>	<b>Age at Death</b>	<b>Sex</b>	<b>Brain Region</b>	<b>Country</b>
C2	None	88	F	Left cortex	Sweden
C23	None	78	F	Substantia nigra	United Kingdom
MSA2	MSA	70.5	M	Substantia nigra	United Kingdom
MSA3	MSA	58	F	Substantia nigra	United Kingdom
MSA6	MSA	61	F	Basal ganglia	United Kingdom
MSA9	MSA	82	M	Pons	Australia
MSA10	MSA	64	M	Pons	Australia
MSA11	MSA	61	M	Pons	Australia
MSA12	MSA	66	F	Substantia nigra	United States
MSA13	MSA	65	M	Substantia nigra	United States
MSA18	MSA	54	M	Substantia nigra	United States
MSA27	MSA	66	F	Pons	Australia
MSA28	MSA	69	M	Pons	Australia