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Seed amplification of MSA alpha-synuclein aggregates preserves the biological and structural properties of brain-derived aggregates

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Abstract

Parkinson's disease (PD), Dementia with Lewy bodies (DLB), and multiple system atrophy (MSA), are characterized by the misfolding and aggregation of alpha-synuclein (αSyn). Compelling evidence showed that αSyn aggregates exist as distinct conformational strains in different synucleinopathies. Recently, we reported that the αSyn Seed Amplification Assay (αSyn-SAA) can amplify and distinguish αSyn strains from PD and MSA. In this study, we investigate whether MSA-seeded, SAA-amplified αSyn fibrils retain the biological and structural properties of the αSyn seeds present in MSA brains. We study the biological activities of both brain-derived and SAA-amplified αSyn aggregates using an αSyn "biosensor" cell model and a synucleinopathy transmission mouse model. Our *in vitro* and *in vivo* findings reveal that the SAA-amplified αSyn fibrils preserve the biological properties of the brain-derived MSA strain. Detailed analyses of the in vivo studies demonstrate that both brain-derived and SAA-generated αSyn aggregates induce a similar disease, with comparable incubation periods, neuropathological damage and clinical manifestations. High-resolution cryo-EM analysis of SAA-amplified αSyn fibrils demonstrates that their conformation at the protofilament level closely resembles one of the αSyn filaments previously identified in MSA patient brains. Our findings suggest that SAA can amplify disease-specific misfolded αSyn conformation while preserving its main biological properties.

Introduction

The accumulation of pathological alpha-synuclein (αSyn) in the brain is a hallmark of a group of neurodegenerative disorders termed synucleinopathies, including Parkinson's disease (PD), Dementia with Lewy Bodies (DLB), and Multiple System Atrophy (MSA)[1-3]. Pathological αSyn aggregates are found predominantly in Lewy bodies (LBs) and Lewy neurites (LNs) in neurons of PD, DLB, and frequently Alzheimer's disease (AD) patients, and as Glial Cytoplasmic Inclusions (GCIs) in oligodendrocytes of MSA patients[2-4]. Nevertheless, it is often possible to observe some LB-like aggregates in MSA patients and some GCI-like deposits in PD[5]. The role of neuronal αSyn aggregates in MSA is not completely understood, but recent data suggest that they may play an important role in neurodegeneration[6]. In recent years, various studies have provided strong evidence for αSyn aggregates adopting distinct structures in different synucleinopathies[7-9]. These unique structures are usually referred to as conformational strains[10,11]. A great body of evidence has suggested pathological αSyn aggregates spread across brain regions as diseases progress[12-15], arguably following the "prion-like" seeding/nucleation mechanism[10,16]. The spread of αSyn pathology has been further supported by transmission studies of patient-derived αSyn aggregates in various model systems, including cells, rodents, and non-human primates[9,17-23]. Recent cryogenic electron microscopy (cryo-EM) structural studies of αSyn filaments extracted from patient brains confirmed that αSyn can misfold differently and adopt the distinct "Lewy fold" and "MSA fold" in PD/DLB and MSA, respectively[24,25].

Taking advantage of the "prion-like" seeding properties of disease-associated αSyn aggregates, our lab has developed an αSyn Seed Amplification Assay (αSyn-SAA) for amplifying and detecting αSyn aggregates from either cerebrospinal fluid (CSF) samples or highly diluted brain tissues of PD, DLB, AD, and MSA patients[26-29]. SAA was previously referred to as either

PMCA (protein misfolding cyclic amplification) or RT-QuIC (real-time quaking-induced conversion), terms originating from the prion field where these technologies started[30,31]. The unified SAA term was coined to highlight the basic principle behind these assays (seeding) and set apart the new assays from PMCA and RT-QuIC, which amplify infectious prions. We and others have demonstrated that αSyn-SAA can detect seeding-competent αSyn aggregates in biological samples with high sensitivity, specificity, and reproducibility[26,27,32-43]. In addition to its great diagnostic value, the αSyn-SAA can also differentiate αSyn strains in PD and MSA[27,43]. The biochemical, biophysical, and biological analyses of αSyn fibrils amplified from PD and MSA patient samples revealed differential binding affinities towards amyloid conformation-specific dyes, distinct protease resistance patterns, unique ultrastructural features, and different levels of toxicity to cultured cells, indicating a fundamental difference in their structures[27]. Interestingly, αSyn aggregates amplified from CSF and brain samples of the same disease (PD or MSA) share the same aforementioned disease-specific characteristics, suggesting that αSyn seeds in CSF faithfully represent those accumulating in the brain[27].

Without αSyn seeds, the intrinsically unstructured recombinant αSyn can spontaneously form fibrils of various morphologies and structures under different *in vitro* aggregation conditions[44-47]. These preformed fibrils (PFFs) can also induce the aggregation of endogenous αSyn and spread αSyn pathology in both cellular and animal models[18,19,48-51]. However, unlike endogenous αSyn aggregates found in LBs, LNs, or GCIs, αSyn PFFs generally display distinct biochemical and biological properties[52-54]. Cryo-EM studies provided the structural evidence to explain the discrepancies observed between brain-extracted αSyn fibrils and unseeded αSyn PFFs. Indeed, αSyn PFFs adopt various conformations different from the native LB and MSA structures[24,25,44,45,55]. Interestingly, it was also reported that αSyn fibrils formed in seeded reactions

exhibit different biochemical, biophysical, and structural features than non-seeded PFFs[56]. To date, few studies have been conducted to compare the properties of αSyn aggregates before and after amplification. Therefore, more research is necessary to evaluate whether αSyn fibrils generated *in vitro* in seeded reactions recapitulate the strain properties of the pathological αSyn aggregates found in patient brains.

The strain phenomenon has been extensively studied in prion diseases. Each prion strain is thought to represent a distinct structure of misfolded prion protein that produces a unique disease phenotype[10,57]. Historically, the biological properties of prion strains have been characterized in animal bioassays by monitoring the incubation period, clinical signs, the brain regions where prion aggregates accumulate, and the morphological, biochemical, and conformational properties of these aggregates[58-61]. The strain characterization criteria have also been applied to compare strain properties of brain-derived prions and PMCA-amplified prions. Compelling evidence supports that PMCA-amplified prions can faithfully maintain the infectivity and strain properties of the original prions used as seeds in PMCA reactions[62,63].

In this study, we investigate whether αSyn-SAA amplified fibrils from the CSF of MSA patients maintain both the biological and structural strain properties of αSyn aggregates present in patient brains. Biologically, we first demonstrate that both αSyn aggregates present in MSA brains and the MSA-seeded, SAA-amplified αSyn fibrils seeded the endogenous αSyn to form aggregates with the MSA-unique morphological features in a cellular model[21]. Furthermore, in a transgenic animal model that has been widely used to characterize αSyn transmission and strain properties[9,19,52,53], both MSA brain-derived and SAA-amplified αSyn fibrils induce a very similar fatal neurological disease characterized by severe motor dysfunctions and extensive αSyn pathology. Finally, we determine the cryo-EM structure of SAA-amplified fibrils and compared it

with previously reported structures for brain-derived MSA aggregates. Our comprehensive functional-structural studies suggest that SAA-amplified αSyn fibrils keep the main biological properties of the brain MSA αSyn strain, as well as exhibit striking similarities in their high-resolution structures. Further studies are warranted to explore similar comparisons with αSyn aggregates amplified from patients with PD and DLB.

Results

Characterization of MSA brains and generation of SAA-amplified αSyn aggregates

For our studies, we used αSyn aggregates derived from the brains of four clinically diagnosed and neuropathologically confirmed MSA cases (M1, M2, M3, and M4, see Table 1). Immunohistochemical analysis using an antibody specific for αSyn phosphorylated at position 129, a widely used biomarker of αSyn deposits, showed the typical GCIs characteristics of MSA (Supplementary Fig. 1). Brain homogenates (BHs) (40%, w/v) were prepared from these brain samples, and insoluble fractions were collected from pre-cleared BH and used for the studies.

Antemortem CSF samples were collected from four MSA (M1, M2, M3, and M5) patients (Table 1) and used as seeds in our αSyn-SAA assay[26,27] to generate αSyn fibrils (Supplementary Fig. 2). We have reported that our αSyn-SAA can, in addition to differentiating PD and MSA αSyn strains in the first-round amplification, maintain the strain properties in subsequent rounds of amplification[27]. To exclude the possibility that the αSyn seeds-containing CSF components that might contribute to the propagation of αSyn aggregates, second-round SAA amplified fibrils were used in both *in vitro* and *in vivo* characterizations in the current study (Supplementary Fig. 2a).

MSA brain-derived and SAA-amplified αSyn fibrils propagate in αSyn "biosensor" cells

In a pioneering study, the Diamond Lab developed a cellular assay utilizing the HEK293T "biosensor" cell line, which expresses human αSyn (A53T)-CFP/YFP fusion proteins to investigate the αSyn strain properties associated with PD and MSA[21]. Brain extracts from PD and MSA patients displayed consistent seeding activities in these biosensor cells. However, the morphology of MSA-seeded αSyn aggregates differed drastically from that of PD-seeded inclusions. While PD brain extracts induced the formation of small and punctate αSyn inclusions, MSA-treated cells produced filamentous and string-like αSyn aggregates[21]. Thus, this biosensor cell line can be employed to differentiate and characterize PD and MSA αSyn conformational strains.

To assess the biological activity of our samples in these biosensor cells, we applied BH extracts from four different MSA patients to the cells. Cells were cultured for 10 days, and confocal images of the treated cells were taken daily to monitor the morphology of the seeded αSyn inclusions. All four MSA brain samples seeded the aggregation of endogenous αSyn monomers, and the resulting expected string-like inclusions were consistently observed across all MSA-treated cells (Supplementary Fig. 3a). In accordance with the previous report[21], our cellular MSA transmission results strongly suggest that the filamentous, string-like αSyn inclusions formed in the biosensor cells represent a distinct characteristic of MSA αSyn seeds. Similarly, SAA-amplified αSyn fibrils from all four CSF samples induced the efficient formation of endogenous αSyn aggregates. Interestingly, the newly formed αSyn inclusions exhibited filamentous, string-like morphological features (Supplementary Fig. 3b), comparable to those induced by brain-derived MSA αSyn seeds. No αSyn aggregation was observed in cells untreated or incubated with control brain homogenate (Supplementary Fig. 3c). Our biosensor cell transmission results suggest

that the MSA-seeded, SAA-amplified αSyn fibrils maintained the unique seeding properties of the brain-derived MSA αSyn seeds.

Transgenic mice inoculated with MSA brain homogenates develop a fatal neurological disease

To investigate the *in vivo* biological properties of αSyn fibrils, we utilized a transgenic mouse model overexpressing human αSyn with the A53T mutation (line M83, hemizygous, referred to as M83+/- in this study)[64], which has been extensively employed for studying human synucleinopathies[9,19,52]. It has been reported by various groups that M83+/- mice, after receiving MSA BHs via intracerebral (i.c.) injections, develop neurological and motor dysfunctions such as kyphosis, ataxia, lethargy, cachexia, and paralysis[9,23,53]. However, i.c. inoculation of αSyn aggregates from PD patients into M83+/- mice has generally produced negative results[9,23].

We injected crude BHs (40%, w/v) from four different MSA cases (M1, M2, M3, and M4) (Table 1) into the dorsal striatum region on both sides of the M83+/- mouse brains (5 µL per hemisphere). All 20 mice (n=5 mice per MSA case, n=20 in total) receiving MSA BHs developed neurological signs of severe motor dysfunctions and reached a moribund stage between 111 and 154 days post inoculation (dpi) with a median survival time of 129.5 dpi (Fig. 1a), in agreement with previous results from similar studies[23,53] (two mice with prolonged survival times were excluded from the survival curve after outlier analysis). All six mice injected with PBS never developed any neurological signs (Fig. 1a). Brain tissues were collected from both groups of mice. The left hemispheres were fixed in formalin for histological analyses, and the right halves were homogenized for biochemical characterizations. Immunohistochemical (IHC) staining with an antibody against the pathological αSyn species phosphorylated at residue Serine129 (pS129)

revealed widespread pS129-positive αSyn deposits in mice induced by different MSA BH inocula (Fig. 1b and Supplementary Fig. 4a). The αSyn pathology was primarily observed in the midbrain, brain stem, hypothalamus, and cerebellum, which is also in agreement with previous reports[52,53]. Consistent with the lack of neurological signs, mice injected with PBS showed no sign of pS129-positive αSyn pathology by IHC staining (Fig. 1b). We further validated the IHC findings by biochemical analyses. BHs of the right hemispheres from mice injected with PBS and MSA BHs were subjected to protease digestion, a widely used biochemical tool for identifying protein aggregates and distinguishing conformational strains[27]. The immunoblotting results clearly demonstrated that protease-resistant αSyn inclusions were present in mice injected with MSA BHs but not in those injected with PBS (Fig. 1c). The protease-resistance core was detected with an antibody recognizing the mid region of the protein (antibody BD42), but, as expected, not by an antibody recognizing the C-terminus (antibody Syn-211), which is outside the aggregate core (Supplementary Fig. 4b). Taken together, our results confirm that αSyn aggregates present in MSA brains produce a fatal neurological disease in M83+/- mice, characterized by severe motor dysfunctions, accompanied by widespread pS129-positive αSyn pathology.

MSA-seeded, SAA-amplified αSyn fibrils induce a similar neurological disease in M83+/- mice

To examine whether the patient sample-seeded SAA-amplified αSyn fibrils can recapitulate the biological activity of brain-derived αSyn aggregates, we used material amplified by SAA from CSF of 4 different MSA patients. Twenty-one M83+/- mice were stereotaxically injected with the SAA-amplified αSyn fibrils (n=5-6 mice per sample) in both brain hemispheres. Injections were done in the same way as mice inoculated with MSA BH, i.e., 5 µL of inoculum

into each side of the dorsal striatum (10 µL per mouse). As controls, we included a group of mice injected with preformed αSyn fibrils (αSyn PFF) generated *in vitro* spontaneously (i.e., non-seeded) (Supplementary Fig. 5). Nineteen mice receiving the SAA-amplified αSyn fibrils developed clinical signs similar to those observed in mice injected with MSA BHs, such as hunchback, unbalanced walking, rapid weight loss, and hind limb paralysis, which led to a moribund stage in a few days after the first symptoms were observed. One mouse was found dead at 63 dpi without any neurological signs, and one mouse was still alive and considered an outlier when the manuscript was submitted. Both mice were excluded from the survival curve analysis. The median survival time for mice injected with SAA-amplified αSyn fibrils was 126.0 dpi, very similar to the 129.5 dpi observed in mice exposed to MSA BH (**Fig. 1a**). Indeed, comparisons of survival curves found no significant difference between the MSA BH and MSA SAA groups (Log-rank (Mantel-Cox) test, Chi square: 0.7314, df: 1, *p* value: 0.3924; Gehan-Breslow-Wilcoxon test, Chi square: 2.971, df: 1, *p* value: 0.0847). IHC analyses revealed widespread pS129-positive αSyn deposits in mice injected with MSA-seeded, SAA-amplified αSyn fibrils, with a similar pathological pattern observed in MSA BH-inoculated mice, primarily in the midbrain, cerebellum, hypothalamus, and brainstem (Fig. 1b and Supplementary Fig. 4a). The morphology of the inclusions observed in both MSA BH and MSA SAA animals was very similar (Supplementary Fig. 4c). Protease-digestion results confirmed that mice injected with SAA-amplified αSyn fibrils contained a significant amount of protease-resistant αSyn aggregates (Fig. 1c and Supplementary Fig. 4b). The control animals injected with αSyn PFF, as well as PBS-inoculated mice, never developed neurological signs when sacrificed between 471 and 493 dpi (Fig. 1a). IHC staining revealed little to no pS129-positive αSyn pathology (Fig. 1b), and Proteinase K (PK) digestion found no protease-resistant αSyn aggregates in PFF-injected mice (Fig. 1c and Supplementary Fig. 4b). Altogether, our data

suggest that similar to MSA BH, the MSA-seeded, SAA-amplified αSyn fibrils were able to seed and propagate endogenous αSyn aggregates, inducing a fatal neurological disease in M83+/- mice.

Both brain-derived and SAA-amplified αSyn aggregates produced the same neuropathological alterations in M83+/- mice.

To further compare the αSyn pathology between mice injected with MSA BH and SAA-amplified materials, we examined different brain regions for pS129-positive αSyn deposits. Extensive αSyn pathology was found in the hypothalamus, midbrain, and brainstem, with less αSyn accumulation in the cerebellum and sparse pS129-positive staining in the cortex, hippocampus, and thalamus in both groups of mice (Fig. 2a). Quantitative comparisons between mice injected with MSA BH and SAA-amplified fibrils revealed no significant difference in the pathological αSyn burdens in matching brain regions (Fig. 2b). Interestingly, in the pathology-ridden brain regions, such as the hypothalamus and midbrain, two morphologically distinct pS129-positive αSyn deposits were often observed: ring-like and Lewy body (LB)-like aggregates. Ring-like αSyn aggregates are circular deposits surrounding the nucleus, and LB-like aggregates are dense αSyn perinuclear inclusions (Fig. 2c). Interestingly, similar ring-like neuronal αSyn inclusions have been reported in atypical MSA patients[65]. Numerical quantification of both types of deposits revealed a similar ring-like dominant deposition pattern in both groups of animals (Fig. 2d). It has been reported that in MSA BH-injected M83+/- mice, the pS129-positive αSyn deposits accumulate predominantly in neurons over oligodendrocytes, which has been attributed to the prion protein promoter that drives the overexpression of mutant αSyn in these mice[9,23,53]. Our immunofluorescence confocal microscopy analyses confirmed that pS129-positive αSyn aggregates were primarily localized in neurons, with no detectable accumulation in

oligodendrocytes, in MSA BH-injected M83+/- mice. A similar cellular tropism of αSyn deposits was also found in MSA-seeded SAA-amplified fibrils-inoculated mice (**Supplementary Fig. 6**).

The remarkable resemblance in αSyn pathologies (Fig. 2) suggests that the αSyn aggregates found in mice injected with MSA BH and SAA-amplified fibrils might be conformationally similar. To further test this hypothesis, we measured the conformational stabilities of αSyn aggregates in the brains of mice from both groups using a commonly used conformational stability assay. In this assay, mouse BH samples from both groups were subjected to denaturation by guanidine hydrochloride (GdnHCl). The amount of GdnHCl-resistant αSyn aggregates was plotted against the concentration of GdnHCl to determine the $GdnHCl_{50}$, i.e., the GdnHCl concentration at which half of the aggregates were denatured. Remarkably, very similar denaturation curves and $GdnHCl_{50}$ values were found for αSyn aggregates in both groups of mice (Fig. 3a). Next, we used our αSyn-SAA to further verify the similarities between the αSyn aggregates in inoculated animals. Highly diluted mouse BHs (10^{-5}) from both groups were subjected to αSyn-SAA. SAA-amplified fibrils from mice injected with either MSA BH or SAA-amplified fibrils displayed the typical MSA ThT fluorescence readings (< 2,000 a.u.) (Fig. 3b), suggesting that the MSA αSyn strain has been propagated in these mice.

Taken together, our *in vitro* and *in vivo* findings strongly support the notion that αSyn-SAA preserves the biological properties of the MSA αSyn strain. This highlights its potential as a powerful platform not only for disease diagnosis but also for elucidating disease mechanisms and advancing the development of novel therapeutics.

MSA-seeded, SAA-amplified αSyn fibrils exhibit a similar structure to the αSyn protofilaments found in MSA brains

In parallel with the biological studies, we set out to obtain and compare structures of the MSA-seeded, SAA-amplified αSyn fibrils with those of the brain-derived MSA strain, using both liquid chromatography-mass spectrometry (LC-MS), and high-resolution cryo-EM. According to the published structures for MSA aggregates, the core of the brain-derived MSA filaments encompasses amino acids G14 to F94 for PF-IA and PF-IIA, K21 to Q99 for PF-IB, and G36 to Q99 for PF-IIB[25]. To biochemically assess the core of the MSA-seeded, SAA-amplified αSyn fibrils, αSyn aggregates amplified from the same four CSF samples used for the biological studies (Table 1) were subjected to limited proteolytic digestion with PK, followed by LC-MS analysis of the PK-resistant fragments. SDS-PAGE analysis of all four PK-digested samples revealed two distinct bands (Supplementary Fig. 7a), consistent with our earlier report[27]. One of the bands had a slightly higher molecular weight than the other, designated as the high molecular weight (HMW) and the low molecular weight (LMW) bands, respectively. The two bands were excised separately and subjected to trypsin digestion, followed by LC-MS analysis, as detailed in the Methods section. LC-MS analyses of both bands revealed the presence of peptides corresponding to the αSyn sequence, spanning from amino acid E13 to K102 (Supplementary Fig. 7b). Notably, the HMW band contained a significant number of peptides starting near (or just before) amino acid Q24 and extending to K96, suggesting that the HMW band corresponds to a fibrillar fold with core residues at least spanning between amino acids 24 and 96, which is reminiscent of the PF-IB filament. The LMW band, on the other hand, contained peptides beginning near (or just before) E35 and ending at K96, implying that it represents a fibrillar fold with core residues spanning between amino acids 35 and 96 (Supplementary Fig. 7b), similar to the PF-IIB protofilament. In summary, these biochemical analyses indicate that the αSyn fibrils seeded by four different MSA CSF samples

exhibit similar structural characteristics, which are reminiscent of those found in the brains of MSA patients.

Next, we aimed to determine the high-resolution structures of the MSA-seeded, SAA-amplified αSyn fibrils using cryo-EM. For cryo-EM helical reconstruction, the fibrils must be well separated and twisted. We screened all four SAA-amplified samples (Table 1) using a Krios cryo-EM system, and the M5 fibrils consistently exhibited superior separation and a well-defined helical periodicity. Based on these observations and the aforementioned LC-MS results (Supplementary Figs. 7b), we selected the M5 sample as representative for the cryo-EM analysis. We imaged the M5 fibrils in a Titan Krios electron cryo-microscope equipped with a K2 Summit direct detector (Fig. 4a). Two-dimensional classification of the cryo-EM images of these fibrils indicated the presence of three main fibril types (MI, MII, and MIII) (Fig. 4b). However, only one type of fibrils (MIII) had a well-defined twist necessary for helical reconstruction analysis. This class of fibrils has a crossover distance of ~70 nm and a width of ~10 nm with 2-fold symmetry (Fig. 4b). Using Relion, we reconstructed the MIII fibrils to a resolution of 3.9 Å (Figs. 4c and 4d). The core of the MIII structure is composed of residues G36-K96 (Fig. 5a) and comprises seven parallel in-register β-sheets (PIRBS) spanning from residue L38 to I88 (Figs. 5b and 5c). Supplementary Figure 8 shows the cryo-EM density map, atomic model fitting, and angular distribution of particles. Interestingly, the size of the PK-resistant LMW band closely matches that of the MIII fold, as well as the PF-IIB filament isolated from MSA patient brains. Indeed, a refined atomic model revealed that the MSA-seeded, SAA-amplified MIII fibrils share a similar fold to the PF-IIB1 and PF-IIB2 protofilaments reported previously from αSyn aggregates extracted from MSA brain[25]. The structural similarity was analyzed by estimating the Root Mean Square Deviation (R.M.S.D.), with values of 2.3 Å (43 pruned atom pairs) for PF-IIB1 (**Fig. 6a**) and 1.7 Å (43 pruned atom pairs) for

PF-IIB2 (Fig. 6b). Across residues T44 to K80, a notable resemblance is apparent between MIII and MSA PF-IIB1, with primary differences occurring within the G36-K43 and T81-K96 segments (Fig. 6a and Supplementary Figure 9a). For PF-IIB2, a significant alignment is observed with MIII spanning residues T44 to V82, whereas the main distinction lies in the G36-K43 and E83-K96 regions (Fig. 6b and Supplementary Fig. 9b). Compared to the αSyn filament isolated from PD and DLB brains, the MIII structure shows no resemblance to the Lewy fold (8A9L) with an R.M.S.D. value of 11.5 (43 pruned atom pairs) (Supplementary Fig. 10).

Despite the striking similarities with the MSA PF-IIB at the protofilament level, the overall packing of the MIII fibrils is different from the brain-derived MSA filaments. Our structure is composed of two homologous protofilaments with a structure reminiscent of PF-IIB, whereas the type II filaments in the MSA brains are asymmetrically packed with PF-IIA and PF-IIB protofilaments. The asymmetrical fold is favored most likely due to the presence of non-proteinaceous molecules, or cofactors, that might act as stabilizers for such structures[25]. Therefore, even though the αSyn-SAA assay is carried out *in vitro* using pure recombinant αSyn protein without any cellular cofactors, the striking degree of structural similarity between the SAA-amplified MIII and the MSA PF-IIB at the protofilament level indicates that our αSyn-SAA assay can replicate the structure of MSA αSyn aggregates.

Discussion

Compelling evidence supports the concept that αSyn aggregates can adopt diverse structures, commonly referred to as conformational strains, which appear to be associated with heterogeneous neuropathologies and diverse clinical manifestations in different synucleinopathies[10,11,66,67]. Distinct αSyn strains can faithfully propagate their properties during

the seeding and spreading of the aggregates in the brain. An important question in the field is whether *in vitro*-seeded fibrils can recapitulate the properties of the patient-derived aggregates. In the present study, we compared the biological and pathological activities of brain-derived and SAA-amplified αSyn aggregates using a biosensor cellular model and a transgenic mouse model of synucleinopathies. Our *in vitro* and *in vivo* findings demonstrated that the MSA-seeded, SAA-amplified αSyn fibrils retain the biological properties of the brain-derived MSA αSyn aggregates, providing support that SAA faithfully replicated strain properties. This conclusion is supported by the findings that both brain-derived and SAA-amplified aggregates: 1) produced similar MSA-specific filamentous, string-like αSyn inclusions in the biosensor cells (Supplementary Fig. 3), 2) induced a similar neurological disease in a mouse model with comparable clinical signs and disease courses (Fig.1); 3) targeted the same brain regions with similar neuropathological profiles (Fig.2); and 4) propagated αSyn aggregates that present similar biochemical properties (Fig. 3).

The M83 mouse model used in our experiments has been utilized in various αSyn transmission and strain characterization studies involving PD and MSA strains[9,23,68], as well as αSyn PFFs[19,53]. The M83+/- mice used in this study overexpress A53T mutant αSyn by three to four times over the level of the endogenous mouse αSyn[64]. Most M83 transmission studies have reported that the inoculation of MSA, but not PD BH[9,23,68], leads to the induction of pathology and disease in these animals. However, some studies provided evidence for the seeding of PD αSyn aggregates in this model[20,68]. This discrepancy may be explained by variations in the quantity and/or quality of PD αSyn seeds across different studies. Another intriguing observation is the neuronal accumulation of αSyn inclusions observed in M83+/- mice injected with MSA αSyn aggregates, which are thought to accumulate mostly in oligodendrocytes. One possible explanation for this result is that M83 mice overexpress human αSyn with the A53T mutation under the control

of the prion protein promoter, which is mostly expressed in neurons[23,53,64]. Nevertheless, this remains speculative and requires further research. Therefore, it is important to highlight that the MSA-infected M83 mice has some limitations and should be considered a synucleinopathy transmission model instead of an MSA disease model, due to the lack of the MSA-unique oligodendroglial αSyn inclusions in these animals. There is an undeniable urgency to develop alternative animal models and establish optimal transmission protocols for the biological characterization of the Lewy fold αSyn strain. Another limitation of this study is the characterization of the neuropathological changes by using an antibody specific for detection of 129 phosphorylated αSyn. Although this is a widely utilized biomarker of αSyn pathology, it may be useful to further characterize these mice using additional αSyn markers, such as the recently reported N-terminal αSyn antibody that detects a greater extent of MSA-related pathology in human brains[69]. Another limitation with the model systems used in this study is that both the M83 mice and the biosensor HEK293T cells might not necessarily propagate the most pathogenic αSyn aggregates present in the human MSA brain, but a specific conformation that replicate better in these models.

The conclusion from our *in vitro* and *in vivo* experiments that αSyn-SAA produces an αSyn strain with similar biological/pathological properties to the aggregates observed in MSA patients' brains is further supported by studies of the high-resolution structure of the SAA-generated fibrils. Indeed, the cryo-EM structure of the SAA-amplified protofilaments shares a high degree of similarity with the PF-IIB protofilaments found in MSA patients' brains (Fig. 6). These results suggest that our αSyn-SAA technology essentially maintains the conformation of the original seed after amplification. Our results contrast with a recent report by Lövestam and colleagues, which shows that most fibrils amplified from MSA CSF have different structures compared to the

aggregates extracted from the brains of MSA patients[70]. This study found seven different types of fibrils, five of which have different structures from the seeds used to generate them. Conversely, the other two types adopted a strikingly similar structure to one of the filaments observed in the MSA patient brain[70]. In our experience, the quality of the αSyn substrate, salt concentration, pH, temperature, and shaking conditions, among other factors, may significantly impact the results of the αSyn-SAA reaction[28,29]. Variations in any of these conditions likely affect the seeding process, and spontaneous aggregation can supersede the seeded aggregation. Therefore, the conditions necessary for the faithful seeding process should be optimized to avoid generating spontaneous aggregation products. For this purpose, it is crucial to thoroughly characterize the aggregates produced in the αSyn-SAA reaction using "low resolution" techniques, such as binding to thioflavin T (ThT) or other conformationally-sensitive dyes, limited protease resistance, antibody mapping, and secondary structure studies. In our previous report, we optimized the αSyn-SAA technology to discriminate MSA and PD strains with 95.4% accuracy, and the products of the reactions were carefully characterized by various techniques[27]. Lövestam and colleagues utilized a substantially different procedure for amplification. For example, their method involved 1 min of shaking and a 1 min resting period for a total of 120 hours or continuous shaking for 72 hours[70]. As described in our previous publications, the principle behind αSyn-SAA is a cyclic amplification with alternating phases of aggregate growth and fragmentation to multiply the number of seeds[28,29]. Cycles of incubation-shaking are crucial to producing aggregates that faithfully maintain the properties of the seeds. In our experience, extensive shaking with no or very short resting phases will likely cause substantial spontaneous unseeded aggregation, resulting in structures that do not resemble the original seeds.

In the current study, we have produced separate SAA-amplified αSyn fibril preparations that were seeded by different MSA (M1, M2, M3, and M5) CSF samples. However, we were only able to solve the high-resolution structure of the fibrils amplified from the M5 CSF sample, due to the lack of enough twisted filaments for the other SAA-amplified fibril samples (M1-M3). The cryo-EM structural studies found that the M5-amplified αSyn fibrils are composed of three sub-classes: MI (47%), MII (23%), and MIII (30%), and both MI and MII fibrils lack the well-defined crossover distance necessary for helical reconstruction. Our LC-MS data suggest that the LMW band aligns well with PF-IIB and, interestingly, the amplified product of M5 was enriched in the LMW band (Supplementary Fig. 7a). On the other hand, the HMW band may align with the PF-IB or PF-IIA, described for the brain MSA aggregates. It is possible that a proportion of the straight filament types may correspond to these structures. Our biological strain characterization results support this hypothesis. If these straight fibrils did not replicate the structure of the brain-derived, twisted MSA filaments, we would have expected distinct biological responses, considering that MI and MII fibrils account for 70% of the SAA-amplified products. Therefore, it is more likely that, through seeded amplification, both straight and twisted αSyn fibrils preserved the pathological MSA folds. Indeed, in the cryo-EM study of αSyn fibrils isolated from patients with Lewy body pathology, it has been suggested that both twisted and untwisted αSyn fibrils adopt the same Lewy-fold[24]. Hence, it is reasonable to propose similar structural preservation in the case of MSA-seeded, SAA-amplified fibrils.

Another important point is that despite the similarities at the protofilament level, brain-extracted and SAA-amplified aggregates showed different packing of the fibrils. Indeed, whereas brain-isolated αSyn fibrils consist of two different protofilaments, SAA-amplified aggregates were composed of two identical protofilaments. SAA conditions, including agitation pattern and speed,

temperature, pH, type of buffer and detergent, salt concentration, and lack of brain cofactors, can be critical for the *in vitro* formation of disease-associated protein aggregates. In this regard, it has been shown for MSA and several of the structures of Tau strains implicated in different tauopathies, the presence of additional electron densities that likely represent cofactor molecules, which might be an integral part of the core structures of brain-derived filaments and could help with the specific packing of the fibrils[25,71-73]. Studies of seeded prion amplification by PMCA have led to the identification of cofactor molecules, such as RNA and lipids, that are crucial for propagating pathogenic prions and maintaining prion strain properties[74]. Interestingly, both lipids and nucleic acids have been shown to modulate the *in vitro* aggregation of αSyn[75-77]. Therefore, cofactors might play a role in the propagation of αSyn strains.

In summary, our results provide both biological and structural evidence supporting that SAA-amplified αSyn fibrils can preserve the main strain properties of the brain MSA αSyn aggregates. These findings strengthen the relevance of using SAA-amplified fibrils as a proxy for the endogenous aggregates present in patients' samples and to use the assay for the identification of novel therapeutic molecules inhibiting αSyn aggregation and seeding.

Methods

Compliance statement. The use of human samples was approved by the institutional review board at The University of Texas Health Science Center at Houston. The use of brain from the Bispebjerg Brain Bank, as well as CSF samples, was approved under the regional ethical committee (j.no.: H-15016232) and Danish data protection agency (j.no.: P-2020-937). The study design and conduct complied with all relevant regulations regarding the use of human study participants and was conducted in accordance to the criteria set by the Declaration of Helsinki. Animal experiments

conducted in this study followed an animal protocol (Protocol #: AWC-22-0097) approved by the Animal Welfare Committee of the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at Houston.

Mice. Hemizygous M83 transgenic mice (M83+/-) were purchase from Jackson Laboratories (Strain #: 004479, B6;C3-Tg(Prnp-SNCA*A53T)83Vle/J). These mice express the full-length human αSyn with the A53T mutation under the mouse prion protein promoter. Animals were kept in cages under standard housing conditions: 22°C, 12-hour light/dark cycles, and unrestricted access to fresh water and chow. For this study we used 18 mice injected with MSA BH (55% females), 19 with MSA-seeded, SAA-amplified αSyn fibrils (43% females), 9 mice injected with αSyn PFF (55% females), and 6 mice injected with 1X PBS (50% females).

Human samples. Human MSA samples (**Table 1**), including postmortem brain tissue and antemortem CSF specimens, were kindly provided by the Bispebjerg Brain Bank, Centre for Neuroscience and Stereology, Department of Neurology, Copenhagen University Hospital, Bispebjerg and Frederiksberg Hospital, Denmark, and Drs. Ann Schmeichel, Wolfgang Singer, and Phillip Low (Mayo Clinic in Rochester). A list of human samples used in this study is provided in Table 1. Forty percent (w/v) brain homogenates (BH) were made in the homogenization buffer containing 1x PBS with 1x protease inhibitor (EDTA free) and 1x phosphatase inhibitor by using a Precellys homogenizer. After homogenization, BH samples were stored at -80 °C until use. Upon arrival, CSF samples were kept at -80 °C until use.

Expression and purification of recombinant αSyn. The expression and purification of monomeric αSyn was done as previously described[26]. Briefly, BL21(DE3)pLysS Escherichia coli cells bearing pET-21b plasmids were cultured and induced to express the wild-type human αSyn protein with a C-terminal His6-tag in the presence of 0.1 mM IPTG (isopropyl β-D- thiogalactoside)

at 25 °C. The bacterial cells were pelleted and lysed in lysis buffer (50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole, 1.0 mM PMSF, 0.1 mM tris-(2-carboxyethyl) phosphine (TCEP) and 1 mg/mL lysozyme) by sonication on ice. After an initial centrifugation at 12,000g for 15 min at 4 °C, the supernatant was then ultra-centrifuged again at 100,000 g for 30 min at 4 °C. The final supernatant was passed through a 0.45-μm filter and loaded onto a pre-packed IMAC column (Ni Sepharose 6 Fast Flow resin, GE Healthcare). Monomeric αSyn protein was eluted with 250 mM imidazole and combined for overnight dialysis in PBS (pH 7.4) at 4 °C. The following morning, the αSyn protein was filtered (Amicon Ultra centrifugal filters, 100-kDa cut-off, Millipore), aliquoted, and stored at -80 °C until use. Protein concentration was determined using a bicinchoninic acid (BCA) assay kit (Pierce).

αSyn Seed amplification assay (αSyn-SAA). Assay conditions previously described as αSyn-PMCA were used to generate the MSA-seeded, SAA-amplified αSyn fibrils for biological and structural studies[26,27]. In brief, monomeric αSyn at the concentration of 1 mg/mL in 100 mM PIPES, pH 6.5, 500 mM NaCl, were placed in opaque 96-well plates (Catalog #: 3916, Costar) in the presence of 5 μM ThT at a final volume of 200 μL, including 40 μL of CSF or diluted BH (10^{-5}) from MSA patients or controls. Samples were subjected to cyclic agitation (1 min at 500 rpm followed by 29 min without shaking) at 37 °C. The increase in ThT fluorescence was monitored at excitation of 435 nm and emission of 485 nm periodically, using a microplate spectrofluorometer Gemini-EM (Molecular Devices, Sunnyvale, CA). For the second round of amplification, a portion of the amplified material was diluted 1:100 into fresh αSyn substrate, and a new αSyn-SAA assay was conducted. The second-round SAA-amplified αSyn fibrils were used in both biological and structural studies.

Generation of preformed αSyn fibrils (αSyn PFFs). αSyn PFFs were produced by subjecting αSyn monomers (1 mg/mL in 1 x PBS) to constant agitation at 500 rpm at 37 °C for four consecutive days. The formation of αSyn PFFs was visually verified using Transmission Electron Microscopy.

Transmission Electron Microscopy. The sample was prepared using a previously described method[78]. Briefly, 5 µL of the sample was applied onto glow-discharged carbon-coated copper grids and allowed to adsorb for 30 seconds at room temperature. Excess liquid was carefully blotted away using filter paper. Immediately after blotting, the grid was washed by gently touching it to a drop of distilled water and blotting again. This washing step was repeated twice. The grid was then stained by applying 5 µL of 2% (w/v) uranyl acetate for 22 seconds. After staining, excess stain was removed by blotting with filter paper, and this step was repeated three times. Finally, the grid was air-dried at room temperature. Grids were imaged using a JEOL JEM-1400 transmission electron microscope operated at 120 kV.

αSyn biosensor cell assay. The αSyn biosensor cells were created by Dr. Marc Diamond (UT Southwestern in Texas) using kidney HEK293 cells expressing αSyn fused to green fluorescence protein[21]. For assays using MSA BH samples, crude 10% (w/v) BH was pre-cleared by centrifugation at 1,000 g for 5 minutes at 4 °C and the resulting supernatant was then ultra-centrifuged at 100,000 g for 30 minutes at 4 °C. The final pellet was resuspended in 1X PBS with one-third of the original volume and used to transduce the αSyn biosensor cells. One hundred thousand biosensor cells/well were plated in 24-well plates and cultured overnight. The next day, SAA-amplified αSyn fibrils or resuspended BH pellets were briefly sonicated immediately before the assay. Five µL of αSyn fibrils (1 mg/mL) or 10 µL of resuspended BH pellet (derived from 30 µL 10% BH) was mixed with 45 or 40 µL Opti-MEM medium (Invitrogen), respectively. Two µL

of Lipofectamine 2000 (Invitrogen) was mixed with 48 µL of Opti-MEM medium, added to the fibril/BH pellet-Opti-MEM mixture, and then incubated at room temperature for 20 minutes. After incubation, the 100 µL of fibril/BH pellet-Lipofectamine 2000-Opti-MEM mixture was added to the cultured cells per well. Treated cells were cultured for 4 to 10 days, and live cell images were obtained daily to monitor the morphologies of endogenous αSyn aggregates.

Stereotaxic injection. Both male and female M83+/- mice were used for inoculation studies. Two- to three-month-old mice were anesthetized with isoflurane, with 5% used in the induction chamber and 2% for maintenance. The hair on the top of the head was clipped to further secure the mouse in a stereotaxic frame. Eye ointment was applied, and 0.25% Bupivacaine at 2.5mg/kg was administered subcutaneously in the incision site for local blocking. Surgical scrubs were applied to the surgical area using Q-tips, alternating between povidone-iodine and 70% alcohol. This procedure was performed three times, extending outward 0.5 to 1.0 cm from the midline incision. A 1cm longitudinal incision in the skin was made to expose the skull, and the soft tissue was cleaned with a sterile Q-Tip. Then, a craniotomy was conducted with a 1 mm diameter surgical drill bit. Various inocula (MSA BH samples (40% BH, 10 µL per mouse), SAA-amplified αSyn fibrils (1 mg/mL, 10 µL per mouse), αSyn PFFs (1 mg/mL, 10 µL per mouse), and PBS (1X, 10 µL per mouse)) were briefly sonicated and injected stereotaxically into the dorsal striatum bilaterally (AP: 0.2 mm, ML: ±2 mm, DV: 2.6 mm, 5 µL per side) using a Hamilton model 80366 syringe mounted in a Stoelting™ Quintessential Injector with an injection rate of 2 µL/min. After inoculation, the skin incision was closed using a simple interrupted pattern with a 6-0 non-absorbable suture, which was removed 10 to 14 days later. Analgesia was provided by the administration of carprofen at a 5 mg/kg dose subcutaneously once a day for three days. After surgery, mice were monitored three times a week for the following clinical signs: kyphosis, ataxia,

lethargy, cachexia, and paralysis. Once mice developed any of the above clinical signs, they were monitored closely every day until the terminal stage of the disease was reached, characterized by severe hindlimb paralysis with difficulties in getting water and food. Then, mice were sacrificed and transcardially perfused with 1X PBS containing 5 mM EDTA before brains were collected. The right sagittal hemispheres were snap-frozen in liquid nitrogen and stored at -80 ºC for biochemical analyses, and the left hemispheres were kept in 10% formalin for histopathological studies. The disease duration was calculated as the number of days between the inoculation and sacrifice. Survival curves were plotted and compared in GraphPad Prism (10.4.1) using the Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. Outlier animals (analyzed at graphpad.com) or mice found dead without clinical signs were not included in analyses.

Immunohistochemistry (IHC) and Immunofluorescence (IF).

MSA brain characterization. For M1, M2, and M3, formalin-fixed paraffin-embedded (FFPE) brain tissue sections were used to evaluate the presence of MSA pathology, in particular the glial cytoplasmic inclusions (GCIs). OCT-embedded frozen brain tissue was used for M4. The anti-phospho-Ser129-αSyn antibody EP1536Y (Catalog #: ab51253, Abcam, 1:2000 dilution) was used to detect phosphorylated αSyn pathology.

Mouse brain tissue IHC. Formalin-fixed left hemispheres of M83+/- mice were dehydrated and embedded in paraffin wax. Formalin-fixed paraffin-embedded (FFPE) tissues were sectioned sagittally at a 10 µm thickness. Two sections 100 µm apart were mounted on the same glass slide. Slides were then rehydrated through Xylene, 100%, 95%, 90%, 80%, and 70% EtOH, and rinsed 3 times with PBS (5 min each). Antigen retrieval was done by incubating the slides at 80 ºC in a sodium citrate buffer (pH 6.0) containing 0.05% Tween-20 for 20 min. After rinsing with PBS (3 times, 5 min each), endogenous peroxidase activity was blocked by incubating slides in 3%

H_2O_2/10% methanol in PBS for 20 min on a rocking platform. The slides were then rinsed 3 times with PBS (5 min each) and blocked with 2.5% normal goat serum for 20 minutes at room temperature. Then the slides were incubated with rabbit anti-phospho-Ser129-αSyn antibody EP1536Y (Catalog #: ab51253, Abcam, 1:2000 dilution in PBS containing 0.2% Triton X-100 and 3% BSA) overnight at room temperature. The next day, the slides were washed 3 times in PBS (5 min each) and processed using ImmPRESS® Goat Anti-Rabbit IgG Polymer kit (Vector Laboratories, Burlingame, CA, USA). Then, slides were developed using a DAB peroxidase substrate kit (Catalog #: SK-4100, Vector Laboratories). For counterstaining, slides were incubated with hematoxylin (Mayer's MHS16, Sigma-Aldrich) for 1 min and rinsed with tap water for 5 min. Finally, the slides were dehydrated, mounted with DPX (Electron Microscopy Sciences, Hatfield, PA, USA), and let dry overnight.

Sagittal brain images were visualized using a Leica Stellaris Microscope with 10X or 63X objective. For each animal, IHC images of three sections with 100 µm intervals were used to quantify the pS129 burden in seven brain regions: cortex, hippocampus, hypothalamus, thalamus, midbrain, cerebellum, and brain stem. The pS129 positive staining area was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and the pS129 burden of each region was expressed as a percentage of a preset area for each brain region: Hippocampus (0.8 mm^2), Thalamus (0.8 mm^2), Hypothalamus (1.2 mm^2), Frontal cortex (0.8 mm^2), Cerebellum (0.2 mm^2), Midbrain (1.6 mm^2), and Brain stem (1.9 mm^2). The pS129 burdens were compared by two-way ANOVA with Sidak's multiple comparisons test in GraphPad Prism (10.4.1), and the values were expressed as means ± S.E.M. Statistical significance threshold was set at p=0.05.

For morphological characterization of the pS129-positive αSyn inclusions, sagittal brain images were obtained with a 40X objective. For each animal, IHC images of three sections with 100 µm

intervals were used to count the numbers of the ring-like and Lewy body (LB)-like αSyn deposits. The numbers of deposits with different morphologies were converted into percentages of total aggregates (ring-like plus LB-like) and compared by one-way ANOVA with Sidak's multiple comparisons test in GraphPad Prism (10.4.1). The final values expressed as means ± S.E.M. Statistical significance threshold was set at p=0.05.

Mouse brain tissue IF to co-localize staining with different cell types. Formalin-fixed left hemispheres of M83+/- mice were dehydrated and embedded in paraffin wax. Formalin-fixed paraffin-embedded (FFPE) tissues were sectioned sagittally at a thickness of 10 µm. Two sections that are 100 µm away were mounted on the same glass slide. Slides were then rehydrated through Xylene, 100%, 95%, 90%, 80%, and 70% EtOH, and rinsed 3 times with PBS (5 min each). To recognize oligodendrocytes, we used the goat anti-Olig2 antibody (Catalog #: AF2418, R&D Systems). Antigen retrieval was performed by incubating the slides at 80 ºC in a sodium citrate buffer (pH 6.0) containing 0.05% Tween-20 for 20 minutes. After rinsing with PBS three times (5 minutes each), a second antigen retrieval was carried out by incubating the slides at 80 ºC in a Tris-EDTA buffer (pH 9.0) containing 0.05% Tween-20 for 20 minutes. To stain for neurons, mouse anti-NeuN antibody (Catalog #: MAB377, Sigma-Aldrich) was used in conjunction with rabbit anti-phospho-Ser129-αSyn EP1536Y (Catalog #: ab51253, Abcam). Antigen retrieval was done by incubating the slides at 80 ºC in a sodium citrate buffer (pH 6.0). The slides were then rinsed three times with PBS (5 minutes each) and blocked with 3% BSA for 1 hour at room temperature. The primary antibodies Goat anti-Olig2 (1:50), Mouse anti-NeuN (1:50), and Rabbit anti-pSer129-αSyn (1:200) were diluted in PBS containing 0.2% Triton X-100 and 3% BSA and incubated overnight at room temperature. The next day, the slides were washed 3 times in PBS (5 min each) and treated using fluorescent secondary antibodies Donkey anti-Goat Fluor™ Plus 488

(Catalog #: A32814, Invitrogen™), Donkey anti-Mouse Alexa Fluor™ 488 (Catalog #: A21202, Invitrogen™), and Goat anti-Rabbit Alexa Fluor™ Plus 594 (Catalog #: A32740, Invitrogen™), respectively, at 1:500 dilution in PBST (Triton 0.2% in PBS 1x) for 2 hours. After two washes, the slides were incubated in DAPI 1X solution for 10 min (Catalog #: D9542, Sigma-Aldrich) and washed in PBS for 5 mins. Finally, the samples were mounted with Fluorsave (Catalog #: 345789, Millipore) and let dry overnight. IF images were visualized using a Leica Stellaris Microscope with a 40X objective.

Protease digestion assays. 10% (w/v) mouse brain homogenates (BH) were prepared in PBS with a protease inhibitor cocktail (Roche) using a Dounce tissue homogenizer. Halt Phosphatase Inhibitor (ThermoFisher) was added to a 1X final concentration. Then, BH samples were aliquoted and stored at -80 °C until use. For protease digestion assays, samples were first incubated with 0.5% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 1mM MgCl2 and 1U/µL Benzonase (EMD Millipore). After 20 minutes, the mixture was centrifuged at 1,000 g for 5 minutes. The supernatants were collected and treated with 1 mg/mL proteinase K (PK) for 30 minutes at 37 °C with agitation (800 rpm). Digestion was terminated with 1 mM Pefabloc and the samples were centrifuged at 200,000 g for 30 minutes at 4 °C. The supernatants were aspirated, and the pellets were resuspended in PBS. Pellet samples were boiled in NuPage sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose membranes. After transfer, membranes were blocked with 5% milk in PBS-T (0.05%) for 1 hour at room temperature and incubated with mouse anti-αSyn antibody (BD clone 42, 1:2,000 diluted in blocking buffer) overnight at 4 °C. The next day, membranes were washed 3 times with PBS-T (0.1%) (10 min each) and incubated with anti-mouse secondary antibody (Sigma, 1:10,000 diluted in blocking buffer). Membranes were then washed 3

times with PBS-T (0.1%) (10 min each), developed with Amersham ECL Prime Western Blotting Detection Reagent (Cytiva), and imaged using BioRad ChemiDoc imager.

Conformational stability assay for αSyn aggregates in mouse brain extracts. 10% (w/v) mouse brain homogenates (BH) were prepared in PBS with a protease inhibitor cocktail (Roche) using a Dounce tissue homogenizer. Halt Phosphatase Inhibitor (ThermoFisher) was added to 1X final concentration. Then, BH samples were aliquoted and stored at -80 °C until use. For conformational stability assay, samples were first incubated on ice with 0.5% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 1mM $MgCl_2$ and 1U/µL Benzonase (EMD Millipore) for 30 minutes, followed by centrifugation at 1,000 g for 5 minutes at 4 °C. The supernatants were collected and subjected to ultracentrifugation at 100,000 g for 1 hour at 4 °C. The resultant supernatants were discarded, and the pellets were resuspended in the starting volumes of 1X PBS with protease inhibitor (PI). 10 µL of mouse brain extracts were diluted to 40 µL by adding 30 µL 1X PBS+PI, then incubated with equal amounts of GdnHCl solutions of 0 to 6 M to have final GdnHCl concentrations of 0, 0.5, 1, 1.5, 2, 2.5, and 3 M. The mixtures were incubated at room temperature for 2h with gentle vortexing every 30 minutes. After incubation, samples were ultracentrifuged at 100,000g for 1 hour at 23 °C. The supernatants were discarded, and the pellets were washed with 100 µL of 1X PBS+PI, followed by ultracentrifugation at 100,000g for 1 hour at 23 °C. The supernatants were discarded, and the pellets were resuspended in 10 µL 1X PBS+PI. The suspended samples were subjected to SDS-PAGE and western blot analysis as described above, using the BD42 antibody. Western blot images were used in ImageJ to quantify the amount of GdnHCl resistant αSyn at different concentrations of GdnHCl, which were then normalized to the sample incubated without GdnHCl. The normalized residual αSyn levels were then plotted against different concentrations of GdnHCl and fit with curves using nonlinear regression in GraphPad

Prism, yielding $GdnHCl_{50}$ values for each animal. An unpaired, two-tailed t-test was used to compare $GdnHCl_{50}$ values.

Mass-spectrometric analysis of the PK-digested MSA-seeded, SAA-amplified samples. MSA CSF-seeded αSyn fibrils were treated with 1 mg/mL of Proteinase K (PK) and incubated for 1 hour on a thermomixer (37 °C, 250 rpm). The digestion reaction was then stopped by the addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). PK-digested samples underwent ultracentrifugation at 100,000g for 1 hour at 4 °C. The supernatant was discarded, and the pellet was washed with 150 µL of PBS buffer, followed by re-centrifugation under the same conditions. The final pellet was resuspended in the sample buffer and loaded onto a 16% Tricine gel for SDS-PAGE analysis. Two proteolytically digested bands were visualized using Coomassie Brilliant Blue staining.

Each gel band was cut into small pieces (1 mm^2) and placed in microcentrifuge tubes. A 25 mM ammonium bicarbonate/50% acetonitrile solution was added to cover the gel pieces and vortexed for 10 minutes. The supernatant was then removed and discarded. This process was repeated twice, after which 100% acetonitrile was added and vortexed for 10 minutes. The supernatant was discarded, and the gel pieces were air-dried. Next, 10 mM dithiothreitol in 25 mM ammonium bicarbonate was added to the dried gel pieces, and the mixture was incubated at 56°C for 1 hour. After removing the supernatant, 55 mM iodoacetamide was added, and the reaction proceeded in the dark at room temperature for 45 minutes. The supernatant was again discarded, and the gel pieces were washed with 25 mM ammonium bicarbonate by vortexing for 10 minutes. After discarding the supernatant, the gels were dehydrated using 25 mM ammonium bicarbonate/50% acetonitrile. The gel pieces were then fully dried using a 100% acetonitrile wash and air drying. To rehydrate the dried gel pieces, 12.5 ng/mL trypsin (Pierce™, MS-grade, ThermoFisher) was added just enough to cover them, and the samples were incubated on ice for 30 minutes. Additional

25 mM ammonium bicarbonate was added to cover the gel pieces, and the samples were incubated at 37°C overnight. The digested samples were transferred to clean tubes, and the gel pieces were treated with 50% acetonitrile and 5% formic acid, followed by vortexing for 30 minutes, then 5 minutes of sonication. The supernatant was collected, and this step was repeated. The combined supernatant was dried in a speed vac, and the dried samples were resuspended in 0.1% formic acid. An aliquot of the tryptic digest was analyzed by LC/MS/MS using an Orbitrap FusionTM TribridTM mass spectrometer (Thermo ScientificTM) interfaced with a Dionex UltiMate 3000 Binary RSLCnano System. Peptides were separated with a C18 reversed-phase column (100 μm ID x 25 cm, five μm / 18Å Reprosil-Pur C18-AQ beads from Dr Maisch, Ammerbuch-Entringen, Germany) at a flow rate of 350 nl/min. Gradient conditions were: 2%-22 % B for 90 min, 22%-35% B for 15 min, 35%-90% B for 5 min, followed by 90% B for 15 min (solvent A, 0.1 % formic acid in water; solvent B, 0.1% formic acid in acetonitrile). The peptides were analyzed using a data-dependent acquisition mode. The survey scan was performed with 120K resolution from 350 to 1550 m/z with an AGC target of 2e5 and a maximum injection time of 50 msec. The DDA cycle was limited to 3 seconds. Monoisotopic masses were then selected for further fragmentation for ions with 2 to 6 plus charge within a dynamic exclusion range of 30 seconds. Fragmentation priority was given to the most intense ions. Precursor ions were isolated using the quadrupole with an isolation window of 1.6 m/z. HCD was applied with a normalized collision energy of 35%, and the resulting fragments were detected using the rapid scan rate in the ion trap. The AGC target for MS/MS was set to 1e4, and the maximum injection time was limited to 35 msec.

The raw data files were processed using Thermo Scientific Proteome Discoverer software. The spectra were searched against the Uniprot-Homo sapiens database using Sequest. The database search was restricted to the following parameters. Trypsin was set as the enzyme with maximum

missed cleavages set to 2. The precursor ion tolerance was set to 10 ppm, and the fragment ion tolerance was set to 0.8 Da. Carbamidomethylation on cysteine was set as a static modification. Variable modifications were set to oxidation of methionine. The search results were validated and trimmed to a 1% FDR for strict conditions and 5% FDR for relaxed conditions using Percolator.

Cryo grid preparation and cryo-EM imaging. SAA-amplified αSyn fibrils were concentrated by ultracentrifugation at 100,000 g for 30 min and resuspension at 500 μM αSyn in 50 mM PIPES buffer. A 3 μL aliquot of the sample was applied to the glow-discharged 1.2/1.3 holey carbon-coated copper grids (Quantifoil R1.2/1.3, 200 mesh, Cu) and incubated for 60 seconds. Grids were then blotted for 3.5 seconds with filter paper and plunge-frozen in liquid ethane using a Vitrobot Mark IV (ThermoFisher Scientific). Cryo-EM Imaging was performed on a Titan Krios G3 TEM (ThermoFisher Scientific) operating at 300 keV equipped with a post-column BioQuantum Energy Filter. Images were recorded in super-resolution mode at a nominal magnification of 130,000X using a K2 Summit direct detector (Gatan Inc.). Each movie stack was recorded over 7 s, split into 35 frames, with a total accumulating electron dose of ~49 $e^{-}/Å^{2}$. Acquisition details are given in Supplementary Table 1.

Helical reconstruction. Fibrils were reconstructed in RELION-3.1[79,80]. Movie frames were corrected for beam-induced motions and dose-weighted in RELION using its own motion-correction implementation[81]. Non-dose-weighted micrographs were used for CTF estimation with CTFFIND-4.1[82]. Approximately thirteen thousand fibrils were picked manually. Particle segments were extracted using a box size of 1,500 pixels and an interbox distance of 14. 25 Å and downscaled to 700 pixels for 2D classification. Three types of fibril classes were observed. However, only one class had well-defined crossover distances, which were measured manually. The initial estimate for the helical twist of that class was -1.2°, assuming a helical rise of 4.75Å

and a crossover distance of around 700 Å (estimated from the MIII 2D class). Relion_helix_inimodel2d program was then used to reconstruct the de novo 3D initial model from the 2D classes[79], and segments were re-extracted without down-sampling in boxes of 640x640 pixels for use in 3D classifications and auto refinements. Final reconstructions of all the maps were performed after CTF refinement and Bayesian polishing, followed by 3D auto-refinement and standard RELION post-processing with a soft solvent mask that extended to 20% of the box height. The helical twist and rise after final reconstruction were found to be -1.07° and 4.8Å, respectively.

Model building. Initial segmentation of individual subunits for each type of fibril was done using Segger in UCSF Chimera[83,84]. Known αSyn structures were then fit to the individual subunits. In fibrils where the known structures appeared to agree with the overall fold observed in the density, the known structures were trimmed to the core residues that fit into the density. Where the folds of the known structure and density appeared to be different, Pathwalker[85] was run to generate an initial backbone trace; the density was seeded with 100 pseudoatoms, and a threshold at which point the overall fold could be seen was provided for each run of Pathwalker. As with the fibrils that fit the known structures, models based on Pathwalker were trimmed to contain just the core portions of the fold. These initial models, both from known structures and Pathwalker, were then completed in Coot[86] using the density map and the Baton Build option. The initial models were then converted into full-atom models in Coot and refined with Phenix real-space refinement[87]. Refined models were then imported back into Coot and adjusted manually to improve their fit on the map. For each fibril, 10 copies of the corresponding individual subunit were then propagated into the density map at adjacent positions. Phenix real-space refinement and Coot manual optimization were iterated, maximizing the model quality, model clash, and fit to density. Final

models were then analyzed using the comprehensive validation tools in Phenix. Model visualization and interpretation were carried out in Coot and UCSF Chimera.

Statistical analysis. All statistical analyses were performed using GraphPad Prism (10.4.1 version). The statistical significance threshold was set at p=0.05. Data were compared using the Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests, two-way ANOVA with Sidak's multiple comparisons test, one-way ANOVA with Sidak's multiple comparisons test, or unpaired two-tailed t-tests, as stated for different experiments.

Data Availability Statement.

Most data is included in the article and supplementary figures and raw data are in the source Data file. The Cryo-EM data for polA generated in this study have been deposited in the EMDB and RCSB databases under accession codes 8UKA [https://doi.org/10.2210/pdb8UKA/pdb] and EMD-42350 [https://www.ebi.ac.uk/pdbe/entry/emdb/EMD-42350]. Additional requests for data or materials will be provided without restrictions by contacting the corresponding author. Source Data are provide it with this work.

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Author contributions

F.W. was responsible for the *in vivo* studies, including data analysis and preparation of the final version of the figures, and, together with V.B., wrote the first draft of the manuscript. V.B. performed most of the cryo-EM studies, including data analysis. He also prepared the figures and wrote the first draft of the paper. C.B. monitored the animals, evaluated the disease progression, and performed all histological studies of injected animals. S.R. and N.D.C. performed the animal surgeries. M.P. and S.P. participate in performing some of the αSyn-SAA studies. T.A., D.G., and H. E. participated in the biochemical characterization of αSyn from mouse brain, including stability and protease-digestion studies. Q.N. performed the αSyn biosensor cell assay and obtained the cell images. S.K. and R.A. performed the histological characterization of the human brains used in this study. J.F. and S.A. contributed to patient material and postmortem tissue handling. D.H. prepared the samples for Mass/Spec analysis, screened samples in negative stain electron microscopy, and collaborated with data acquisition for cryo-EM studies. V.M. collaborated with data acquisition for cryo-EM studies. L.C.-M. provided advice regarding αSyn-SAA. M.L.B. was responsible for building the atomic model of the MIII αSyn fibril. M.S. generated the amplified materials using the αSyn-SAA assay. I.M. helped to characterize some of the amplified materials. I.S. provided expert advice on the cryo-EM studies. C.S. supervised the entire project, acquired funding, and was responsible for the final version of the manuscript. All authors reviewed and corrected the manuscript.

Competing interest

Claudio Soto is a Founder, Chief Scientific Officer, consultant and shareholder of Amprion Inc., a biotechnology company that focuses on the commercial use of seed amplification assays for high-sensitivity detection of misfolded protein aggregates involved in various neurodegenerative diseases. Luis Concha is an employee of Amprion Inc. and Sandra Pritzkow also has a conflict in relation to Amprion. The University of Texas Health Science Center at Houston has licensed patents and patent applications to Amprion. The remaining authors declare no competing interests.

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Case	Clinical diagnosis	Age (Years)	Sex	Disease Duration (Years)	Brain regions analyzed	CSF seeded αSyn-SAA	Used for inoculation
M1	MSA-C	65	Male	5	Putamen	Yes	BH (putamen), CSF-SAA fibrils
M2	MSA-C	59	Female	8	Putamen, midbrain	Yes	BH (putamen), CSF-SAA fibrils
M3	MSA-P	69	Female	8	Putamen, cerebellum	Yes	BH (putamen), CSF-SAA fibrils
M4	MSA-P	71	Female	10	Frontal cortex	No	BH (frontal cortex)
M5	MSA-C	47	Male	1.5	-	Yes	CSF-SAA fibrils

Table 1. Demographic and clinical characteristics of patient samples

Figure legends

Figure 1. MSA BH and MSA-seeded, SAA-amplified αSyn fibrils induce a similar neurological disease in M83+/- mice. (**a**) Kaplan-Meier survival curves of mice injected with MSA BH (M1 BH: n=5, M2 BH: n=5, M3 BH: n=5, and M4 BH: n=3), MSA-seeded, SAA-amplified αSyn fibrils (simplified as MSA SAA. M1 SAA: n=4, M2 SAA: n=4, M3 SAA: n=5, and M5 SAA: n=6), αSyn PFF (n=9), or 1X PBS (n=6). (**b**) Representative IHC images (10X) of sagittal brain sections of mice inoculated with MSA BH (n=18), MSA SAA (n=19), PFF (n=9), or PBS (n=6). pS129-positive αSyn deposits were observed in the brains of all 18 mice inoculated with MSA BH and all 19 mice inoculated with MSA SAA, but not in any of the 9 mice injected with PFF, or any of the 6 mice injected with PBS. Scale bar: 1 mm. (**c**) Representative western blot image of protease digestion of BHs of mice inoculated with MSA BH (n=18), MSA SAA (n=19), PFF (n=9), and PBS (n=6). BHs of all 18 mice inoculated with MSA BH and all 19 mice inoculated with MSA SAA, and no BHs of the 9 mice injected with PFF and the 6 mice injected with PBS, contain PK-resistant αSyn aggregates. The undigested (PK -) BH sample was loaded into the gel as a migration control. Mouse anti-total αSyn antibody (Fisher Scientific: BDB610787) was used to detect αSyn. Numbers on the left indicate molecular weight markers.

Figure 2. MSA brain-derived and MSA-seeded αSyn aggregates produced the same neuropathological changes in M83+/- mice. (**a**) Representative IHC images (10X and 40X) of pS129-positive αSyn deposits in the Hippocampus, Thalamus, Hypothalamus, Cortex, Cerebellum, Midbrain, and Brain stem of mice inoculated with MSA BH and MSA SAA. Insets in the 10X images correspond to the 63X images on the right panels. Scale bars: 100 µm. (**b**) Quantification

of pS129-positive αSyn burden in different brain regions. The values were expressed as means ± SEM (standard error of the mean). No significant difference in the amount of pS129 αSyn burden was found in the indicated brain regions between mice injected with MSA BH (n=10) and mice injected with MSA SAA (n=11) by two-way ANOVA with Sidak's multiple comparisons test. The statistical significance threshold was set at p=0.05. (**c**) Representative IHC images (40X) of pS129-positive αSyn deposits in midbrains of mice injected with MSA BH and MSA SAA. Both LB-like and ring-like pS129-positive αSyn deposits were found in both groups of mice. Scale bar: 10 μm. (**d**) Quantification of LB-like vs ring-like pS129-positive αSyn pathology in midbrains of mice injected with either MSA BH (n=10) or MSA SAA (n=11). The values were expressed as means ± SEM. No significant difference in the distribution of distinct pS129 αSyn pathology was found in the midbrain between mice injected with MSA BH and mice injected with MSA SAA by one-way ANOVA with Sidak's multiple comparisons test. The statistical significance threshold was set at p=0.05.

Figure 3. Conformational characterization of αSyn aggregates in the brains of inoculated mice. (**a**) Representative western blot images and the corresponding denaturation curves for the conformational stability analysis of αSyn aggregates from mice injected with MSA BH and MSA SAA. The western blots show the amount of aggregated αSyn that remained in the pellet after centrifugation following treatment with different concentrations of GdnHCl. For this study, we use the BD42 antibody. The values shown in the curves were expressed as means ± SEM. Statistical analysis by a two-tailed t-test revealed no significant difference in $GdnHCl_{50}$ values between mice injected with MSA BH (n=5) and mice injected with MSA SAA (n=5). The statistical significance threshold was set at p=0.05. (**b**) Representative SAA kinetic curves of diluted BHs (10^{-5}) of mice

injected with MSA BH (left panels, two animals) and MSA SAA (right panels, two animals). Experiments were done in triplicate. Error bars indicate the SEM. The ThT fluorescent values of seeded amplification of αSyn fibrils were consistent with the ThT reading pattern for the MSA αSyn strain (**Supplementary Figure 2**).

Figure 4. Cryo-EM characterization of SAA-amplified fibrils. (**a**) Cryo-electron microscopy image of MSA-seeded, SAA-amplified αSyn fibrils. Scale bar: 50 nm. (**b**) 2D class averages of MI, MII, and MIII fibrils in a box spanning 794 Å. (**c**) FSC curve for final reconstructed MIII map. (**d**) Local resolution map for MIII fibrils, with the legend indicating resolutions in Å.

Figure 5. Cryo-EM structure of MSA-seeded, SAA-amplified αSyn fibrils. (**a**) αSyn primary sequence depicting β-strands and loop regions. The NAC region is highlighted in orange. (**b**) Cryo-EM density map (transparent grey) and fitted atomic model. (**c**) Cartoon view of five successive rungs of the amplified fibril.

Figure 6. Comparison of SAA-amplified fibrils with protofilaments PF-IIB present in the MSA patient brain. Atomic model of the amplified fibril (blue) overlaid with the model of protofilament (**a**) PF-IIB1 (yellow) and (**b**) PF-IIB2 (red). The R.M.S.D. values for 43 pruned atom pairs are (**a**) 2.3 Å and (**b**) 1.7 Å.

Brief Summary

Alpha-synuclein (αSyn) aggregates implicated in synucleinopathies can be amplified by a seed amplification assay (SAA). Here, the authors show that SAA amplified αSyn aggregates from the

CSF of a multiple system atrophy patient retain the biological and structural properties of the aggregates deposited in the brain

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