Structures of α-synuclein filaments from multiple system atrophy

A causal link between α-synuclein assembly and disease was established by the findings that missense mutations in SNCA (the gene that encodes α-synuclein) and multiplications of this gene give rise to rare inherited forms of Parkinson’s disease and Parkinson’s disease with dementia. Some mutations also cause DLB. The missense mutations in SNCA that result in G51D and A53E substitutions can give rise to atypical synucleinopathies, with a mixture of Parkinson’s disease and MSA pathologies. Sequence variation in the regulatory region of SNCA is associated with an increased expression of α-synuclein and a heightened risk of developing idiopathic Parkinson’s disease, which accounts for over 90% of cases of this disease.

MSA is a sporadic synucleinopathy of adult onset, with symptoms of parkinsonism, cerebellar ataxia and autonomic failure. Cases of MSA are classified as MSA-P, which show predominant parkinsonism caused by striatonigral degeneration, and MSA-C, which show cerebellar ataxia associated with olivopontocerebellar atrophy. Autonomic dysfunction is common to both subtypes. In neuropathological terms, MSA is defined by regional nerve cell loss and the presence of abundant filamentous α-synuclein inclusions in nerve cells. The mean duration of the disease is 6–10 years, but survival times of 18–20 years have been reported. The late appearance of autonomic dysfunction correlates with prolonged survival.

α-Synuclein is a 140-amino-acid protein, over half of which (residues 7–87) consists of 7 imperfect repeats, with the consensus sequence KTEGV. These residues encompass the lipid-binding domain. The repeats partially overlap with a hydrophobic region (residues 61–95) known as the non-β-amyloid component, which is necessary for the assembly of recombinant α-synuclein into filaments. The carboxy-terminal region (residues 96–140) is negatively charged, and its truncation results in the formation of, filamentous inclusions of α-synuclein in brain cells.

Seeded assembly of α-synuclein, propagation of inclusions and nerve cell death have been demonstrated in a variety of systems. Assemblies of recombinant α-synuclein with different morphologies account for over 90% of cases of this disease.
have displayed distinct seeding capacities. Moreover, α-synuclein from glial cytoplasmic inclusions has previously been reported to be approximately three orders of magnitude more potent than α-synuclein from Lewy bodies in seeding the aggregation of α-synuclein. Indirect evidence has also suggested that distinct conformers of assembled α-synuclein may characterize MSA and disorders with Lewy pathology. Solubility in sodium dodecyl sulfate (SDS) distinguishes α-synuclein filaments of MSA from those of DLB.

**Neuropathological characteristics**

We used sarkosyl to extract filaments from the putamen of five individuals with a neuropathologically confirmed diagnosis of MSA (hereafter referred to as MSA cases 1–5). In MSA cases 1, 2, 3 and 5, filaments were also extracted from the frontal cortex; the same was true of the cerebellum for MSA case 1. Most sarkosyl-insoluble α-synuclein phosphorylated at S129 was soluble in SDS. More than 90% of α-synuclein inclusions are phosphorylated at S129. For MSA case 1, the individual was diagnosed as MSA-P and had an age at death of 85 years; for MSA cases 2, 3, 4 and 5, the individuals were diagnosed as MSA-C and had ages at death of 68, 59, 64 and 70 years, respectively. The disease durations were 9, 18, 10 and 19 years for MSA cases 1, 2, 3, 4 and 5, respectively.

The abundant glial cytoplasmic inclusions and neuronal inclusions were stained by an antibody specific for α-synuclein phosphorylated at S129. Negative-stain electron microscopy, all five cases of MSA showed a majority of twisted filaments, which had a diameter of 10 nm and a periodicity of 80–100 nm (Fig. 1b, Extended Data Fig. 1b). Immunogold negative-stain electron microscopy with the anti-α-synuclein antibody PER4 showed decoration of MSA filaments (Extended Data Fig. 1c, d), consistent with previous findings. Immunoblotting sarkosyl-insoluble material from the putamen with the antibodies Syn303 and PER4 revealed evidence of monomeric α-synuclein and high-molecular-weight aggregates (Extended Data Fig. 1e). Truncated α-synuclein was also present. When the antibody pS129 was used, full-length α-synuclein was the predominant species. Consistent with the results of immunostaining (Fig. 1a), in MSA cases 1 and 3 the putamen contained lower levels of α-synuclein than it did in MSA cases 2, 4 and 5.

We observed the seeded aggregation of expressed wild-type human α-synuclein in SH-SY5Y cells after the addition of sarkosyl-insoluble seeds from the putamen of MSA cases 1–5 (Extended Data Fig. 2). Seeds from MSA case 3 were the most potent, and those from MSA case 2 were least effective at inducing seeded aggregation. Seeds from MSA cases 1, 4 and 5 had intermediate seeding potencies.

**Two types of MSA filament**

We imaged the sarkosyl-insoluble filaments using cryo-electron microscopy (cryo-EM) (Extended Data Fig. 3). These filaments looked identical upon visual inspection of the micrographs, but reference-free 2D class averaging revealed two types of filament (Extended Data Fig. 3b, d). Type I filaments were less symmetrical than the type II filaments. In the putamen, the ratios of type I to type II filaments were 80:20 in MSA case 1 and 20:80 in MSA case 2. MSA cases 3 and 4 had mostly type I filaments, and MSA case 5 had only type II filaments (Fig. 2a, b).

This suggests that the duration of MSA may correlate with the ratio of filament types in putamen, but additional cases of disease are required to establish this more firmly. What is true for the putamen may not be true of α-synuclein filaments from other affected regions of the brain. We identified predominantly type I filaments in the putamen in MSA cases 1 and 3 (Fig. 2), whereas we found almost exclusively type II filaments in the cerebellum in MSA case 1 and in the frontal cortex in MSA cases 2, 3 and 5 (Extended Data Fig. 3d). It remains to be seen whether the MSA type I and type II filaments are common to both nerve and glial cells.

**Protofilaments adopt extended folds**

We determined the cryo-EM structures of MSA type I and type II filaments from the putamen to resolutions sufficient for de novo atomic modelling (Fig. 2, Extended Data Table 1). The best structures were resolved to a resolution of 2.6 Å for type I filaments in MSA case 1, and a resolution of 3.1 Å for type II filaments in MSA case 2 (Extended Data Fig. 4). Type I and type II filaments are each made of two protofilaments, which consist of an extended N-terminal arm and a compact C-terminal body (Fig. 2, Extended Data Fig. 5). Both the type I and the type II filaments are asymmetrical. The larger protofilament of the type I filaments (PF-IA) comprises residues G14–F94 of α-synuclein, and the smaller protofilament (PF-IB) comprises residues K32–Q99 (Fig. 2c). For type II filaments, PF-IIA and PF-IIB comprise residues G14–F94 and G36–Q99, respectively (Fig. 2d). Protofilament folds differ from each other within and between filament types. MSA type I and type II filaments are thus collectively made of four distinct protofilaments (Figs. 2, 3a).

PF-IA comprises 12 β-strands. The N-terminal arm of PF-IA consists of a cross-β hairpin (residues G14–G31) and an extended one-layered L-shaped motif at residues K32–K45. The C-terminal body of PF-IA adopts a three-layered L-shaped motif. The outer layer (residues E46–V66) is the longest of these layers, and packs against the outside of the central layer (residues G67–E83). A salt bridge between E46 and K80 stabilizes this interaction. The shorter inner layer (residues G84–F94) packs against the inside of the central layer. Glycine-rich turns connect the layers. PF-IB comprises 10 β-strands. The N-terminal arm of PF-IB consists only of a cross-β hairpin at residues G25–K45. The three-layered L-shaped motif of the C-terminal body of PF-IB is topologically similar to that in PF-IA. Nevertheless, the two motifs differ in structure—most notably in the packing of the inner layer against the central layer by the residues that follow G86. The body of PF-IA ends at F94, whereas the body of PF-IB extends to Q99.
PF-IIA also comprises 12 β-strands and spans residues G14–F94. The PF-IA and PF-IIA protofilaments have similar N-terminal arms (Fig. 3). Although the C-terminal body of PF-IIA adopts a three-layered L-shaped motif, its conformation differs from that of PF-IA. In type I protofilaments residues G47–V52 from the outer layer pack against residues A76–K80 from the central layer, whereas in PF-IIA this packing is shifted by two residues and involves residues V74–A78 in the central layer. This creates a sizeable cavity between the central layer and the L-shaped bend at E57 in the outer layer. This shift also increases the distance between the Ca atoms of E46 and K80 by 5 Å, but a salt bridge may still form between their side chains. PF-IIB is the smallest protofilament core and comprises 9 β-strands. The N-terminal arm of PF-IIB is made of a single L-shaped conformation at residues G36–K45. The C-terminal body of PF-IIB forms a three-layered L-shaped motif, which exists in two conformations: PF-IIB1, which is virtually identical to PF-IB, and PF-IIB2, which has a different backbone conformation at residues T81–A90 (Fig. 2f–h). On the basis of the number of classified helical segments, the ratio of type II filaments containing PF-IIB1 (type II1) to type II filaments containing PF-IIB2 (type II2) was 20:80 (Fig. 2b, Extended Data Table 1).

Filaments enclose additional molecules
In MSA type I and type II filaments, two non-identical protofilaments pack against each other through an extended interface that forms a large cavity surrounded by the side chains of K43, K45 and H50 from each protofilament.

**Fig. 2** Cryo-EM maps and atomic models of type I and type II filaments of α-synuclein from MSA. a, b, Cryo-EM maps of type I filaments from the putamen in MSA cases 1, 2, 3 and 4 (a) and of type II filaments from the putamen in MSA cases 1, 2 and 5 (b). For MSA case 2, enlarged views of the different regions in type II, and type I, filaments are also shown. c, d, Schematic of the primary structure of human α-synuclein, indicating the cores of PF-IA, PF-IB, PF-IIA and PF-IIB. The non-β-amyloid component (NAC) domain (residues 61–95) is also shown. C, C terminus; N, N terminus. e, f, Sharpened, high-resolution cryo-EM maps of type I (e) and type II (f) filaments of α-synuclein from MSA, with overlaid atomic models. Unsharpened, 4.5 Å low-pass-filtered maps are in grey. The high-resolution maps show weaker densities that extend from the N- and C-terminal regions, a peptide-like density in PF-IIA, and weaker densities that border the solvent-exposed chains of K32 and K34 in PF-IA, PF-IB and PF-IIA. Weaker densities that border the solvent-exposed chains of K58 and K60 in PF-IA and PF-IIA are also present. g, h, Cryo-EM structures of A78–Q99 of PF-IIB, illustrating heterogeneity (PF-IIB1 and PF-IIB2). There is strong density at the protofilament interfaces of type I and type II filaments, which is surrounded by the side chains of K43, K45 and H50 from each protofilament.
of the density remains to be established. The observations that it is
disconnected from the density of the α-synuclein polypeptide chains
and that it would need to compensate four positive charges for every
β-sheet rung suggest that this density is non-proteinaceous. The
cavity is larger in type I filaments than in type II filaments and contains
additional, smaller densities between H50, G51 and A53 of PF-IA, and
V37 and Y39 of PF-IB. Although we used sarkosyl to extract filaments,
the central cavity is not large enough to accommodate one sarkosyl
molecule per rung. Moreover, the negative charge of the headgroup of
sarkosyl (−1) cannot compensate for the positive charge (+4 per rung)
of the central cavity, and the polar nature of the cavity is not compatible
with the fatty-acid tail of sarkosyl.

Besides the density in the large cavity at the interface of the proto-
filaments, several other densities are visible at lower intensities. At the
N and C termini of the ordered cores of all four protofilaments, fuzzy
densities probably correspond to less-well-ordered extensions of the
core. The longest extensions are seen for PF-IA and PF-IIA. Unlike PF-IA,
a peptide-like density of unknown identity is packed against residues
K80–E83 of PF-IIA. This density may correspond to an extension of the
C-terminus of α-synuclein in PF-IIA, or to an unknown protein that
is bound to the filament core. Additional unconnected densities are
observed in front of pairs of lysines on the exterior of the filaments—that
is, in front of K32 and K34 of PF-IA, PF-IB and PF-IIA, as well as in front
of K58 and K60 of PF-IA and PF-IIA. Similar densities have previously
been observed in front of pairs of lysines on the exterior of tau filaments
from Alzheimer’s disease4, Pick’s disease5, chronic traumatic encephalopathy6
and corticobasal degeneration7, although the molecules that
form these densities remain unknown.

In the structures of MSA type I and type II filaments, residues G51 and
A53 of α-synuclein form part of the interfaces of the protofilaments,
and are located close to K43, K45 and H50. The mutations in SNCA that
lead to G51D and A53E substitutions are the only known disease-causing
mutations that increase the negative charge of α-synuclein8–15. All four
protofilaments of the MSA filaments can accommodate the side chains of
D51 or E53 without substantial structural changes (Extended Data
Fig. 6c, d). The presence of D51 or E53 may thus give rise to similar
type I and type II filament structures. However, the changes in charge
of the residues that surround the central cavity may lead to a different
molecular composition of the additional density in cases of MSA with
G51D and A53E substitutions, as compared to sporadic MSA.

Using mass spectrometry of sarkosyl-insoluble α-synuclein from the
putamen, we found that N-terminal acetylation, C-terminal truncation
and ubiquitination of K6 and K12 were common to MSA cases 1–5. In
the sequences of the filament cores, K21 was ubiquitinated in all cases.
Despite having identical structures of type I and type II filaments, in
only some cases of MSA did sarkosyl-insoluble α-synuclein also show
ubiquitination of K23, K60 and K80, acetylation of K21, K23, K32, K34,
K45, K58, K60, K80 and K96, as well as phosphorylation of Y39, T59,
T64, T72 and T81. With the exception of ubiquitination of K80, the
percentage of α-synuclein molecules modified at a given residue was
low, which suggests that these post-translational modifications are not
responsible for the additional densities in the cryo-EM maps. Some of
these modifications have previously been described1, but others are
newly described here.

Ubiquitination of K80 was detected in sarkosyl-insoluble α-synuclein
in MSA cases 2 and 5, which show a preponderance of type II filaments.
This bulky post-translational modification, which is compatible with
the structure of PF-IIA, clashes with the surroundings of the K80 side
chain in PF-IA, PF-IB and PF-IIA. Moreover, one end of the peptide-like
density—which is specific to type II filaments—is located next to K80
of PF-IIA. This density, which does not appear to be connected to the
side chain of K80, may consist of a mixture of different sequences and
ubiquitination might possibly have a role. Phosphorylation of T72 may
favour PF-IIA over PF-IA. The side chain of T72 is buried in PF-IA, whereas
it borders a large cavity between the outer and central layers in PF-IIA.
Phosphorylation of T81 may distinguish PF-IIA from PF-IA, as the side
chain of this residue is buried in PF-IIB, but is solvent-exposed in PF-IIB2.
Post-translational modifications in only one protofilament may favour
the formation of asymmetrical type I and type II filaments. Thus, in the
structures of PF-IA and PF-IIA, the side chain of K60 is solvent-exposed
and can carry a bulky modification. By contrast, in the structures of
PF-IB and PF-IIIB this side chain is buried in the interfaces between
protofilaments.

**DLB filaments**

Our results show that α-synuclein filaments adopt the same structures
in different individuals with MSA. Similar observations have previously
been made for tau filaments from the brains of individuals with Alzhei-
mer’s disease16–19, Pick’s disease20, chronic traumatic encephalopathy21.
and corticobasal degeneration. Tau filaments adopt an identical fold in individuals with the same disease, but different tauopathies are characterized by distinct folds. To assess whether the same is true of synucleinopathies, we used cryo-EM to examine α-synuclein filaments that were isolated from the brains of three individuals with a neuropathologically confirmed diagnosis of DLB.

In the frontal cortex and amygdala, abundant Lewy bodies and Lewy neurites were stained by the pS129 antibody (Extended Data Fig. 7a). Following sarkosyl extraction, α-synuclein filaments from the brains of individuals with DLB did not appear to twist and were thinner than those from the brains of individuals with MSA (Extended Data Fig. 7b, d). Similar differences between α-synuclein filaments from the brains of individuals with Lewy pathology and those with MSA have previously been described. Unlike MSA, most sarkosyl-insoluble α-synuclein phosphorylated at S129 from the brains of individuals with DLB was SDS-insoluble, consistent with previous findings. The lack of twist precluded the determination of the three-dimensional structure of α-synuclein filaments from DLB by cryo-EM, but on the basis of reference-free 2D class averages (Extended Data Fig. 7e) we conclude that the structures of α-synuclein filaments of DLB are different from those of MSA.

Synthetic filaments

We next compared the structures of filaments from the brains of individuals with MSA with those assembled in vitro from recombinant wild-type and mutant α-synucleins. As with all of the MSA protofilaments, some recombinant α-synuclein protofilaments also contain three-layered L-shaped motifs (Extended Data Fig. 9). One feature that the recombinant α-synuclein filaments with the three-layered L-shaped motif have in common is that they were assembled in the presence of polyanions, such as phosphate, of MSA filaments and most recombinant filaments are made of either one protofilament or two identical protofilaments related by helical symmetry.

As with all of the MSA protofilaments, some recombinant α-synuclein protofilaments also contain three-layered L-shaped motifs (Extended Data Fig. 8). The largest differences are in the extended sizes of the MSA protofilaments, and in the asymmetrical packing of these protofilaments. None of the recombinant α-synuclein filaments contain the long N-terminal arms of MSA filaments and most recombinant filaments are made of either one protofilament or two identical protofilaments related by helical symmetry.

Outlook

Here we establish the presence of two types of α-synuclein filament in MSA, and suggest that different conformers or strains of assembled α-synuclein exist in DLB. To understand the causes and spreading of α-synuclein pathology as well as the distinguishing characteristics of synucleinopathies, it will be important to identify the mechanisms of seed formation and subsequent assembly. The presence of post-translational modifications in assembled α-synuclein is well-established, but their relevance for assembly is not understood.

In addition, the structures of α-synuclein filaments in MSA reveal the presence of non-proteinaceous molecules, reminiscent of findings in tauopathies. It will be important to identify the chemical nature of these molecules and to study their effects—alone or in conjunction with post-translational modifications—on α-synuclein and tau assembly. Understanding the structural specificity of filament assembly in disease will facilitate the development of tracers for imaging filamentous amyloid assemblies of α-synuclein in the brain, and of molecules that prevent, inhibit and reverse filament formation.

Online content

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Methods

No statistical methods were used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment.

Clinical history and neuropathology

MSA case 1 was in an 85-year-old woman who died with a neuropathologically confirmed diagnosis of MSA-P following a 9-year history of bradykinesia, rigidity in upper and lower limbs and autonomic failure. MSA case 2 was in a 68-year-old woman who died with a neuropathologically confirmed diagnosis of MSA-C following an 18-year history of cerebellar ataxia, gait disturbance and autonomic failure. MSA case 3 was in a 59-year-old man who died with a neuropathologically confirmed diagnosis of MSA-C following a 9-year history of dystarsia, cerebellar ataxia and autonomic failure. MSA case 4 was in a 64-year-old man who died with a neuropathologically confirmed diagnosis of MSA-C following a 10-year history of cerebellar ataxia, dystarsia and autonomic failure. MSA case 5 was in a 70-year-old man who died with a neuropathologically confirmed diagnosis of MSA-C following a 9-year history of cerebellar ataxia and autonomic failure. DLB case 1 was in a 59-year-old man who died with a neuropathologically confirmed diagnosis of DLB following a 10-year history of resting tremor, bradykinesia, rigidity, postural instability and visual hallucinations. DLB case 2 was in a 74-year-old man who died with a neuropathologically confirmed diagnosis of diffuse Lewy body disease following a 13-year history of bradykinesia, postural instability and visual hallucinations. DLB case 3 was in a 78-year-old man who died with a neuropathologically confirmed diagnosis of diffuse Lewy body disease following a 15-year history of resting tremor, bradykinesia, autonomic symptoms and visual hallucinations.

Extraction of α-synuclein filaments

Sarkosyl-insoluble material was extracted from fresh-frozen brain regions of individuals with MSA and DLB, essentially as previously described. In brief, tissues were homogenized in 20 vol (v/w) extraction buffer consisting of 10 mM Tris-HCl, pH 7.5, 0.8 M NaCl, 10% sucrose and 1 mM EGTA. Homogenates were brought to 2% sarkosyl and incubated for 30 min. Following a 10 min centrifugation at 10,000 g, the supernatants were spun at 100,000 g for 20 min. The pellets were resuspended in 500 μg/ml extraction buffer and centrifuged at 3,000 g for 5 min. The supernatants were diluted threefold in 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 10% sucrose and 0.2% sarkosyl, and spun at 166,000 g for 30 min. Sarkosyl-insoluble pellets were resuspended in 100 μg/ml of 30 mM Tris-HCl, pH 7.4. We used approximately 0.5 mg tissue for cryo-EM and 0.5 g for negative-stain immuno-electron microscopy. In some experiments, sarkosyl-insoluble pellets were resuspended in 30 mM Tris-HCl, 2% SDS, left at room temperature for 30 min and spun at 100,000 g for 30 min. The pellets were resuspended in 8 M urea. Both supernatants and pellets were immunoblotted using anti-pS129 α-synuclein antibody.

Immunolabelling and histology

Immunogold negative-stain electron microscopy and western blotting were carried out as previously described. Filaments were extracted from putamen in MSA cases 1–5, the frontal cortex in MSA cases 1, 2, 3 and 5, and the cerebellum in MSA case 1, the frontal cortex in DLB cases 1 and 2, and the amygdala in DLB case 3. PER445, a rabbit polyclonal serum that recognizes the carboxy-terminal region of α-synuclein, was used at 1:50. Images were acquired at 11,000× with a Gatan Orius SC200B CCD detector on a Tecnai G2 Spirit at 120 kV. For western blotting, the samples were resolved on 4–12% Bis-Tris gels (NuPage) and the primary antibodies diluted in PBS plus 0.1% Tween 20 and 5% non-fat dry milk. Before blocking, membranes were fixed with 1% paraformaldehyde for 30 min. Primary antibodies were: Syn303 (a mouse monoclonal antibody that recognizes the N-terminus of α-synuclein) (BioLegend) at 1:4,000, PER4 at 1:4,000 and pS129 (a rabbit monoclonal antibody that recognizes α-synuclein phosphorylated at S129) (ab51253, Abcam) at 1:5,000. Histology and immunohistochemistry were carried out as previously described56,59. Some brain sections (8 μm) were counterstained with haematoxylin. The primary antibody was specific for α-synuclein phosphorylated at S129 (ab51253).

Seeded α-synuclein aggregation

The ability of sarkosyl-insoluble fractions from the putamen in MSA cases 1–5 to convert expressed soluble α-synuclein into its abnormal form was examined, as previously described46,47. Following the addition of variable amounts of seeds (ranging from 1 to 4,700 pg/ml), transfected SH-SY5Y cells were incubated for three days. Sarkosyl-insoluble α-synuclein was extracted, run on 15% SDS-PAGE and immunoblotted with a mouse monoclonal antibody specific for α-synuclein phosphorylated at S129 (pSyn64 at 1:1,000). Band intensities were quantified using ImageJ software.

Mass spectrometry of sarkosyl-insoluble α-synuclein

Protease digestion and nano-flow liquid chromatography–ion trap tandem mass spectrometry (LC-MS/MS) (Thomast Scientific, Q Exactive HF) were used to identify post-translational modifications in sarkosyl-insoluble α-synuclein extracted from the putamen in MSA cases 1–552. The concentration of α-synuclein was determined using an enzyme-linked immunosorbent assay kit (Abcam). Sarkosyl-insoluble fractions containing approximately 65 ng of α-synuclein were treated with 70% formic acid for 1 h at room temperature, diluted in water and dried. They were digested overnight with trypsin and lysyl-endopeptidase. Peptides were then analysed by LC-MS/MS.

Cryo-EM

Extracted α-synuclein filaments were applied to glow-discharged holey carbon gold grids (Quantifoil R1.2/1.3, 300 mesh) and plunge-frozen in liquid ethane using an FEI Vitrobot Mark IV. Micrographs were acquired using two different Thermo Fisher Titan Krios microscopes that were operated at 300 kV. On the first microscope, at the MRC Laboratory of Molecular Biology, a Gatan K2 Summit direct detector was used in counting mode. On the second microscope, at the UK electron Bio-Imaging Centre (eBIC), a Gatan K3 direct detector in super-resolution mode was used. Inelastically scattered electrons were removed by a GIF Quantum energy filter (Gatan) using a slit width of 20 eV. Further details are given in Extended Data Table 1 and Supplementary Tables 1–3.

Helical reconstruction

Movie frames were corrected for beam-induced motion and dose-weighted using the motion-correction implementation of RELION65. Super-resolution K3 movies were Fourier-cropped during motion correction, and the reported pixel sizes in Extended Data Table 1 and Supplementary Tables 1–3 are the physical pixel sizes. Aligned, non-dose-weighted micrographs were used to estimate the contrast transfer function using CTFIND-4.14. All subsequent image-processing steps were performed using helical reconstruction methods in RELION 3.0. Filaments were picked manually.

MSA datasets

Segments for reference-free 2D classification comprising an entire helical crossover were extracted using an inter-box distance of 14.1 Å. For samples extracted from the putamen, segments with a box size of 750 pixels and a pixel size of 1.15 Å were downscaled to 256 pixels for MSA cases 2–5, and segments with a box size of 900 pixels and a pixel size of 0.83 Å were downscaled to 300 pixels for MSA case 1. For samples extracted from frontal cortex in MSA cases 1, 2, 3 and 5, and cerebellum in MSA case 1, segments with a box size of 750 pixels and a pixel size of 1.15 Å were downscaled to 256 pixels. MSA type I and type II filaments from the putamen were initially separated by reference-free 2D classification, and segments that contributed to suboptimal 2D class averages were discarded. For both types of filaments, an initial helical
twist of −1.4° was calculated from the apparent crossover distances of filaments in micrographs, and the helical rise was fixed at 4.7 Å. Using these values, initial 3D models for both types were constructed de novo from 2D class averages of segments that comprise entire helical crossovers using the relion_helix_inmodel2d program 64. Type I and type II filament segments were then re-extracted using box sizes of 220 pixels for MSA cases 2–5 or 320 pixels for MSA case 1, without downscaling. Starting with these segments and an initial de novo model that was low-pass-filtered to 10 Å, 3D auto-refinement was carried out for several rounds with optimization of helical twist and rise after reconstructions showed separation of β-strands along the helical axis. We then performed Bayesian polishing and contrast transfer function refinement, followed by 3D classification with local optimization of helical twist and rise, but without further image alignment, to remove segments that yielded suboptimal 3D reconstructions. To further separate the subtypes of type II filaments, segments from MSA case 2 were subjected to additional supervised and focused 3D classifications of K45–V95 from P5-H1B; type II, and II, filaments served as references. For all cases, selected segments were used for further 3D auto-refinement. Final reconstructions were sharpened using the standard post-processing procedures in RELION 65. Overall resolution estimates were calculated from Fourier shell correlations at 0.143 between the two independently refined half-maps, using phase-randomization to correct for convolution effects of a generous, soft-edged solvent mask that extended to 20% of the height of the box. Local resolution estimates were obtained using the same phase-randomization procedure, but with a soft spherical mask that was moved over the entire map. Using the relion_helix_toolbox program 66, helical symmetries were imposed on the post-processed maps, which were then used for model building. The reported ratios of MSA type I and type II filament segments in each case were determined by 2D classification of mixed sets of segments, which were re-extracted with box sizes of 750 or 900 pixels, while keeping the alignment parameters fixed to those resulting from the initial 3D refinements.

DLB datasets
DLB filament segments were extracted using an inter-box distance of 14.1 Å. For DLB cases 1–3, segments with a box size of 540 pixels and a pixel size of 1.15 Å were downsampled to 270 pixels. Reference-free 2D classification was performed using standard procedures.

Model building and refinement
Atomic models for type I and type II filaments were built de novo in Coot 67, using the maps of MSA case I and MSA case 2, respectively. Model building was started from the topologically conserved C-terminal bodies using the cryo-EM structure of recombinant α-synuclein filaments (RCSB Protein Data Bank code (PDB) 6A6B) as an initial reference 68. The handedness of the final models was confirmed by the presence of densities for the main-chain carbonyl oxygen atoms in the map of type I filaments at a resolution of 2.6 Å. For turns with weaker densities, models were built at low display thresholds. Models containing five β-sheet rungs were refined in real-space by PHENIX using local symmetry and geometry restraints 69. MolProbity 70 was used for model validation. Additional details are given in Extended Data Table 1.

Ethical review processes and informed consent
The studies carried out at Tokyo Metropolitan Institute of Medical Science and at Indiana University were approved through the ethical review processes of each Institution. Informed consent was obtained from the patients’ next of kin.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this paper.
Extended Data Fig. 1 | Filamentous α-synuclein pathology and immunolabelling of α-synuclein filaments from MSA. a, Staining of inclusions in the frontal cortex in MSA cases 1, 2, 3 and 5 and the cerebellum in MSA case 1 by the antibody pS129 (brown). Scale bar, 50 μm. b, Negative-stain electron microscopy images of filaments from the frontal cortex in MSA cases 1, 2, 3 and 5, and the cerebellum in MSA case 1. Scale bar, 50 nm. c, d, Representative immunogold negative-stain electron microscopy images of α-synuclein filaments extracted from the frontal cortex in MSA cases 1, 2, 3 and 5, and the cerebellum in MSA case 1 and the putamen in MSA cases 1–5. Filaments were labelled with the antibody PER4. Scale bar, 200 nm. e, Immunoblots of sarkosyl-insoluble material from the putamen for MSA cases 1–5, using the anti-α-synuclein antibodies Syn303 (N terminus), PER4 (C terminus) and pS129 (phosphorylation of S129). For gel source data, see Supplementary Fig. 1.
Extended Data Fig. 2 | Aggregation of α-synuclein in SH-SYSY cells after addition of seeds from the putamen for MSA cases 1–5. Quantification of wild-type human α-synuclein phosphorylated at S129 in SH-SYSY cells after addition of variable amounts of α-synuclein seeds from the putamen for MSA cases 1–5. The results are expressed as mean ± s.e.m. (n = 3 experiments).
Extended Data Fig. 3 | Cryo-EM images and 2D classification of MSA filaments. a, c, Representative cryo-EM images of α-synuclein filaments from the putamen in MSA cases 1–5, the frontal cortex in MSA cases 1, 2, 3 and 5 and the cerebellum in MSA case 1. Scale bar, 100 nm. b, d, Two-dimensional class averages spanning an entire crossover of type I and type II filaments extracted from the putamen in MSA cases 1–5, the frontal cortex in MSA cases 1, 2, 3 and 5, and the cerebellum in MSA case 1.
Extended Data Fig. 4 | Evaluation of the resolution of cryo-EM maps and of refined models. a–c, For type I (a), type II, (b) and type II, (c) filaments of α-synuclein from MSA: Fourier shell correlation (FSC) curves of two independently refined half-maps (black line); FSC curves of final cryo-EM reconstruction and refined atomic model (red); FSC curves of first half-map and the atomic model refined against this map (blue); FSC curves of second half-map and the atomic model refined against the first half-map (yellow dashes). d–f, Estimates of local resolution of the reconstructions of type I (d), type II, (e) and type II, (f) filaments of α-synuclein from MSA.
Extended Data Fig. 5 | Type I and type II filaments of α-synuclein from MSA.

a. Schematic of a type I filament, showing asymmetric PF-IA and PF-IIB. The non-proteinaceous density at the protofilament interface is shown in light red.

b. Schematic of a type II filament, showing asymmetric PF-IIA and PF-IIB. The non-proteinaceous density at the protofilament interface is shown in light red.
Extended Data Fig. 6 | The inter-protofilament interfaces of MSA type I and type II α-synuclein filaments. a, b, Rendered view of secondary structure elements in MSA type I (a) and type II (b) protofilament folds perpendicular to the helical axis of inter-protofilament interfaces, depicted as three rungs. Because of variations in the height of both polypeptide chains along the helical axis, each α-synuclein molecule interacts with three different molecules in the opposing protofilament. If one considers the interaction between two opposing molecules to be on the same β-sheet rung in the central cavity, the N-terminal arm of PF-IA or PF-IIA interacts with the C-terminal body of the PF-IB or PF-IIB molecule, which is one rung higher, while the C-terminal body of PF-IA or PF-IIA interacts with the N-terminal arm of the PF-IB or PF-IIB molecule, which is one rung lower. c, d, Compatibility of mutant α-synuclein (G51D and A53E) with MSA type I and type II filaments. Close-up views of atomic models of type I (c) and type II (d) α-synuclein folds containing D51 (cyan) and E53 (green). Each mutation adds two negatively charged side chains per rung in the second shell of residues around the central cavity, thus reducing the net positive charge of the shell.
Extended Data Fig. 7 Filamentous α-synuclein pathology in DLB. a, Staining of inclusions in the frontal cortex in DLB cases 1 and 2 and the amygdala in DLB case 3 by the antibody pS129 (brown). Scale bar, 50 μm. b, Negative-stain electron microscopy images of filaments from the frontal cortex in DLB cases 1 and 2 and the amygdala in DLB case 3. Scale bar, 50 nm. c, Representative immunogold negative-stain electron microscopy images of α-synuclein filaments extracted from the frontal cortex in DLB cases 1 and 2 and the amygdala in DLB case 3. Filaments were labelled with the antibody PER4, which recognizes the C terminus of α-synuclein. Arrowheads point to an unlabelled tau paired helical filament. Scale bar, 200 nm. d, Representative cryo-EM images of α-synuclein filaments from the frontal cortex in DLB cases 1 and 2, and the amygdala in DLB case 3. Scale bar, 200 nm. Arrowheads point to a tau paired helical filament, as evidenced by a three-dimensional reconstruction (inset), calculated as previously described. e, Two-dimensional class averages of α-synuclein filaments extracted from the frontal cortex in DLB cases 1 and 2 and the amygdala in DLB case 3.
Extended Data Fig. 8 | Structures of α-synuclein protofilament cores. a, Schematic of secondary structure elements in the α-synuclein protofilament cores of MSA. Red arrows point to the non-proteinaceous density (in light red) at protofilament interfaces. b, c, Secondary structure elements in the α-synuclein protofilament cores assembled from recombinant wild-type (b) and mutant (c) α-synuclein. β-Strands are shown as thick arrows. d, Schematic depicting the first 100 amino acids of human α-synuclein, comparing secondary structure elements in protofilament cores from MSA with those in protofilament cores assembled from recombinant α-synuclein. As observed previously for tau filaments, the arrangement of residues in β-strands is largely conserved among protofilament cores. This is especially the case for residues that adopt the conserved three-layered L-shaped motif, and less so for residues in the N-terminal arms.
Extended Data Fig. 9 | MSA filaments differ from those assembled with recombinant α-synuclein. a, Overlay of the three-layered L-shaped motifs of MSA α-synuclein filaments (yellow, orange, pink and purple) and filaments assembled in vitro using recombinant α-synuclein that contain a similar motif (grey). Despite topological similarities, none of the three-layered L-shaped motifs in recombinant α-synuclein protofilaments is identical to those of MSA protofilaments. The closest similarity to an in vitro structure is between PF-IIB2 and PDB 6PEO52, which differ only in the bend positions in the outer layer (between E57 and K58 for PF-IIB2 and between T59 and K60 for PDB 6PEO).
b, Overlay of MSA and recombinant α-synuclein structures on the basis of the turn at residues K43–V52, revealing a conserved interface between residues E46–V49 and V74–A78 or A76–K80 (red highlight), including the formation of a salt bridge between E46 and K80.
c, Overlay of MSA and recombinant α-synuclein structures on the basis of the conserved turn at residues V63–T72, revealing a second conserved turn (V63–T72) and a conserved packing through tight interdigitations of small side chains between residues A69–T72 and residues on the inner layer (green highlight). In MSA PF-IA and PF-IIA filaments, as well as in PDB 6OSM58, these residues are A89 and A91; in MSA PF-IB and PF-IIB filaments, as well as in PDB 6PEO, they are G93 and V95; in several recombinant α-synuclein structures, they are A91 and G93.
Extended Data Table 1 | Cryo-EM data collection, refinement and validation

| Data collection and processing | Case 1 | Case 1 | Case 2 | Case 2 | Case 2 |
|-------------------------------|--------|--------|--------|--------|--------|
| Magnification                 | 105,000| 105,000| 105,000| 105,000| 105,000|
| Voltage (kV)                  | 300    | 300    | 300    | 300    | 300    |
| Electron exposure (e^-/Å^2)   | 49.2   | 49.2   | 47.5   | 47.5   | 47.5   |
| Defocus range (µm)            | -1.7 to -2.8 | -1.7 to -2.8 | -1.7 to -2.6 | -1.7 to -2.6 | -1.7 to -2.6 |
| Pixel size (Å)                | 0.829  | 0.829  | 1.15   | 1.15   | 1.15   |
| Symmetry imposed              | C1     | C1     | C1     | C1     | C1     |
| Initial particle images (no.) | 329,477| 329,477| 386,301| 386,301| 386,301|
| Final particle images (no.)   | 120,501| 34,239 | 10,067 | 23,983 | 93,137 |
| Map resolution (Å)            | 2.60   | 3.68   | 3.61   | 3.29   | 3.09   |
| FSC threshold                 | 0.143  | 0.143  | 0.143  | 0.143  | 0.143  |
| Helical twist (°)             | -1.44  | -1.36  | -1.40  | -1.41  | -1.34  |
| Helical rise (Å)              | 4.72   | 4.75   | 4.71   | 4.72   | 4.72   |
| Map resolution range (Å)      | 2.29 to 24.12 | -    | -    | 3.05 to 28.11 | 2.84 to 23.00 |

Refinement

| Refinement | Case 1 | Case 1 | Case 2 | Case 2 | Case 2 |
|------------|--------|--------|--------|--------|--------|
| Initial model used (PDB code) | 6A6B | - | - | 6A6B | 6A6B |
| Model resolution (Å) | 2.60 | - | - | 3.27 | 3.1 |
| FSC threshold | 0.5 | - | - | 0.5 | 0.5 |
| Map sharpening B factor (Å^2) | -46.75 | - | - | -62.5 | -60.28 |
| Model composition | | | | | |
| Non-hydrogen atoms | 5,490 | - | - | 4,955 | 4,955 |
| Protein residues | 800 | - | - | 725 | 725 |
| Ligands | | | | | |
| B factors (Å^2) | | | | | |
| Protein | 57.82 | - | - | 73.68 | 69.86 |
| Ligand | | | | | |
| R.m.s. deviations | | | | | |
| Bond lengths (Å) | 0.008 | - | - | 0.008 | 0.008 |
| Bond angles (°) | 0.739 | - | - | 0.643 | 0.706 |
| Validation | | | | | |
| MolProbity score | 1.43 | | | 1.59 | 1.72 |
| Clashscore | 5.78 | | | 8.09 | 9.08 |
| Poor rotamers (%) | 0.93 | | | 0 | 0 |
| Ramachandran plot | | | | | |
| Favored (%) | 97.44 | - | - | 97.16 | 96.45 |
| Allowed (%) | 100 | - | - | 100 | 100 |
| Disallowed (%) | 0 | - | - | 0 | 0 |
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
- Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

EPU v1.11.1 and v2.3.079

Data analysis

RELION v3.0, CTFFIND v4.1, COOT v0.9-pre, phenix-1.17.1-3660, MOLPROBITY v4.2, PyMOL v2.3.2, Chimera v1.8.1, ImageJ, GraphPad Prism

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Raw cryo-EM micrographs are available in the Elecron Microscopy Public Image Archive (EMPIAR), entry numbers EMPIAR-10357 for MSA case 1 and EMPIAR-10358 for MSA case 2. Cryo-EM maps have been deposited in the Electron Microscopy Data Bank (EMDB) under accession numbers EMD-10650 for Type I filaments of MSA case 1, EMD-10651 and EMD-10652 for Type II1 and Type II2 filaments of MSA case 2, respectively. Refined atomic models have been deposited in the Protein Data Bank (PDB) under accession numbers 6XYO for Type I filaments for MSA case 1, 6XYP and 6XYQ for Type II1 and Type II2 filaments of MSA case 2, respectively. LC-MC/MS data have been deposited in the Japan Proteome Standard Repository/Database (JPOST) under I.D. PXD018434.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

For MSA: Putamen samples from 5 cases, frontal cortex samples from 4 cases and cerebellum sample from 1 case, chosen based on availability of tissue (maximum available sample size). For DLB: Frontal cortex samples from 2 cases and amygdala sample from 1 case, chosen based on availability of tissue (maximum available sample size).

Data exclusions

Pre-established common image classification procedures (S.H.W. Scheres, J. Struct. Biol. 180: 519-530, (2012)) were employed to select particle images with the highest resolution content in the cryo-EM reconstruction process. Details of the number of selected images are given in Extended Data Table 1 and Supplementary Tables 1-3.

Replication

All attempts at replication were successful. At least three independent biological repeats per experiment where representative data is shown.

Randomization

Not relevant to study. Samples were allocated into two experimental groups (putamen, frontal cortex and cerebellum samples from cases of MSA and frontal cortex and amygdala samples from cases of DLB) based on neuropathological examination.

Blinding

Not relevant to study. Samples were allocated into two experimental groups (putamen, frontal cortex and cerebellum samples from cases of MSA and frontal cortex and amygdala samples from cases of DLB) based on neuropathological examination.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|----------------------------------|---------|
| n/a | Involved in the study |
| ☐ | Antibodies |
| ☒ | Eukaryotic cell lines |
| ☐ | Palaeontology |
| ☐ | Animals and other organisms |
| ☐ | Human research participants |
| ☒ | Clinical data |
| ☒ | Involved in the study |
| ☐ | ChIP-seq |
| ☒ | Flow cytometry |
| ☒ | MRI-based neuroimaging |

**Antibodies**

Antibodies used

Primary antibodies used are presented in the Methods section with validation referenced. They are:

- Syn303 (BioLegend MMS-5085; diluted 1:4,000 for immunoblotting),
- PER4 (Diluted 1:4,000 for immunoblotting and 1:50 for immunogold negative-stain EM),
- pS129 (Abcam ac51253; diluted 1:1,000 for immunohistochemistry, 1:5,000 for immunoblotting and 1:50 for immunogold negative-stain EM),
- pSyn64 (Diluted 1:1,000 for immunoblotting).

Validation

Syn303 validated against human α-synuclein N-terminus in (Giasson et al. 2000 Science 290, 985-989); PER4 validated against human α-synuclein C-terminus in (Spillantini et al. 1998 PNAS 95, 6469-6473); pS129 validated against human α-synuclein pS129 in manufacturer’s datasheet (abcam); pSyn64 validated against human α-synuclein pS129 in (Fujiiwara et al. 2002 Nat Cell Biol 4, 160-164).

**Eukaryotic cell lines**

Policy information about cell lines

Cell line source(s)

Human neuroblastoma SH-SYSY cell line was obtained from ATCC.

Authentication

We declare that none of the cell lines used were authenticated.
Mycoplasma contamination  The cell line used was mycoplasma-free

Commonly misidentified lines  
(See ICLAC register)  
No commonly misidentified cell lines were used.

Human research participants

Policy information about  studies involving human research participants

Population characteristics  See Methods section. Age at death: 85, 68, 59, 64, 70, 59, 74 and 78; Gender: 2x female, 6x male; Diagnoses: 5x MSA, 3x DLB.

Recruitment  
Selected based on neuropathological examination. No bias was present.

Ethics oversight  
The studies carried out at Tokyo Metropolitan Institute of Medical Science and at Indiana University and the University of Kansas were approved through each university’s Institutional Review Board (IRB). Informed consent was obtained from the patients’ next of kin.

Note that full information on the approval of the study protocol must also be provided in the manuscript.